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IN VITRO CULTURE AND TRANSFORMATION STUDIES OF SPINACH (*SPINACIA OLERACEA* L.)

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A Thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy.

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DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's individual contribution. The author has attended appropriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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(Candidate)

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ABSTRACT

The objective of the present study was to develop a comprehensive and reproducible regeneration system for spinach (*Spinacia oleracea* L.) from commercially important cultivars and to assess the potential use of spinach for *Agrobacterium tumefaciens*-mediated transformation.

Tissue cultures of spinach were initiated from seed material. Axenic shoot cultures of spinach were established on MS-based medium containing 1.0 µM NAA at a temperature of 15°C and under a 16 h photoperiod. These three parameters were found most suitable for the establishment of shoot cultures and the encouragement of axillary shoot growth.

Attempts to enhance axillary shoot production of spinach were investigated by the use of a double phase culture system, employing semi-solid and liquid culture media. The application of liquid medium was feasable with a volume of 5 ml for a duration of 7 or 14 d or with a volume of 10 ml for a duration of 7 d, but the multiplication rate of spinach was not increased.

Adventitious shoot production was initiated from cultured spinach root explants derived from axenic shoots or hypocotyl explants. Sections from root tips and middle segments exhibited the highest shoot regeneration capacity when cultured on Nitsch and Nitsch (1969) medium supplemented with 20 µM NAA and 5.0 µM GA_3. Histological analysis demonstrated that the regenerating shoots originated directly from the root explants. Adventitious shoots were rooted on MS-based medium containing 1.0 µM NAA and transferred to the glasshouse, where the plants were grown to maturity. Seeds collected from regenerated plants were 95 % viable, producing a homgenous, fertile R_1-generation. Flow cytometric analysis was used to determine ploidy levels of regenerated plants and their progenies and showed that spinach leaf tissue from all generations displayed an even proportion of G_0/G_1 cells and G_2/M cells, which may be characteristic for this species.

Transformation studies using *in vitro* derived spinach explants demonstrated a positive response using two strains of *Agrobacterium tumefaciens*. The highest transformation rate was achieved with 25 % of explants being GUS-positive, therefore confirming susceptibility of spinach to the binary vector containing both T-DNA border sequences. It was found that best results were obtained with root explants which had been incubated for 8 weeks prior to co-cultivation with *Agrobacterium* and *in vitro* material which had been maintained in culture for up to 2 years.

This reproducible regeneration system for spinach and the demonstration that spinach is amenable to *Agrobacterium*-mediated transformation provides the basis for potential commercial application within spinach breeding, aiming to generate an improved crop plant.

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ABBREVIATIONS

BA	benzyladenine
BAP	6-benzylaminopurin
4-Cl-IAA	4-chloroindolyle-3-acetic acid
d	day
5,6-Cl_2-IAA	5,6-dichloro-indole-3-acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	gibberellic acid
GUS	β-glucuronidase
h	hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	2-isopentyladenine
µE	micro-Einstein
µM	micromole
MS	Murashige and Skoog (1962)
NAA	α-naphthaleneacetic acid
npt II	neomycin phosphotransferase II
SD	standard deviation
Ti	tumor-inducing
TIBA	2,3,5-triiodobenzoic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 The crop plant spinach (*Spinacia oleracea* L.)

1.1.1 The economical importance of spinach

Spinach (*Spinacia oleracea* L.) is an annual leafy vegetable belonging to the family *Chenopodiaceae*. The genus is native to South-West Asia and was introduced to the Far East and the United States, and spread from Spain to other European countries by the 16th century. Spinach is now widely cultivated in all temperate regions. The modern cultivars have been developed for their abundance of edible leaves. As well as to the fresh market, spinach has become important to the processing industries in the form of canned or frozen products (George, 1985).

Leaf vegetables are important sources of minerals and, in spinach, leaf minerals comprise 1.8 % of the dry weight. The specific nutritive value of spinach lies in the relatively high iron content of 4 - 6 mg per 100 g dry weight (Neskovic and Culafic, 1988). Spinach is also rich in vitamin A and contains appreciable quantities of ascorbic acid, riboflavin and a small quantity of thiamine. The estimation of calcium content is not possible, owing to the fact that it unites with oxalic acid to form calcium oxalate (Thompson and Kelly, 1957). A disadvantage of spinach lies in its high nitrate content of up to 3000 mg NO_3/kg. During transport and storage, nitrite arises from nitrate by reduction, in quantities which may cause methemoglobinemia (Breimer, 1982). Consequently, spinach along with other leaf vegetables, has been included in the 1993 EC-directive on nitrate levels in vegetable crops, which necessitates reducing the nitrate level in the crop.

In the UK, spinach is regarded as a minor speciality crop. Only a few commercial vegetable growers include spinach in their production programme for supplies to local canning and freezing industries (ADAS, 1995). The majority of spinach used in Britain is imported. The marketing company 'Wesspak' (Woodsford, UK) distributes up to 18 - 20 tonnes of imported spinach per week to seven major supermarkets and to the wholesale trade, taking up a 30 % share of the market (Vale, 1994). Figure 1.1 shows imports for the fresh market into the UK in 1993. During this year, 1412 tonnes of spinach with a farmgate value of 1.5 million pounds sterling were imported (Food for Britain, 1995).

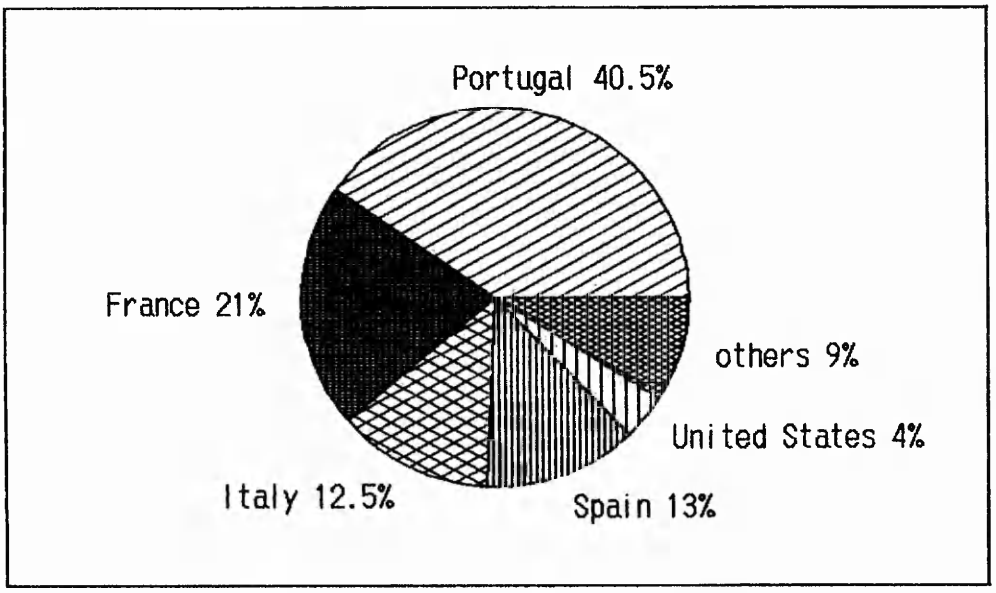


Figure 1.1: Imports of spinach into the Uk in 1993 expressed in % market share of the importing countries (Food for Britain, 1995).

In other European countries, particularly in Portugal and Spain, spinach is grown as a standard vegetable. In Asia, spinach ranks amongst the 10 most important vegetables (AVRDC, 1988) and the spinach crop is also cultivated throughout the United States, where it is utilized for canning and freezing. Even in 1982, the amount of spinach frozen came to 69000 tonnes (Luh and Kean, 1988).

Other green leafy vegetables resembling spinach in growth, culture and usage are discussed by Thompson and Kelly (1957). They include New Zealand spinach (*Tetragania expansa*, *Aizoaceae*), orach (*Artiplex hortensis*, *Chenopodiaceae*) and Swiss chard (*Beta vulgaris* var. *cycla*, *Chenopodiaceae*). However, these are less important market vegetables than spinach.

1.1.2 The conventional culture of spinach

Spinach cultivars are mainly classified according to their seed type and leaf characters. Seed types are round or prickly, and leaf characters are distinguished by their colour, shape, smooth or crinkled texture, position to the stem, petiole length and petiole colour (MAFF, 1979).

Spinach is a typical qualitative long day plant with a critical day length of 14 h. Initially, the plant develops a rosette of leaves soon after germination. Under favourable conditions, the stem begins to elongate and lateral branches arise from the axils of the rosette leaves. The flower clusters appear axially on the stems. Six to twelve flowers develop in each cluster. The male plant dies after flowering, while the female continues to develop, which is important for seed maturation (Thompson and Kelly, 1957).

Spinach is a cool season crop, that takes 6 - 10 weeks to reach marketable size. It can be grown virtually throughout the year, but is usually sown in the UK in the open at 10 - 14 days intervals from March to July to provide harvestable leaves from May to October. For winter production, different varieties are sown in August/September and protected under glass from October. The seeds are sown 1-2 cm deep into alkaline soil, pH 6.5-7.5 (Bleasdale *et al.*, 1991). A fertilzer application of N : P : K = 1 : 2 : 2 is recommended, with supplementary top dressings of nitrogen when necessary. Irrigation is important to obtain a high quality vegetable (George, 1985).

Yields of 12 tonnes spinach per ha are common, but up to 18 tonnes per ha are obtainable (MAFF, 1979). For mechanical harvesting spinach varieties should have an upright growth habit. Gonzales *et al.* (1989) suggested that a GA_3-treatment two weeks before harvest induces upright growth and petiole elongation, which facilitates easier mechanical harvesting. After the harvest, spinach leaves are ideally stored at 0°C at the highest obtainable humidity. Once the vegetables are cooled, the water content is best conserved by wrapping in polyethylene with storage at -0.5°C. The maximum storage life is 14 days (Bleasdale *et al.*, 1991).

An alternative culture method is suggested by Holbrook *et al.* (1993) by using indoor hydroponic cultivation. Optimization of the spinach productivity was achieved under a carbon dioxide-enriched environment, facilitating rapid production with a harvest after 28 days of growth.

1.1.3 Diseases

Spinach is frequently the host of several parasitic fungi which interfere with commercial plant or seed production. *Peronospora* can cause severe damage under moist conditions and low temperatures. Diseases cause serious losses to farmers and create unstability in the volume of leafy material produced for the processing industry.

Seed-borne fungi cause diseases such as leaf spot (*Cladiosporium variabile* and *Phyllosticta spinaciae*), anthracnose (*Colletotrichum dematium* f. *spinaciae*), damping off (*Colletotrichum spinaciicola* and *Rhizoctonia solani*) and wilt (*Verticillium dahliae*, *Verticillium* sp.) (George, 1985). Soil-borne fungi preventing germination include *Phythium* sp., *Phytophthora* sp. and *Phoma* sp.. Vegetative plants also show susceptibility to *Fusarium oxysporum* f.sp. *spinaciae*, *Peronospora farinosa* and *Peronospora spinaciae* (Neskovic and Culafic, 1988).

Several viruses have been identified, which infect spinach and these can also have a negative impact on the harvest. They include cucumber mosaic virus (CMV), turnip mosaic virus (TuMV), tomato bushy stunt virus (TBSV), spinach latent virus (SpLV) and lettuce mosaic virus (LMV) (Neskovic and Culafic, 1988). Other pathogens causing problems in the spinach production are the beet leaf miner (*Pegomyia hyoscyami*) and green aphid (*Myzus persicae*) (Thompson and Kelly, 1957).

1.1.4 Genetics and breeding

Spinach cultivars are mostly diploid (2n = 12). Cultivated spinach is usually considered to be dioecious, with a continuous range of monoecious types with respect to the proportion of pistillate (female) to staminate (male) flowers per plant. Most monoecious plants belong to the female late-flowering leafy type, which are considered superior to the male type. A single gene (X^m) determines monoecism independent of the sex-controlling XY factors (Janick and Stevenson, 1955).

Female plants are characterised by XX, male plants by XY and X^mY, while monoecious plants are determined by X^mX or X^mX^m. The X^mX^m-type is true breeding monoecious, while the X^mX-type is physiologically unstable and may be readily shifted by environmental conditions. The presence of X^m results in a shift towards maleness (Janick and Stevenson, 1955; Frankl and Galun, 1977). The degree of femaleness and maleness in monoecious plants may depend on environmental factors.

Sex expression of spinach can be modified by spacing of the plants, planting date and temperature. High temperature and short days increase the number of male flowers (Thompson, 1954).

Spinach is mainly wind pollinated. For the simplification of the growing of commercial seed it is recommended to use a round-seeded female variety and a prickly-seeded male variety. All plants can be harvested and threshed at the same time, since the prickly seed can be mechanically separated from the round commercial hybrid seed (Sneep, 1958). The hybrid seed production yield is approximately 800 kg seeds per ha (George, 1985).

First-generation hybrid seed can be produced by single-cross of a highly female, monoecious inbred with a highly male, monoecious inbred. In three-way hybrids, highly female monoecious F_1-generations and highly male monoecious parents are used. In the latter method, a large quantity of hybrid seed is produced (Kalloo, 1988). Crossing is also often done using a tetraploid female plant and a diploid pollen parent. This results in production of triploid plants, which seem to be superior to diploids. Spinach breeding practice includes the production of hybrid cultivars which may display heterotic effects. The selection of good inbred parent lines is complicated by the dioecious character of spinach. This can be circumvented by self-pollinating monoecious plants or cross-pollinating such plants with an extreme female or male individual (Thompson, 1954).

1.1.5 Breeding objectives for spinach cultivars

Sneep (1958) formulated three principal aims for producing F_1-hybrid seed of spinach which are still important today. These are to produce a heterosis effect, the combination of favourable characteristics that are governed by dominant genes and to obtain protection of the breeding product.

Crop improvement in spinach is approached from different directions. One important breeding objective is greater disease resistance, in particular, resistance to downy mildew. Other selection criteria for spinach include flower formation and bolting behavior, which are determined by the daylength and, thereby, the rate of growth. Later flowering facilitates a later harvest and, therefore, more leaf mass (Parlevliet, 1968).

For the processing industry, spinach must suffice certain quality standards to ensure suitability for canning and freezing. Great emphasis is placed on achieving low nitrate levels in the harvested product. Quality improvement in any crop plant is a consistent concern for the breeder. One approach described by Kalloo (1988) is the production of tetraploid spinach induced by colchicine treatment. The resulting tetraploid spinach plants were greater in fresh and dry weight and showed an increased iron and mineral content.

The application of tissue culture methods could be useful in spinach breeding. The development of suitable micropropagation methods could facilitate the multiplication and maintenance of certain genotypes of spinach, such as monoecious lines, parental homozygous lines, inbred lines or F_1-hybrids.

1.2 The use of spinach in experimental plant sciences

Spinach has been frequently used as a suitable object in plant biochemical and physiological research. Detchon and Possingham (1975) reported that various antibiotics had an inhibitory effect on ribosomal RNA synthesis in cultured spinach leaf discs. Studies on chloroplast and nuclear DNA of spinach have been investigated by several researchers. For example, Scott and Possingham (1980) investigated the relationship of chloroplast DNA per cell and the increase of the chloroplast population per cell in expanding spinach leaves. The amount of chloroplast DNA and nuclear DNA in the cells of spinach leaf discs cultured under different light regimes was compared by Tymms *et al.* (1982). In a more detailed study, Timmis and Scott (1983) found that the spinach nucleus contains integrated sequences that are homologous to chloroplast DNA sequences.

Spinach is a plant with definite photoperiodic requirements and is, therefore, a popular object to study events during photosynthesis. Oelmüller *et al.* (1993) described, in this context, a genomic DNA segment from spinach, that bears part of a single copy gene for ferredoxin-$NADP^+$-oxidoreductase, playing a crucial role in the photosynthetic energy distribution. Related to the subject of photosynthesis is the occurrence of photoinhibiton at cold temperatures. Somersalo and Krause (1989) stated that cold-hardened spinach plants exhibited a greater resistance to photoinhibition at low temperature in comparison to non-hardened plants, and the susceptibility to photoinhibition was significantly increased when high light intensity was combined with low temperature.

Attention has been paid to explore the stages involved in the development of freezing tolerance of spinach. Kazuoka and Oeda (1992) conducted studies on cold acclimation of spinach and associated cold-regulated proteins with the freezing tolerance in spinach. Guy and Guy (1994) identified and isolated cDNAs for three genes involved during cold acclimation in this plant. In plant transformation experiments, a transfer of these cDNAs was attempted in order to improve the freezing tolerance of petunia and tobacco.

Different experiments concern the current topic of achieving low nitrate levels in leaf vegetables. Spinach has been used for model studies to determine factors leading to nitrate accumulation in leaves and to establish the mode of action of nitrate reductase. Huckelsby and Blanke (1991) observed the accumulation of nitrate in the vacuoles of spinach under low light conditions and short days. Nitrate reductase

catalyses the reduction of nitrate to nitrite, which is the first enzymatic step in nitrate assimilation. Seith *et al.* (1991) found that the amount of nitrate available in spinach determines the level of nitrite-reductase mRNA, while light affects the synthesis of this enzyme.

Earlier publications dealing with *in vitro* studies on spinach were aimed at studying physiological events and were not intended to be an attempt for crop improvement in this species. For *in vitro* studies with spinach, cell suspensions were initiated from calli induced on stem sections or plumules. Protoplasts were usually obtained from fresh green leaves. For example, Dalton and Street (1976, 1977) investigated the importance of the constituents of the gas phase in the greening process of a cell suspension culture and studied the influence of carbohydrates on chlorophyll synthesis in suspension cultures. Fry and Street (1980) examined GA_3-sensitivity in suspension cultures and found the main effect of these treatments was the promotion of cell expansion. In another study, Sticher *et al.* (1982) observed the release of peroxidases of spinach cell suspension cultures in their extracellular medium under the influence of calcium and light. Factors that influence yield of protoplasts from fresh leaves, their stability in culture and the ability to regenerate cell walls of isolated spinach mesophyll protoplasts have been studied by Rose (1980). Protoplasts isolated in the presence of Ca^{2+} and low light intensity showed a greater capacity for cell wall regeneration and protoplast-derived cells could be maintained for 7 days in culture. Nishimura *et al.* (1985) isolated leaf protoplasts and used glycolate oxidase activity as an index for their intactness. Nakagawa *et al.* (1985) succeeded in the isolation of protoplasts from actively growing suspension cultures initiated from plumule-derived calli. The isolated protoplasts were capable of cell wall regeneration and of forming cell clusters. By employing somatic techniques, a fusion product of spinach mesophyll protoplasts with carrot root parenchyma protoplasts was obtained and the resulting heterokaryons were viable for at least 5 days (Hodgson and Rose, 1984).

1.3 Tissue culture techniques for plant production

Plant tissue culture technology has evolved into a powerful technique for rapid clonal multiplication of selected varieties, for the recovery of disease-free clones, for the preservation of valuable germplasm, and for genetic improvement of agricultural, horticultural and forest species. Other economic activities through tissue culture are directed to the production of pharamaceuticals, food colours and flavourings. Plant tissue culture methods, combined with molecular techniques, provide the foundation for the rapidly developing techniques in genetic transformation of crop plants.

1.3.1 Methods of plant propagation by tissue culture

Plants may be propagated asexually by two main pathways. One is the multiplication of existing apical and axillary shoot meristems by means of shoot tip culture or node culture. The other is the formation of adventitious meristems, either directly from organ explants in the form of shoot buds, or indirectly from callus or suspension cultures also in the form of shoot buds or as somatic embryos (Hussey, 1986). Plants from existing meristems result in the production of genetically stable plants, whereas plants from *de novo* meristems can result in progenies which are not true-to-type and in the production of somaclonal variants (Short, 1986).

Regeneration is based on the concept of totipotency of plant cells, which are capable of giving rise to a new plant, since each can carry all the genetic information contained in the zygote (Krikorian and Berquam, 1969).

Gamborg (1975) classes different types of cultures into five categories:

1. Organ cultures

   Aseptic culture of, for example, embryos, anthers, ovaries, buds, roots, flowers, for the study of morphogenesis.

2. Meristem and tissue cultures

   Shoot meristems, leaf and stem meristems or other tissue explants for regeneration and multiplication.

3. Callus cultures

   Initiation and culture of undifferentiated cell masses initiated from seedlings or other tissue sources on agar media.

4. Cell suspension cultures

   Culture of cells in liquid medium (shake cultures or mass cultures in fermenters).

5. Protoplast cultures

   Culture of protoplasts isolated directly from plant organs or from cultured cells.

Preference is often given to systems which enable direct organogenesis to be obtained to maximise genetic uniformity. Genetic stability is best preserved in organized tissue. The probabilily of the occurrence of aberrant cells increases with the age of explants. Explants are, therefore, taken from the youngest possible plant material (Binding and Krumbiegel-Schroeren, 1984).

Factors that determine the success of any culture method are the explant origin, the composition of the nutrient medium and the culture environment. Murashige (1974) suggested three sequential steps to follow in the establishment of tissue cultures. The first step is to obtain an aseptic tissue culture of the chosen plant explant in question. The second is the induction of shoot buds and the multiplication of the propagula while the third step is rooting of the shoots and the re-establishment of plants in soil.

The requirements of the stages *in vitro* are characterized by the nutrient medium composition and the quality of the culture environment. An empirical approach has to be taken in order to establish adequately the needs for the initiation of tissue cultures and the regeneration of plants.

The major constituents in tissue culture media required for successful growth and organogenesis are inorganic macro- and micronutrients, carbon sources, vitamins, reduced nitrogen and phytohormones. These five classes of compounds are usually sufficient for most plant species. Natural complexes are also often added to the medium (Thorpe and Patel, 1984). Table 1.1 lists the major constituents of tissue culture media. The salt mixtures named have formed the basis for fulfilling the salt requirements of most plant tissue and organ cultures. Today, modifications of these basic media are available designed to meet specific culture needs.

Table 1.1: Major constituents of nutrient media (Murashige, 1974).

Inorganic salt mixtures

White (1943)

Hildebrandt, Riker and Duggar (1946)

Heller (1953)

Murashige and Skoog (1962)

Organic substances

Carbohydrate: sucrose, glucose, fructose

Vitamins: thiamine, inositol, nicotinic acid, pyridoxin

Amino acids and amides: arginine, aspartic acid and asparagine, glutamic acid and glutamine, tyrosine

Nitrogen base: adenine

Plant growth regulators:

Auxin: IAA, IBA, NAA, CPA, 2,4-D

Cytokinin: BA, BPA, Kinetin, 2iP, Zeatin

Others: ABA, GA_3

Natural complexes

Hydrolysed protein preparations: casein and lactoalbumin hydrolysates, soy peptone

Brewer's by-products: malt and yeast extracts

Endosperm fluids: coconut, corn

Fruit pulp and juice: banana, orange, tomato

Animal by-products: fish emulsion

The most critical organic components of the tissue culture media are auxins and cytokinins. Organogenesis is determined by the relative concentrations of auxin to cytokinin. Auxins induce cells to divide and promote root initiation, whereas cytokinins reduce apical dominance and thereby allows the production of axillary shoots. Cytokinins are used jointly with auxins to initiate and to promote cell division (Gamborg, 1975). Other phytohormones, including gibberellins and abscisic acid, have been shown to play a role in organ formation. Gibberellins are used to induce plant development from cultured meristems and aid cell elongation. The addition of abscisic acid has shown to be beneficial for the production of embryos from somatic cells (Thorpe, 1980).

Another important factor is the physical quality of the culture medium. The choice of a liquid or gel medium depends on the culture method and the plant species. The gel concentration and the agar quality are important when a semi-solid medium is required. Inoculated liquid nutrient media are usually subjected to a degree of agitation. However, liquid nutrient media can also be used in a stationary state, with the tissue explant submerged or supported by, for example, filter-paper bridges (Murashige, 1974). More recently, cellulose rods have been used for the culture of apical and axillary meristems of cauliflower and chrysanthemums in liquid medium (Short *et al.*, 1987).

The pH of the culture medium is also a critical factor. The usual practice is to adjust the pH value between 5.0 and 6.0. However, Martin and Rose (1976) found that the biomass yield of suspension cultures of *Ipomoea* was significantly influenced by pH-levels of the nutrient medium between 4.8 and 7.1. Therefore, tests for the optimun pH for different cultures are advisable.

1.3.2 Tissue culture systems for plant improvement

The ongoing development of existing tissue culture methods and the progress in the application of molecular techniques provide crop improvement programmes with the potential of exploiting useful genetic changes.

Approaches to plant improvement by somatic cell genetics require reproducible protocols for the regeneration of intact, fertile plants from single cells. Culture methods utilizing protoplasts have been developed for many plant species as a powerful tool for producing both somatic hybrids and somaclonal variants. In addition, these isolated, naked cells provide an amenable system for the uptake of large particles

and macromolecules for plant transformation. Protoplasts can be isolated from most plant tissues and also from callus and cell suspensions. The basic techniques of protoplast isolation, culture and fusion have been derived, primarily, from model systems using Solanaceaous plants as *Nicotiana*, *Petunia*, *Datura* and *Solanum* (Chung, 1987). Regeneration from cultured protoplasts has now been achieved from a wide variety of cereals, legumes, woody plants, medicinal plants and ornamentals (Davey and Power, 1988).

The exposure of plant protoplasts to short, high voltage pulses has been shown to stimulate division of protoplasts and to increase production of cell colonies derived from protoplasts of both herbaceous (Gupta *et al.*, 1988) and woody species (Rech *et al.*, 1987). However, regeneration of plants from protoplasts and cells often produces plants which are not true-to-type, exhibiting physiological and morphological changes. Mosseau (1970) found distinct differences in a number of characters such as leaf shape, leaf surface and chlorophyll content in a population of tobacco plants obtained from single cell cultured *in vitro*. For genetic and plant improvement aspects, the ability to regenerate plants from cell cultures means that genetic manipulations at the cellular level can be evaluated in mature plants and the latter utilized, if required, in conventional breeding programmes.

The fusion of protoplasts by chemical or electrical methods has led to the production of several somatic hybrids, enabling the transfer of genetic information from one species to another. Particularly valuable are somatic hybrids between different plant species which cannot be hybridized by conventional means. For example, Ochatt *et al.* (1989) regenerated a fruit tree somatic hybrid by fusion of cell suspension protoplasts of the sexually incompatible rootstocks wild pear (*Pyrus communis* var. *pyraster* L.) and Colt cherry (*Prunus avium* x *pseudocerasus*), thereby combining valuable traits for environmental stress and disease tolerance.

The production of a somatic hybrid between common buckwheat (*Fagopyrum esculentum*) and wild buckwheat (*Fagopyrum tatricum*) was attempted in order to overcome breeding difficulties due to heteromorphic self-incompatibility. The aim was to obtain a hybridisation product to which *Fagopyrum esculentum* contributed high nutritional value and a short vegetative period and *Fagopyrum tatricum* provided other genetic resources such as self-fertility and high productivity (Lachmann and Adachi, 1990).

Another application of tissue culture in the context of plant improvement is the *in vitro* selection for mutants carrying desirable traits, which is based on somaclonal variation. Somaclonal variation is thought to arise from somatic changes at the gene level in somatic cells, which is enhanced during adventitious shoot formation involving a callus phase and during consecutive subcultures (Larkin and Scowcroft, 1981).

The plant material available for selection varies from single cells to complex differentiated tissues. The production and selection of useful somaclonal variants may be increased by mutagenesis or the application of various selection pressures, such as exposure to phytotoxins, herbicides, heavy metals or extreme temperatures. Plants have been regenerated from NaCl-tolerant tobacco cells and salt tolerance was retained in the regenerated plants (Nabors *et al.*,1980). In another example, Connell and Heale (1986) selected and regenerated plants from hop callus culture resistant to *Verticillium* wilt.

The production of haploids occupies an important position within plant breeding programmes. Haploids can be generated from cultures of anthers, pollen grains or isolated ovules. Plants regenerated from such cells carry the chromosome complement of only one parent. This complement must be doubled in order to produce a fertile plant, which is assumed to be homozygous and to breed true. Such a rapid method for the generation of homozygosity has created considerable interest amongst plant breeders. The production of haploids has been successful in over 200 plant species, many of them belonging to the *Solanaceae* and *Gramineae*. Key factors in the culture of haploid plants are the developmental stage of the reproductive organs, pretreatment of the explants, and composition of and additives to the culture medium (Dunwell, 1986).

Finally, the genetic engineering of plant material contributes greatly to crop improvement programmes. Initially, a desired trait is chosen and the genes encoding the trait need to be identified and isolated from the donor organism. The functional gene must include regulatory regions in order to be correctly expressed in the plant. The isolated gene may be transferred directly into target cells and tissues, or inserted into biological vectors which deliver the genes into the plant cells (Perani *et al.*, 1986).

The most widely applied method for gene transfer is the *Agrobacterium tumefaciens*-mediated transformation system. *Agrobacterium* is a natural pathogen of dicotyledons and, therefore, as a vector system often successfully applied to this group of plants. In contrast, monocotyledons are limited in their susceptibility to transformation by *Agrobacterium*. Thus, other methods for direct gene transfer are being developed to be applied, particularly to monocots. They involve chemically-mediated or silicon carbide fiber-mediated DNA uptake, electroporation, microinjection and biolistic methods.

Examples for successfully applied direct gene transfer methods are the stable transformation of pea and lentils by electroporation of nodal buds (Chowria *et al.*, 1993), the engineering of transgenic rapeseed plants obtained by microinjection of DNA into microspore-derived embryoids (Neuhaus *et al.*, 1987), or the production of fertile, transgenic maize plants from embryogenic maize suspension cultures using silicon carbide fiber-mediated transformation (Frame *et al.*, 1995).

1.4 Tissue culture approaches within the family *Chenopodiaceae*

1.4.1 *Beta vulgaris*

Work on *in vitro* methods as applied to the *Chenopodiaceae* has been very limited. Most studies have been conducted on sugar beet (*Beta vulgaris*), which, as a source of sucrose, is agronomically very important. Several workers have reported axillary and adventitious shoot production in sugar beet. Hooker and Nabors (1977) obtained shoot buds from cotyledon and hypocotyl callus, which was promoted by addition of BAP and TIBA to the Linsmaier and Skoog (1965) basal medium. Various sugar beet explants, petioles, roots, cotyledons, flower buds and shoot tips have been used by Tetu *et al.* (1987) to induce green friable calli. Shoot formation was achieved when the MS-based culture medium contained the anti-auxin TIBA combined with cytokinin. The first regeneration system at high frequency was established by Ritchie *et al.* (1989). *In vitro* shoot regeneration was either obtained from white friable leaf callus incubated on MS-based medium supplemented with 2.22 µM BAP or organogenesis was directly induced in petiole explants by culture on MS-based medium containing 5.0 µM BAP. Haploid sugar beet plants were produced from ovule callus by Galatowitsch and Smith (1989) providing the opportunity to select a homozygous genome. Lindsey and Gallois (1990) succeeded in the genetic transformation of basal stem tissue using *Agrobacterium tumefaciens* strain LBA 4404 and regenerated transgenic plants from sugar beet.

1.4.2 *Atriplex canescens* and *Chenopodium quinoa*

Other species within the *Chenopodiaceae* introduced into culture are *Atriplex canescens* and *Chenopodium quinoa*. *Atriplex canescens* is a perennial, drought and salt tolerant forage plant and is particulary important in arid zones. Wochok and Sluis (1980) initiated shoot tip cultures of *Atriplex* on MS-based medium supplemented with 0.47 µM kinetin and 0.57 µM IAA. A treatment of 1.4 µM GA_3 stimulated shoot elongation and enhanced shoot multiplication.

Chenopodium quinoa, which is rich in protein, has potential as a grain crop. Shoot tip cultures from seedlings and adult plants have been established on modified B5-based medium supplemented with 0.22 mg l^{-1} BA and 0.018 mg l^{-1} NAA (Burnouf-Radosevich and Paupardin, 1985). Callus could be initiated on epicotyl segements by applying 2,4-D and could be maintained for over two years in culture. Shoot regeneration, however, was not achieved (Burnouf-Radosevich, 1988).

1.4.3 *Spinacia oleracea*

Several workers have reported axillary and adventitious shoot production in spinach, but a comprehensive culture system resulting in recurring plant regeneration with the possibility to apply plant transformation techniques is currently not available.

Organogenesis in cultured spinach tissue was first reported by Neskovic and Radojevic (1973). They found that spinach is very recalcitrant to the usual hormonal stimuli for organogenesis, but callus growth and proliferation could be easily obtained. Assuming that spinach tissue had an absolute requirement for IAA and kinetin, initial shoot regeneration from callus tissue was obtained, but could not be repeated.

Sasaki (1989) initiated shoot buds on hypocotyl explants from various Japanese cultivars on MS-based culture medium containing IAA. Enhanced bud formation was achieved by addition of GA_3 to the medium. In different studies, Al-Khayri *et al.* (1991 a, 1991 b, 1992 b) obtained shoot regeneration from callus derived from leaves or germinating seeds. The MS-based culture medium was supplemented with kinetin, 2,4-D and GA_3. A genotype-dependent response of the spinach cultivars was found with the rate of shoot regeneration ranging from 2.4 % to 32.4 %. However, information was not provided on the number of shoots produced.

The need for a high auxin supply in tissue cultures of spinach was highlighted by Mii *et al.* (1992). Hypocotyl explants were treated with the auxin 5,6-dichloro-indole-3-acetic acid. 5,6-Cl_2-IAA is a derivate from a naturally occurring auxin, 4-Cl-IAA, found in *Pisum sativum* (Marumo, 1968). In a comparative study of IAA and 5,6-Cl_2-IAA, Mii *et al.* (1992) found that 5,6-Cl_2-IAA possessed stronger activity and stability than IAA and induced a shoot regeneration response of 82 % in 1 out of 9 cultivars tested.

More recently, Xiao and Branchard (1993) reported somatic embryogenesis induced in hypocotyl derived callus. Plants were regenerated on MS-based medium containing IAA and GA_3 followed by transfer to glasshouse conditions.

Attempts have been made to improve spinach tissue cultures by addition of various compounds. Neskovic *et al.* (1976) showed that low concentrations of abscisic acid increased the fresh and dry weight of callus. Coconut water added to the culture medium improved the growth of callus and shoots (Al-Khayri *et al.*, 1992 d).

A different area of interest within the tissue culture of spinach concerns the flowering and sex expression of cultured apical buds. Culafic and Neskovic (1980) found that, under long day conditions, 75 % of the plantlets flowered and under short days 40 % of the plantlets produced inflorescences when treated with gibberellin. Abscisic acid had no effect on flowering and applications of cytokinins delayed and reduced flower initiation.

The influence of external factors on the sex expression of spinach has been well documented. However, little is known about sex shifts in regenerated plants as a possible result of tissue culture procedures. Al-Khayri *et al.* (1992 b) found in their study, that callus induced from female plants developed shoots with female flowers, while callus from male plants regenerated shoots carrying male flowers. A sex shift among regenerated females was not observed. Male regenerants, however, experienced a shift towards monoecism.

1.5 Objectives of the present study

Little research work has been performed in the field of tissue culture within the *Chenopodiaceae.* Most work has been conducted on sugar beet and a regeneration system for this crop has been developed.

Spinach is an important leafy vegetable and is in demand worldwide, but few attempts have been undertaken to initiate tissue cultures from this species. Spinach may be viewed as a recalcitrant plant species which has been found, to date, to be difficult to introduce and to maintain *in vitro*.

The prime objective of the present study was therefore, to develop a reliable regeneration system for spinach with the ultimate aim to exploit cultured material for crop improvement programmes.

The first main task was the initiation of tissue cultures from commercially important spinach varieties. Once aseptic cultures had been established, the emphasis was placed on the clonal propagation of this species, testing various hormone combinations and adjusting and refining culture conditions. During these investigations, the growth pattern and the development of the *in vitro* grown spinach plants was monitored. The development of a micropropagation system should facilitate the maintenance of valuable breeding and commercial lines and should also provide readily available source material for further experimentation.

The second main objective was directed to the induction of adventitious shoots, thereby establishing a system that allowed for cell-to-plant regeneration. The development of regenerants and variations within the regenerating progenies were screened and plants were reintroduced to ambient glasshouse conditions completing the whole regeneration cycle.

Transformation methods necessitate the establishment of a shoot regeneration system for spinach. The use of *in vitro* cultures of spinach for transformation was also evaluated, by assessing an *Agrobacterium*-mediated transformation system.

Overall, the prime objective of this project was to establish a reproducible culture system for spinach that removed the constraints of genotypic differences and which had the potential to be generally applicable to commercially valuable spinach cultivars.

CHAPTER 2

The initiation of tissue cultures from spinach (*Spinacia oleracea* L.)

2.1 Introduction

Plant tissue cultures have typically been initiated from existing meristems of seedlings and mature plants or from plant organs such as leaves, flowers, stems and roots. Al-Khayri *et al.* (1991a, 1992a) chose for the initiation of tissue cultures from spinach *in vivo* grown leaf material and germinating seeds.

In the present study, tissue cultures of spinach have been initiated from germinating seeds under aseptic conditions. Sterility problems arose through bacterial and fungal infestation of the seeds, particularly of seeds of commercial cultivars. In order to obtain uninfected plant material, preliminary tests were carried out with respect to the sterilization method, incubation medium and incubation temperature.

Cross contamination was prevented by germination of single seeds in McCartney bottles. The germination medium used was half or full strength semi-solidified MS-based medium (Murashige and Skoog, 1962). In addition, vermiculite, perlite and cotton wool plugs were employed, which were moistened with sterile, distilled water, or liquid half or full strength MS medium, respectively. With regards to the germination medium, differences in the seedling quality could not be detected. Therefore, sterile distilled water was used in all subsequent experiments. The incubation temperature was 25°C during the first tests, which was also chosen for the initiation of tissue cultures by Mii *et al.* (1992) and Xiao and Branchard (1993).

Roeggen (1989), however, determined a minimum germination temperature for spinach of -3.1°C. In a different study, it was shown that the germination decreased at temperatures above 12°C (Roeggen, 1984). Additionally, Roeggen's study showed that an amount of 2 ml water per Petri dish was found to be sufficient to aid the germination process.

In order to improve on the seedling quality, the germination temperature in the following experiments was reduced to 5°C. It was expected that lowering the germination temperature would inhibit fungal and bacterial growth.

The aim of the following experiments was to determine the most effective sterilization method for spinach seeds, which provided a high germination rate and eliminated seed-borne infections at the same time. A screening method for bacterial infection in germinated seedlings was developed.

2.2 Materials and Methods

2.2.1 Plant material and sterilization

Seeds of various commercial spinach cultivars and breeding lines were used for the initiation of tissue cultures. The commercial cultivars were 'Longstanding' (Sutton Seeds Ltd., Kentford, UK), 'Medania' and 'Virkade' (both obtained from A.L. Tolzer Ltd., Cobham, UK). Four breeding lines were supplied by Royal Sluis (Wageningen, The Netherlands). The breeding lines were described as follows:

A: Female spinach with resistancy to *Peronospora* race 1, 2, 3

B: Female spinach with resistancy to *Peronospora* race 1, 2

D: Monoecious spinach, resistant to *Peronospora* race 1, 2, 3

E: Monoecious spinach, resistant to *Peronospora* race 1, 3

The seeds were surface sterilized by immersion in 70 % (v/v) ethanol for 1 min, followed by different treatments with 'Domestos' bleach solution (Lever Brothers Ltd., Kingston-upon Thames, UK). The bleach concentrations tested were 15 % (v/v), 20 % (v/v) and 25 % (v/v) and seeds were immersed for a duration of 15 min, 20 min, 25 min or 30 min. Following these treatments the seeds were washed with sterilized 3 % (v/v) 'Tween 80' (Sigma, Chemical Company Ltd., Poole, UK) for 10 min, prior to 2 washes with sterile distilled water for 5 min each. 'Tween 80' and the distilled water were sterilized by autoclaving at 121°C for 15 min.

Sterilized seeds were germinated on sterile filter paper moistened with 2 ml of sterile distilled water in 9 cm Petri dishes under a 16 h photoperiod using 36 W Hybec White fluorescent tubes giving 45 ± 10 $\mu E\ m^{-2}s^{-1}$ of irradiance at 5°C for 21 d. Twelve seeds were placed into each dish and 100 - 150 seeds were used per treatment. Each treatment was repeated 2 - 3 times. Seedlings that had grown both radicles and hypocotyls were scored as having germinated.

In a second experiment, spinach seeds of the commercial cultivars 'Longstanding', 'Virkade' and 'Medania' were sterilized with a 25 % (v/v) 'Domestos' solution for 20 min. The seeds were germinated on sterile filter paper moistened with 2 ml of sterile distilled water or with sterile water containing 25 mg l^{-1}, 50 mg l^{-1} or 100 mg l^{-1} of the filter sterilized antibiotic 'Baypen' (Bayer, Newbury, UK). 'Baypen' contains mezlocillin, a broad-spectrum anti-bacterial agent for the control of both gram-positive and gram-negative pathogens. One hundred seeds were used per treatment and each treatment was repeated twice. Significant differences between values expressed as percentages were established by using the Chi-Sqare test.

2.2.2 Screening for bacterial infection in spinach seedlings

Apical stem explants (10 mm in length) of 28 d old seedlings were screened for any bacterial infection by transfer to 9 cm Petri dishes containing 20 ml of agar-solidified (0.75 % w/v; Oxoid) MS-based medium lacking growth regulators. The pH-indicator Methyl Red (BDH, Poole, UK) was included in the screening medium. Four ml of the 1 % (w/v) aqueous solution of Methyl Red were added to 1 l of culture medium. Methyl Red reacts to the lowering of the pH value with a colour change from orange-red to yellow, which can be caused by bacterial activity. The apical explants were incubated for 2 d at 25 °C in the dark on medium containing Methyl Red, before being used for experimental work.

2.3 Results

The influence of sodium hypochloride ('Domestos') on the reduction of seed infection and hence the production of surface sterilized seeds of Breeding Line A is illustrated in Figure 2.1 A. Figure 2.1 B illustrates the effect of the concentration of sodium hypochloride on the germination rate of seeds.

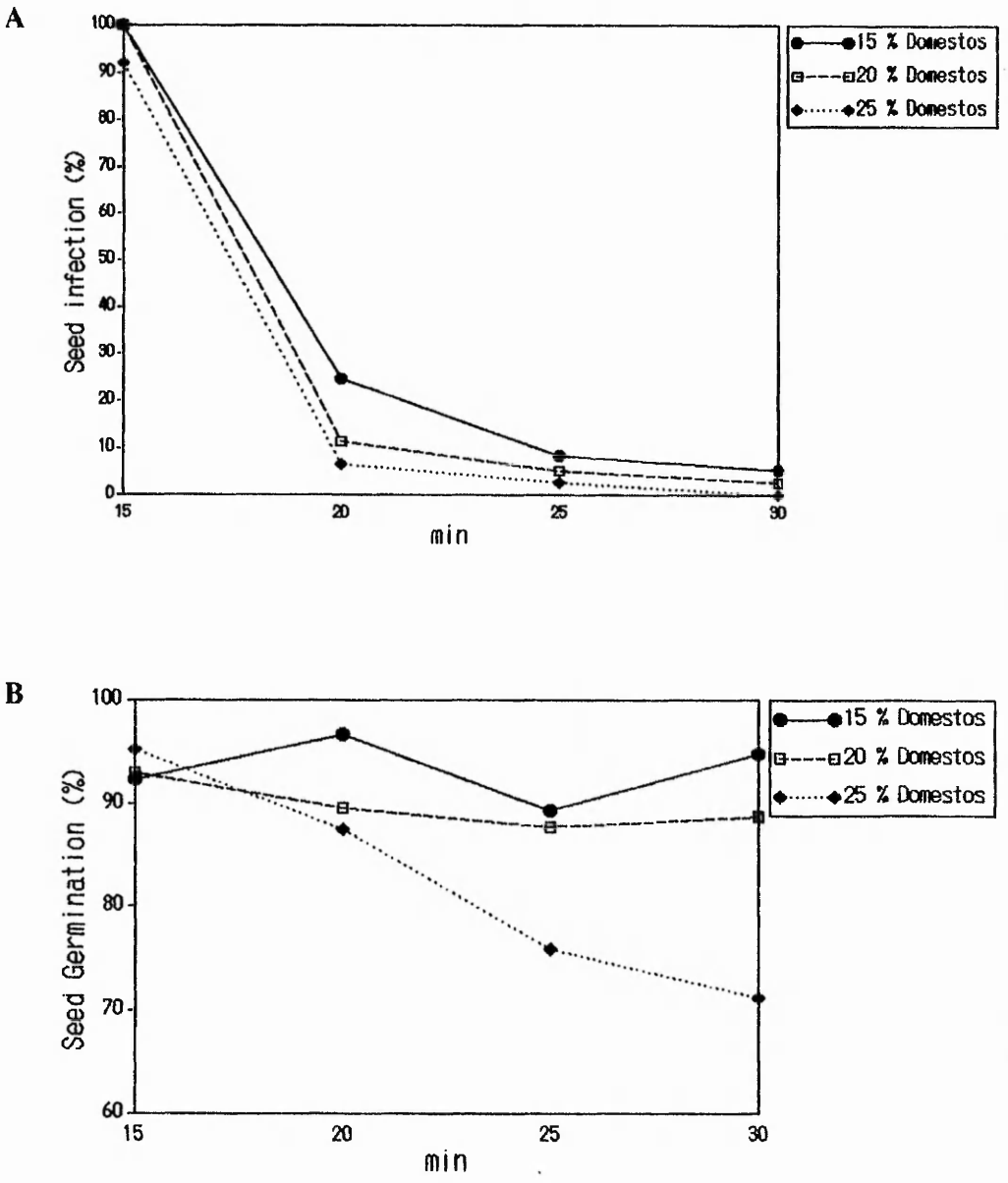


Figure 2.1: (A) Infection of seedlings of Breeding Line A and (B) germination of seedlings of Breeding Line A after sterilization with various concentrations of 'Domestos' for 15, 20, 25 or 30 min.

The infection was strongly reduced when the immersion time was increased from 15 to 20 min. An increase of the immersion time up to 30 min lead to a further improvement of the seed sterility and was found for all concentrations of sodium hypochloride tested. However, with increasing concentrations of 'Domestos' from 15 % to 25 % a further reduction of the infection rate within each tested time interval could be achieved. The lowest infection rate occurred when the seeds were sterilized in a 25 % (v/v) 'Domestos' solution for 30 min.

The germination rate was hardly affected when the concentration of the disinfectant was 15 % or 20 %. However, at the higher concentration of 25 % the germination rate was reduced when the immersion time was increased from 15 min to 30 min. The germination rate decreased from 95 % to 72 %, probably due the toxicity of the disinfectant. A treatment of a 25 % (v/v) 'Domestos' solution for 20 min was selected as the most appropriate sterilization method for seeds of spinach. With this treatment, a noticable loss of viable seeds was avoided and infection was limited.

The same treatments were applied to the other breeding lines and to the commercial cultivars. Comparable results as shown for Breeding Line A were obtained for the other breeding lines, regarding germination rate and infection rate.

Studies on the sterilization of commercial cultivars demonstrated that the seeds still carried a very high proportion of bacterial infection. However, tests with the antibiotic 'Baypen' showed, when added to the germination medium in various concentrations, that bacterial infection was significantly lowered. Table 2.1 demonstrates that, with the addition of the antibiotic 'Baypen' to the germination medium used for the commercial spinach cultivars, a significant improvement of the seed sterility was achieved. The infection rate of 'Longstanding' and 'Virkade' was reduced by approximately 50 %, using a concentration of 100 mg l^{-1} 'Baypen'. The germination rate of 'Longstanding' increased with every applied concentration of 'Baypen' and with the addition of 100 mg l^{-1} of the compound the germination increased from 36.0 % to 65.0 %. 'Medania' was very sensitive to 'Baypen' treatments, the germination rate being reduced from 67.0 % without 'Baypen' addition to the germination medium, to a germination rate of 27.0 % when 100 mg l^{-1} 'Baypen' were added. The seed infection, however, could be successfully reduced from 60.9 % to 5.6 % at the same concentrations.

Table 2.1: Germination and infection rates of three commercial cultivars of spinach in sterile water containing different concentrations of the antibiotic 'Baypen'.

Commercial cultivars		Baypen concentration (mg l^{-1}) 0	25	50	100
Longstanding	germination	36.0	52.0 ***	59.0 ***	65.0 ***
	infection	97.7	61.5 ***	62.1 ***	46.2 ***
Virkade	germination	81.0	78.0	79.0	83.0 ns
	infection	43.9	36.9	31.7	23.4 *
Medania	germination	67.0 ***	54.0 ***	39.0	27.0
	infection	60.9	33.6	29.4 *	5.6 ***

*** = P(0.001) ** = P(0.01) * = P(0.05)
ns = not significant

The screening method described proved to be very reliable for detecting any bacterially infected seedlings. Plate 2.1 shows clearly the difference between healthy and infected plant material. The screening medium remained red-orange in the absence of infection, but became pink, if the seedlings carried bacterial infection. In addition, a yellow corona appeared, surrounding the hypocotyls of infected seedlings.

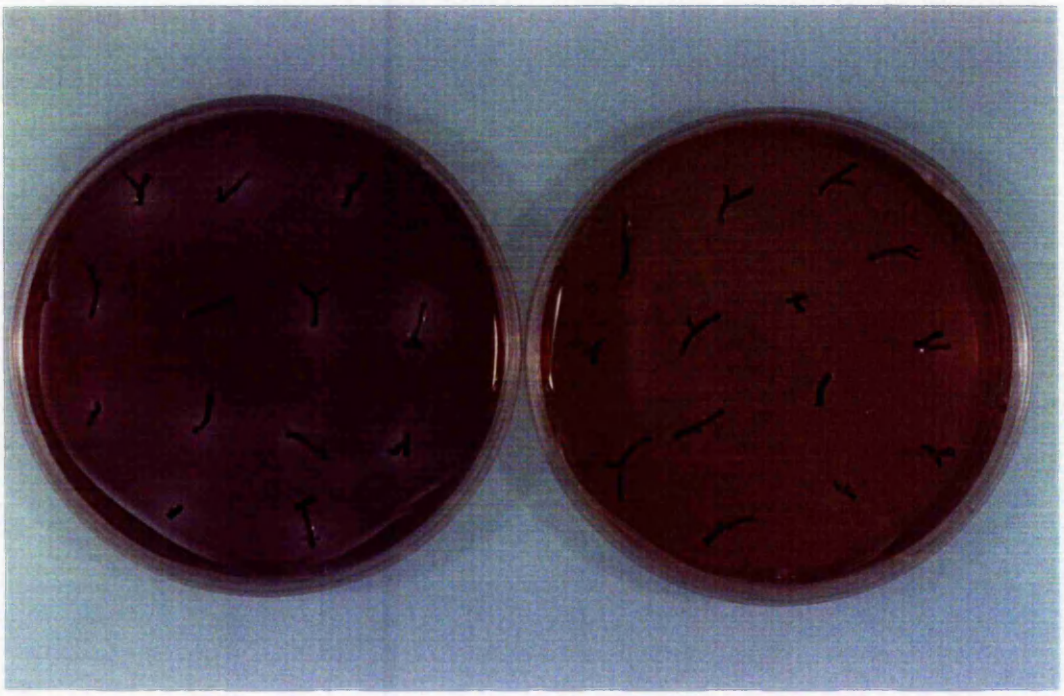


Plate 2.1: Screening for bacterial contamination in apical explants of spinach seedlings. The Petri dish (9 cm Ø) on the right contains healthy plant material, the dish on the left has contaminated plant material.

2.4 Discussion

Spinach tissue cultures could be initiated from germinating seeds, but initially this proved to be difficult due to heavy fungal and bacterial contamination of the seeds, especially of commercial seeds.

Ritchie *et al.* (1989) found that seed sterilisation in sugar beet, which is also a member of the *Chenopodiaceae*, was also problematic due to bacterial and fungal contamination. Immersion of sugar beet seeds in 15 % (v/v) 'Domestos' solution for two 15 min periods was found to be the most adequate, but, depending on the cultivar, only a limited number of sterile seedlings could be produced.

In the present study on spinach a sterilsation method has been developed using a 25 % (v/v) 'Domestos' solution for a single period of 20 min. This method offers a compromise between an acceptable high germination frequency and limited contamination. Fungal contamination was fully eliminated by the sterilisation method used, whereas bacterial infections still persisted. For the reduction of bacterial infection in mung bean seedlings, Tripepi and George (1991) soaked the seeds before sowing in a solution containing 10 mg l^{-1} tetracycline and 10 mg l^{-1} cephalexin for 30 min. Soaking in antibiotic solutions before sowing was not found to be successful for spinach seeds, but bacterial infection of the commercial spinach cultivars could be reduced by adding the antibiotic 'Baypen' to the germination medium. The treatment with 100 mg l^{-1} 'Baypen' in the germination medium was considered to be the most effective with the seeds being germinated at a temperature of 5 °C.

The seeds of the breeding lines proved to be less contaminated than the commercial seed material and bacterial infection was no immediate problem for the initiation of tissue cultures.

This study demonstrated, that before taking the spinach seedlings into culture, screening for any remaining bacterial infection was found to be essential. Ritchie *et al.* (1989) were able to identify bacterially infected apical explants of sugar beet by transfer to agar-solidified MS culture medium for 4 days. Bacterial infection is also a well known problem in tissue cultures of *Maranthacea*. Debergh (1988) suggested that the screening of tissues on a very rich medium, developed by Saglio *et al.* (1973), for the detection of bacterial contaminants, produced reliable results.

The screening method developed in this study, using MS culture medium supplemented with the pH-indicator Methyl Red, proved to be satisfactory for the detection of contaminated seedling explants. The change of temperature from 5°C to 25°C facilitated the detection of latent contamination. The basis for further experimental work involving tissue cultures of spinach was provided by access to sterile seedling material obtained by the application of an efficient sterilization and screening method as developed in the present Chapter.

CHAPTER 3

The culture of spinach (*Spinacia oleracea* L.)

3.1 Introduction

The establishment of spinach cultures provides the basis for clonal propagation and the development of a plant regeneration system. Apical and axillary meristems have been used to initiate regenerating cultures, with the aim of obtaining proliferating shoot cultures. It is well known that external factors, e.g. the medium and culture conditions, play an important role in the establishment of tissue cultures.

The objective of this chapter was to identify an appropriate combination of plant growth regulators in order to induce multiple axillary shoot growth for the establishment of a micropropagation system for spinach. In addition, external factors of light and temperature were investigated to determine the most suitable culture conditions for the propagation and maintenance of spinach cultures.

3.1.1 The influence of plant growth regulators on the establishment of cultures of spinach

Three major classes of growth promoting hormones are recognized. These are auxins, gibberellins and cytokinins. Auxins promote cell division and cell elongation. Gibberellins largely influence cell elongation and, in combination with auxins, control cell differentiation. Cytokinins promote cell division and have an impact on other physiological processes, such as senescence and apical dominance (Nultsch, 1982). Shoot and root initiation is controlled by the auxin-cytokinin balance. High auxin concentrations favour root initiation, while repressing shoot formation. Cytokinins aid shoot initiation and suppress rooting (Murashige, 1974).

Multiple shoot proliferation in most cultures is induced by a high cytokinin and low auxin ratio. For example Paek (1987) found that the addition of 46.5 µM kinetin and 1.5 µM IBA to MS-based culture medium induced shoot proliferation from axillary buds in chinese cabbage. IAA at 1.0 mg l^{-1} and 20 mg l^{-1} 2iP are used for the propagation of globe artichoke (Debergh, 1981). Griga (1994) applied 20 µM BAP and 0.1 µM NAA for the long-term micropropagation of *Pisum sativum* L. . *In vitro* shoot development of the *Prunus* rootstock GF 655-2 required 2.7 µM benzyladenine for the formation of axillary shoots (Baraldi *et al.*, 1988).

Within the *Chenopodiaceae*, **sugar beet** exhibited the best leaf production of seedling apices when established on MS culture medium supplemented with 1.0 µM BAP (Ritchie *et al.*, 1989). A potentially new crop plant, *Chenopodium quinoa*, is described by Burnouf-Radosevich (1988), which is micropropagated through shoot tip culture with 0.22 mg l^{-1} benzyladenine and 0.018 mg l^{-1} NAA.

Neskovic and Culafic (1988) reported that the culture of apical buds from spinach seedlings on hormone-free medium resulted in the production of elongated leafy shoots and even the addition of high cytokinin levels did not cause multiple branching. More recent reports on spinach do not deal with micropropagation, but rather with shoot regeneration from different explant sources. Regenerated shoots, however, were grown and rooted on hormone-free medium or on medium supplemented with 1.0 mg l^{-1} IBA (Al-Khayri, 1991a).

In this study, various combinations and concentrations of auxins and cytokinins have been tested in order to determine the most suitable combination of plant growth regulators for the induction of multiple axillary shoot production in spinach.

The objective of this study was to establish a micropropagation system for spinach so that a continuous source of plant material could be maintained for plant modification by somatic techniques. In addition, from the commercial point of view, an established micropropagation system is important for mass propagation and the maintenance of stable genetic lines.

3.1.2 The role of the growth factor temperature in the culture of spinach

Spinach is a cool season crop plant. Neskovic and Culafic (1988) describe field spinach to develop the most abundant foilage in spring or autumn at a temperature of 15°C to 18°C. Numerous experiments have been conducted on cold acclimation and photo-inhibition of spinach plants. Various authors, such as Guy (1985) or Fennel and Li (1985), found that the freezing tolerance in spinach was greatly increased by exposure to cold acclimating conditions.

Cultures of spinach were grown by Al-Khayri *et al.* (1991a,b) at a continuous temperature of 20°C or at a day/night temperature of 20°C/16°C. However, under field conditions, spinach is cultivated under low temperatures. Therefore, the application of low temperatures to tissue cultures of spinach may prove to be beneficial. In this study, three different temperatures were tested in order to determine improvements on spinach tissue cultures with regard to enhanced axillary shoot production and general plant quality.

3.1.3 The influence of photoperiod on the development of spinach in culture

Spinach is an obligate long-day plant with a critical daylength of 14 h. In early field experiments of this crop, the main focus of research was directed to the production of spinach hybrid seed and the external factors, which play an important role concerning the initiation of flowers and hence seed. Thompson (1954) found that under short day conditions of 8 h to 9 h daylength, spinach plants hardly elongated or produced flowers, whereas under a daylenght of 14 h or more the flower and seed production increased. Similar observations were made by Parlevliet (1968), who studied earliness in spinach cultivars, which coincides with the rate of flower formation. The light intensity did not interfere with earliness in the cultivars tested, but daylenght had an profound effect on increasing the flower formation.

Different approaches were used for *in vitro* cultures of spinach. Sasaki (1989) kept all spinach cultures under continuous light, whereas Al-Khayri *et al.* (1991a, 1992a) chose for both *in vitro* and *in vivo* cultures a daylength of 10 h; for flower induction the light duration was extended to 14 h.

In the present study, the objective was to maintain spinach cultures in the vegetative phase, thereby, allowing maximum production of axillary shoots which could result in an improved micropropagation system for this crop.

3.2 Materials and Methods

3.2.1 Growth medium and culture conditions

MS-based culture medium was used for all experiments. The MS nutrient salts and vitamins were used at full strength, supplemented with 30 g l^{-1} sucrose and solidified with 7.5 g l^{-1} Oxoid agar. The pH was adjusted to 6.2 prior to autoclaving (121 °C for 15 min). The antibiotic Baypen (Bayer, Newbury, UK) was filter sterilized and added to the culture medium at 40 °C to give a final concentration of 25 mg l^{-1}. All plant growth regulators used in the following experiments were also filter sterilized (Acrodisc 0.45 µm syringe filter, Gelman Sciences) and included in the growth medium at the required concentrations.

The spinach seeds were sterilized and germinated according to the protocol developed in Chapter 2. Stem apices, 10 mm in length, were excised from axenic seedlings and transferred to 100 ml capacity screw-capped polystyrene containers (Sterilin), with 15 ml of culture medium per vessel. Two shoot explants were cultured in each container. All cultures were grown under a 16 h photoperiod using 36 W White Pluslux 3500 fluorescent tubes giving 42 ± 14 µE $m^{-2} s^{-1}$ of irradiance at 25 °C.

After 42 d of culture, the plants were harvested and assessed for their height, number of leaves (including senescent leaves), the percentage of plants producing axillary shoots and number of axillary shoots per plant. Flower production, callus and root formation and symptoms of hyperhydricity were also recorded. Each experiment and treatments were conducted in latin square design.

3.2.2 The influence of kinetin in combination with various auxins on axillary shoot production in spinach varieties

I. Kinetin in combination with IBA

Breeding Lines A, B, D, E and the commercial variety Medania were used for the following test. The applied concentrations of plant growth regulators were 1.0, 5.0 or 10.0 µM kinetin combined with 1 µM IBA. Control plants were grown without plant growth regulators.

The number of plants per treatment was as follows: Breeding Lines A: n = 26, B: n = 20, D: n = 29, E: n = 32; Medania: n = 13.

ii. Kinetin in combination with IAA

Breeding Lines A and B were tested with a combination of kinetin and IAA. The applied concentrations of kinetin were 0, 1.0, 2.5 and 5.0 µM; the concentrations of IAA were 0 and 1.0 µM. The culture containers used in this experiment were 60 ml capacity screw-capped polystyrene containers (Sterilin). The number of plants (n) for each breeding line and treatment was 12.

iii. Kinetin in combination with NAA

Kinetin in combination with NAA was tested only on Breeding Line A. The concentrations of kinetin were 0, 1.0, 2.5 and 5.0 µM; the concentrations of NAA tested were 0, 0.5, 1.0 and 1.5 µM. Eighteen plants were used per treatment.

3.2.3 The influence of BAP combined with TIBA, IAA or NAA on axillary shoot production from spinach

i. BAP in combination with TIBA

This hormone combination was tested on Breeding Lines A, B, D and E. BAP was applied at concentrations of 0, 0.1, 0.5 1.0 and 2.0 µM and the TIBA concentrations were 0, 0.5 or 1.0 µM. The number of plants (n) for each breeding line and treatment was 10.

ii. BAP in combination with IAA

Only Breeding Line E was submitted to various combinations of BAP at 0, 1.0, 2.5 and 5.0 µM and IAA at 0, 0.5 and 1.0 µM. The number (n) of plants per treatment was 18.

iii. BAP in combination with NAA

BAP and NAA were tested on Breeding Line A. BAP was applied at 0, 1.0, 2.5 and 5.0 µM; NAA was used at 0, 0.5 or 1.0 µM. Sixteen plants were used per treatment.

3.2.4 The influence of 2iP in combination with NAA on axillary shoot production of spinach

The concentrations of 2iP tested were 0, 1.0, 2.5 or 5.0 µM. NAA was added to the medium at 1.0 µM or was omitted from the medium. These concentrations were tested only on Breeding Line E. Twenty two plants were used per treatment.

3.2.5 The influence of NAA on the growth and development of spinach Breeding Lines A and E

I. Breeding Line A

In this experiment, the NAA concentrations tested were 0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 µM, with 12 plants per treatment.

After 6 weeks of culture, axillary shoots were excised and transferred to fresh MS-based medium supplemented with 1.0 µM NAA. The plants were grown to maturity over an 8 week period under the same conditions as described above. Other parameters assessed were axillary shoot production, the formation of inflorescences and seed set.

II. Breeding Line E

The NAA concentrations applied were 0, 0.25, 0.5, 0.75, 1.0, 2.5 and 10.0 µM, with 18 plants per treatment.

Axillary shoots were excised after 6 weeks of culture and transferred to MS-based culture medium containing 1.0 µM NAA. The plants were grown to maturity over a culture period of 8 weeks and further axillary shoot production, formation of inflorescences and seed set were assessed.

3.2.6 The influence of IAA on Breeding Line A

The tested concentrations of IAA were 0, 0.5, 0.75 and 1.0 µM, with 21 plants per treatment.

3.2.7 The influence of temperature on the development of spinach *in vitro*

I. Culture conditions

Breeding Line E and the commercial cultivar 'Longstanding' were used to determine the most suitable temperature for *in vitro* grown spinach. MS-based culture medium, as described in 3.2.1, was used to establish the axenic cultures from seedling shoot tips. The growth medium was supplemented with 1.0 µM NAA.

The temperature regimes tested were 5°C, 15°C and 25°C. The cultures were grown in incubators under a 16 h photoperiod using 36 W Hybec White fluorescent tubes, giving an irradiation of 45 ± 10 µE $m^{-2}s^{-1}$. This experiment was repeated twice with 24 replicates in each treatment.

The development of leaves and plant height was monitored over a growth period of 6 weeks. Leaf area, fresh weight, root and flower formation and chlorophyll content were assessed after a culture period of 6 weeks.

II. Determination of the chlorophyll content

Chlorophyll content was determined after a modified method used by Lichtenthaler and Wellburn (1983). Before the leaves were used for the pigment assay, the leaf area was measured with a leaf area meter (Delta-T-Devices, Cambridge, UK). The leaves were then cut lengthways and placed in 5 ml of 80 % (v/v) acetone in McCartney bottles to extract the chlorophyll. The McCartney bottles were kept for 24 h at 5°C in the dark. The plant extracts were used undiluted and measured by spectrophotometry (Cecil, Cambridge, UK). The readings for chlorophyll a were taken at a wavelength of 663 nm and for chlorophyll b at a wavelenghts of 645 nm.

The amount of chlorophyll per leaf area was calculated using the following formula (Lichtenthaler and Wellburn, 1983):

$$\text{Chl a+b} = (Y \times v/l) \; / \; \text{mm}^2 \text{ leaf area}$$

$$Y = \begin{cases} \text{Chl a } (\mu g\ ml^{-1}) = 12.21\ A_{663} - 2.81\ A_{645} \\ \text{Chl b } (\mu g\ ml^{-1}) = 20.13\ A_{645} - 5.03\ A_{663} \end{cases}$$

v = extraction volume

l = pathway of cuvette (=1cm)

3.2.8 The influence of light on the growth of spinach *in vitro*

Breeding Line A and the commercial variety 'Virkade' were used to investigate the optimum photoperiod for the growth of spinach *in vitro*.

Cultures from seedling shoot tips were established on MS-based medium as described in 3.2.1. The growth medium was supplemented with 5.0 µM BAP.

The cultures were maintained in incubators at 25°C under 3 different photoperiodic regimes using 36 W Hybec White fluorescent tubes, giving an irradiation of 45 ± 10 $\mu E\ m^{-2}s^{-1}$. The periods were 12 h, 14 h and 16 h.

This experiment was conducted with 26 replicates in each treatment for Breeding Line A and with 21 replicates in each treatment for 'Virkade'. After 6 weeks of culture, the number of leaves, fresh weight, number of axillary shoots produced and number of flowers formed, were determined.

3.2.9 Statistical analysis

The data obtained for plant height, number of leaves and number of axillary shoots per plant for all treatments were tested for significant differences by analysis of variance. Individual treatments were compared with one another by application of the 2-sample T-test for confidence limits of 5.0 % (*), 1.0 % (**) and 0.1 % (***).

The Chi-square test was chosen for establishment of significant differences between values expressed as percentages. Values for the least significant differences were computed for confidence limits of 5.0 % (*), 1.0 % (**) and 0.1 % (***).

3.3 Results

3.3.1 The influence of kinetin, in combination with different auxins, on axillary shoot production of spinach varieties

I. Kinetin in combination with IBA

The plant height of both breeding lines and the commercial cultivar was clearly affected by IBA and kinetin. Figure 3.1 shows that the plant height was significantly greater on culture medium devoid of growth regulators. Plant height was reduced by culture on medium supplemented with IBA and kinetin. Higher concentrations of 5.0 µM and 10.0 µM kinetin resulted in retarded growth in all varieties.

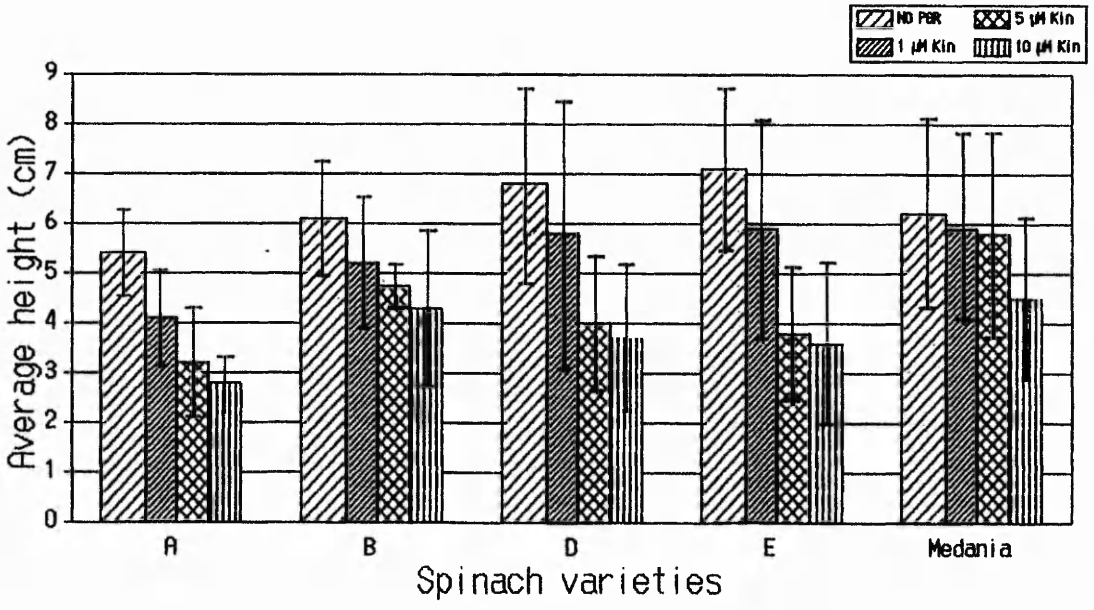


Figure 3.1: Average plant height of Breeding Lines A, B, D, E and the commercial cultivar 'Medania' achieved with 1.0 µM IBA combined with various concentrations of kinetin (mean values ± SD).

Plants produced in culture on medium supplemented with growth regulators were characterized by very short internodes, long petioles and a rosette type of growth, whereas plants cultured on hormone-free medium had long internodes and short petioles (Plate 3.1).

The effect of different levels of kinetin on axillary shoot production varied greatly between the individual spinach varieties. The control and 1.0 µM kinetin treatment induced significantly more axillary shoots in Breeding Lines A and D than the 5.0 µM or 10.0 µM treatments, whereas the 1.0 µM kinetin treatment produced the least axillary shoots in Breeding Line E and 'Medania' (Table 3.1). The results for Breeding Line B were not very conclusive because of plant death, due to recurring bacterial infection, during the course of the experiment.

The development of inflorescences commenced after 6 weeks in culture. With increasing kinetin levels, more callus was produced at the stem bases and the degree of hyperhydricity also increased.

Table 3.1: Influence of kinetin on the percentage of axillary shoots produced by spinach cultivars.

	w/o PGR	1.0 µM KIN	5.0 µM KIN	10 µM KIN
Cultivar				
A	55.26 *	53.85 *	30.43	33.33
B	42.38	37.50	0.00	30.00
D	58.21 *	62.96 *	16.67	36.00
E	46.52	29.73	32.14	51.72
Medania	63.48 *	15.38	61.54 *	65.81 *

w/o PGR - lacking plant growth regulators; * = P(0.05)

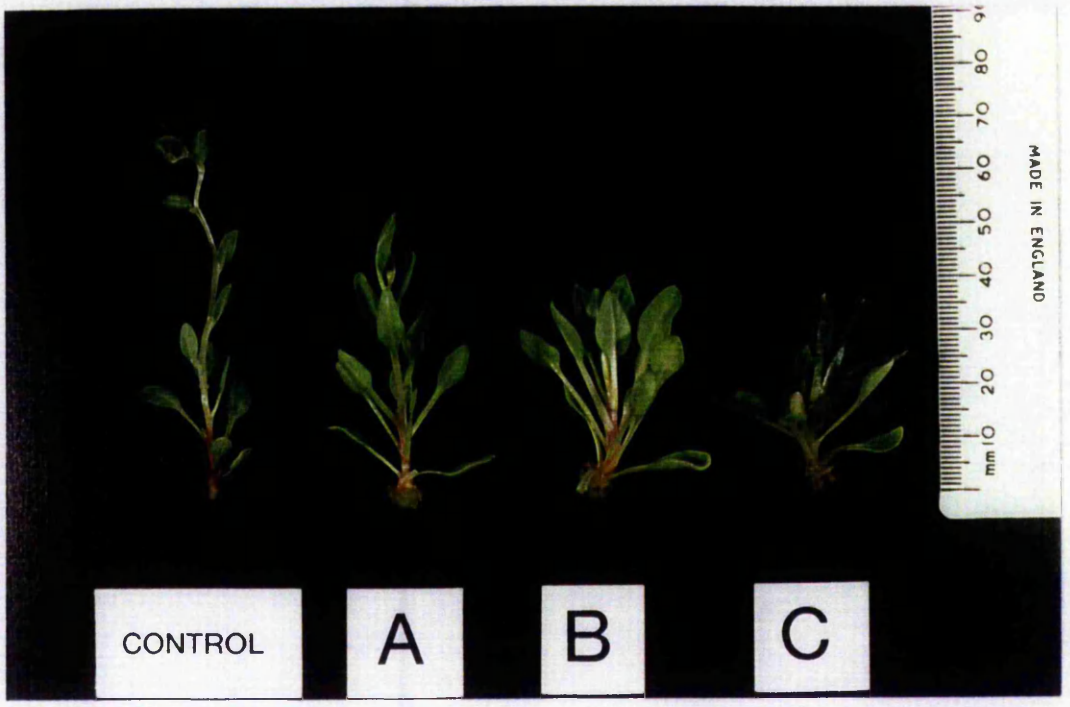


Plate 3.1: Breeding Line E cultured on MS-based culture medium devoid of plant growth regulators (control) and on culture medium containing 1.0 µM IBA combined with 1.0 µM (A), 5.0 µM (B) or 10 µM (C) kinetin.

ii. Kinetin in combination with IAA

Plant height and growth characteristics of Breeding Lines A and B were influenced by increasing amounts of kinetin in a similar way as described in the previous experiment. The retarding effect of kinetin was reduced by IAA included in the medium. The combination of 1.0 µM IAA with 0 or 1.0 µM kinetin produced larger plants than the same level of IAA in combination with 2.5 µM or 5.0 µM kinetin.

The number of leaves produced in both breeding lines was not affected by any hormone combination in comparison to the control plants. However, leaf senecence was significantly enhanced in the presence of IAA in the culture medium (Figures 3.2 and 3.3).

The highest percentage of plants producing axillary shoots was found on a medium containing 1.0 µM IAA only or on medium supplemented with 1.0 µM IAA and 1.0 µM kinetin. This response was found in both breeding lines, although Breeding Line A being more responsive to the applied treatments than Breeding Line B (Table 3.2). Only 1 or 2 shoots per plant were produced, when axillary shoot production occurred.

Table 3.2: The influence of kinetin and IAA on the percentage of plants of Breeding Lines A and B producing axillary shoots.

Breeding Line A

Kinetin (µM)	0	1.0	2.5	5.0
IAA (µM)				
0	0.00	0.00	9.09	7.27
1	54.55 *	72.72 *	0.00	18.18

* = P(0.05)

Breeding Line B

Kinetin (µM)	0	1.0	2.5	5.0
IAA (µM)				
0	0.00	0.00	0.00	0.00
1	37.50	37.50	10.00	25.00

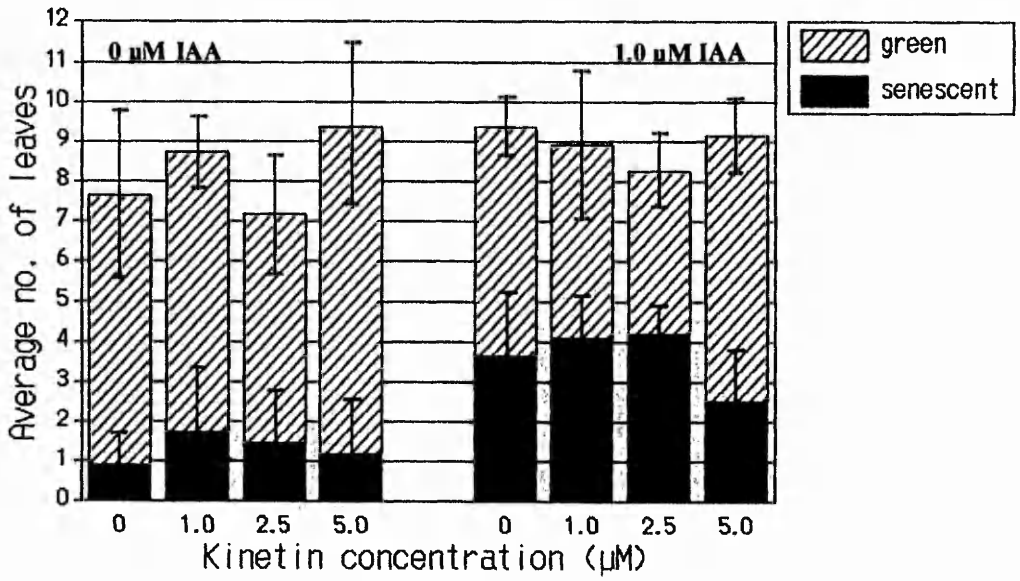


Figure 3.2: The influence of kinetin and IAA on leaf production and leaf senescence of Breeding Line A (mean values ± SD).

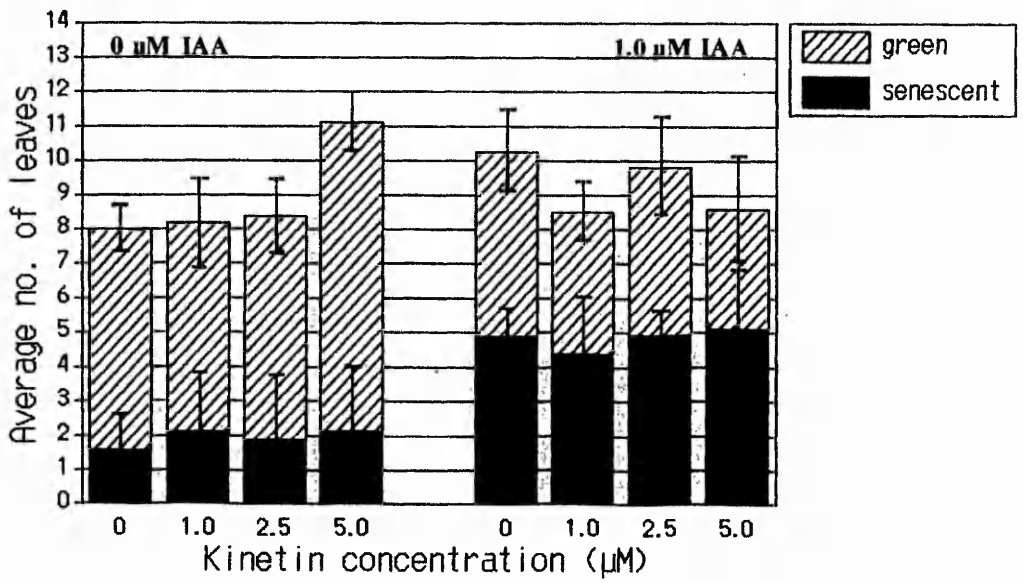


Figure 3.3: The influence of kinetin and IAA on leaf production and leaf senescence of Breeding Line B (mean values ± SD).

iii. Kinetin in combination with NAA

Plant height of Breeding Line A was found to be greatest in kinetin treatments in combination with 0.5 µM or 1.0 µM NAA. No significant differences were be obtained between treatments, when kinetin was included in the culture medium. Plant height was significantly greater when the plants were grown on kinetin-free medium containing 0.5 µM or 1.0 µM NAA in comparison to any other treatment, as demonstrated in Figure 3.4. Plant height was reduced in each treatment when 1.5 µM NAA was added to the culture medium.

Most axillary shoots were produced by plants grown on medium containing 0.5 µM NAA, with 46.67 % of the plants producing axillary shoots, or on medium containing 0.5 µM NAA and 1.0 µM kinetin, with 42.8 % of the plants developing axillary shoots. Any combination of the highest hormone concentrations did not stimulate any axillary shoot production in cultured spinach explants (Table 3.3). The number of axillary shoots ranged between 1 and 3 shoots per plant.

Table 3.3: The influence of kinetin and NAA on the percentage of plants producing axillary shoots.

Kinetin (µM)	0	1.0	2.5	5.0
NAA (µM)				
0	0.00	33.33	20.00	0.00
0.5	46.67 *	42.80 *	8.30	0.00
1.0	33.33	14.29	0.00	0.00
1.5	0.00	0.00	0.00	0.00

* = P(0.05)

Plate 3.2 illustrates the influence of the various hormone combinations on the development of the plants. An unusual observation was the production of large amounts of green hard stem based callus when the plants were submitted to 2.5 µM or 5.0 µM kinetin in any combination with NAA. Roots developed on culture medium containing only NAA.

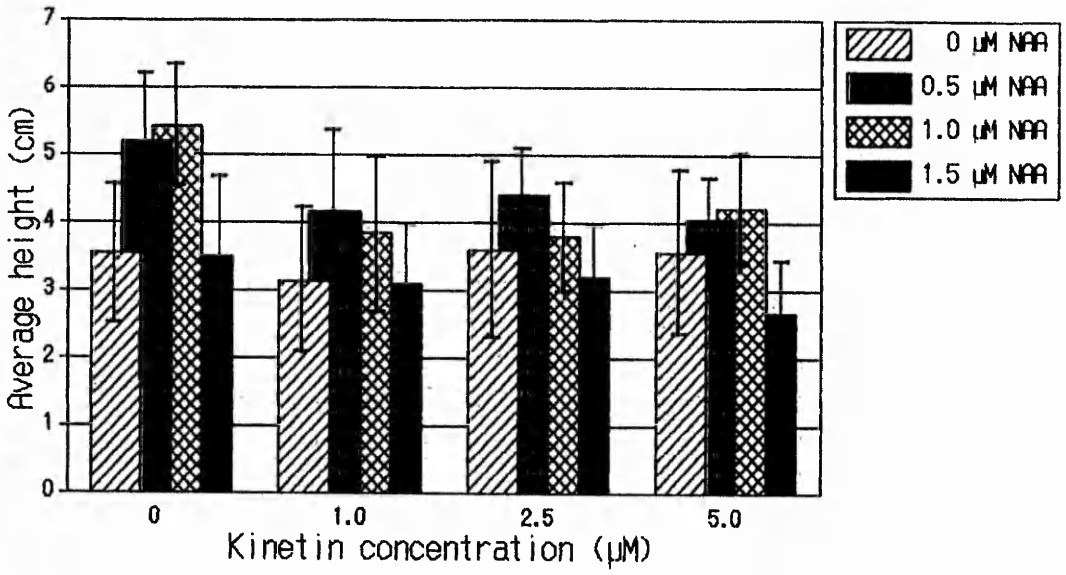


Figure 3.4: The effect of kinetin and NAA on the plant height of Breeding Line A (mean values ± SD).

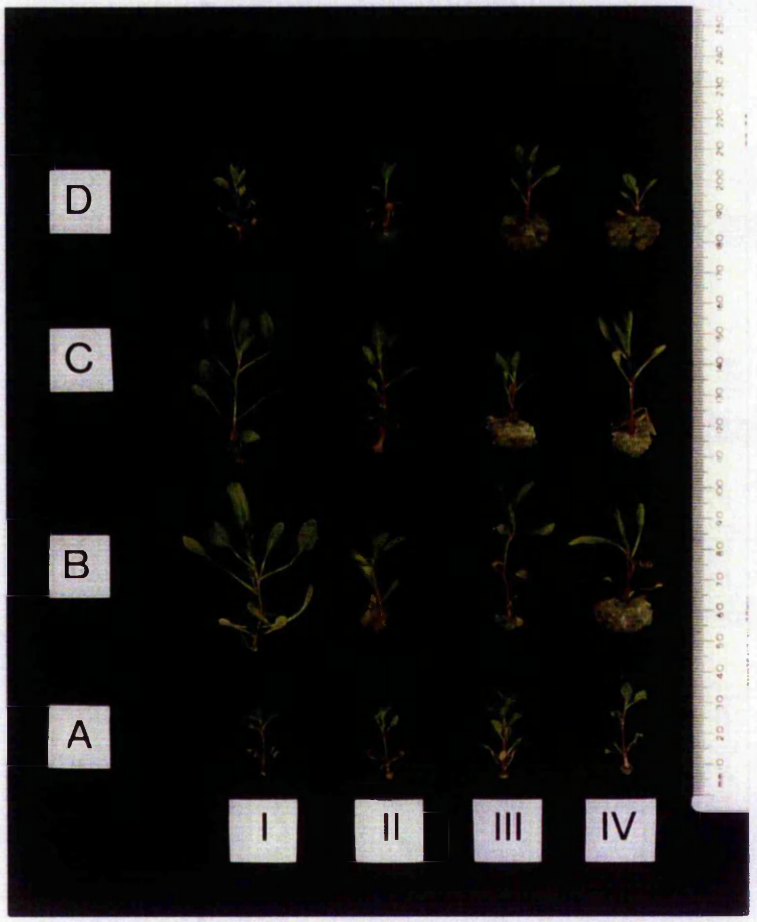


Plate 3.2: The influence of kinetin and NAA on the development of Breeding Line A.

Key to treatments: A = 0 µM NAA I = 0 µM kinetin
B = 0.5 µM NAA II = 1.0 µM kinetin
C = 1.0 µM NAA III = 2.5 µM kinetin
D = 1.5 µM NAA IV = 5.0 µM kinetin

3.3.2 The influence of BAP, in combination with different auxins, on axillary shoot production of spinach

i. BAP in combination with TIBA

This hormone combination was applied to all breeding lines. Most plants exhibited hyperhydric characteristics and a very high percentage of the plants died under the BAP-TIBA treatment. Because of the poor plant quality produced, TIBA was excluded from any further experimentation.

ii. BAP in combination with IAA

The effect of BAP on the plant height of Breeding Line E when grown on 2.5 µM BAP and 5.0 µM BAP was very different to that found in other treatments and both produced significantly smaller plants than the 1.0 µM BAP treatment and very significantly smaller plants than those grown without BAP. The only interaction between IAA and BAP concentrations were found with 1.0 µM BAP. In combination with 0.5 µM IAA, significantly smaller plants were produced than in combination with 0 µM IAA or 1.0 µM IAA (Figure 3.5).

The total number of leaves produced was not influenced by either BAP or IAA. However, the number of leaves exhibiting senescence increased when BAP was added to the culture medium and was increased significantly when IAA and BAP were present in combination (Figure 3.6).

A change of the plant growth character was seen to be influenced by the various hormone combinations. BAP broke the apical dominance and shortened the petioles. Increasing concentrations of BAP produced large amounts of callus at the stem base, whereas on medium containing only IAA, root development was initiated (Plate 3.3). Most axillary shoots, however, were produced by plants grown without BAP in the culture medium (Table 3.4). Seventy seven % of the plants grown on medium containing 1.0 µM IAA developed significantly more axillary shoots than those influenced by any other hormone treatment. The average number of axillary shoots did not exceed 3 per plant. The percentage of plants forming inflorescences was also influenced by the applied hormone combinations. One µM IAA or 1.0 µM IAA combined with 1.0 µM BAP stimulated significantly more inflorescences than other hormone combinations (Table 3.5).

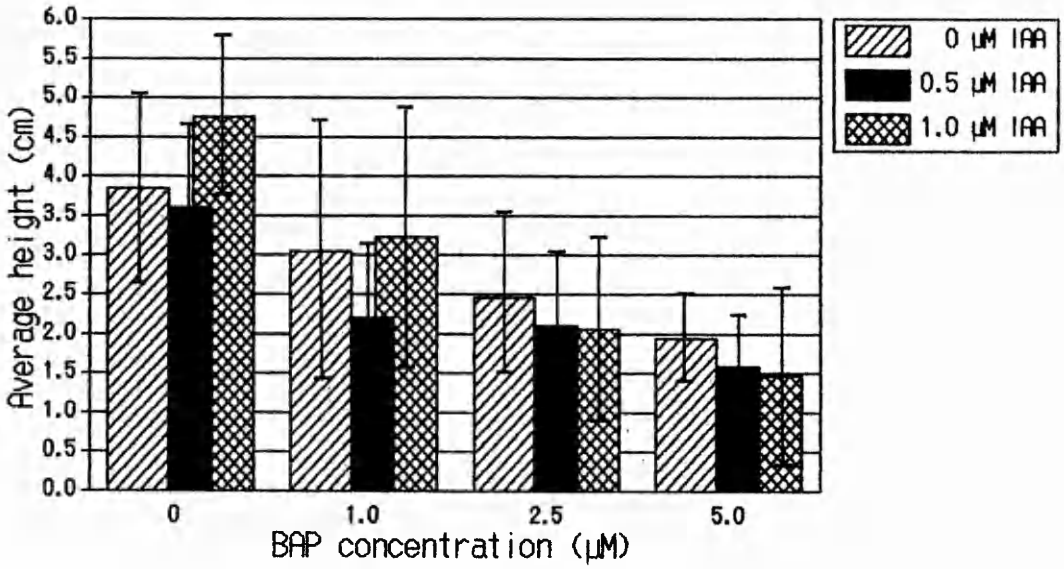


Figure 3.5: The influence of various concentrations of BAP and IAA on the height of Breeding Line E (mean values ± SD).

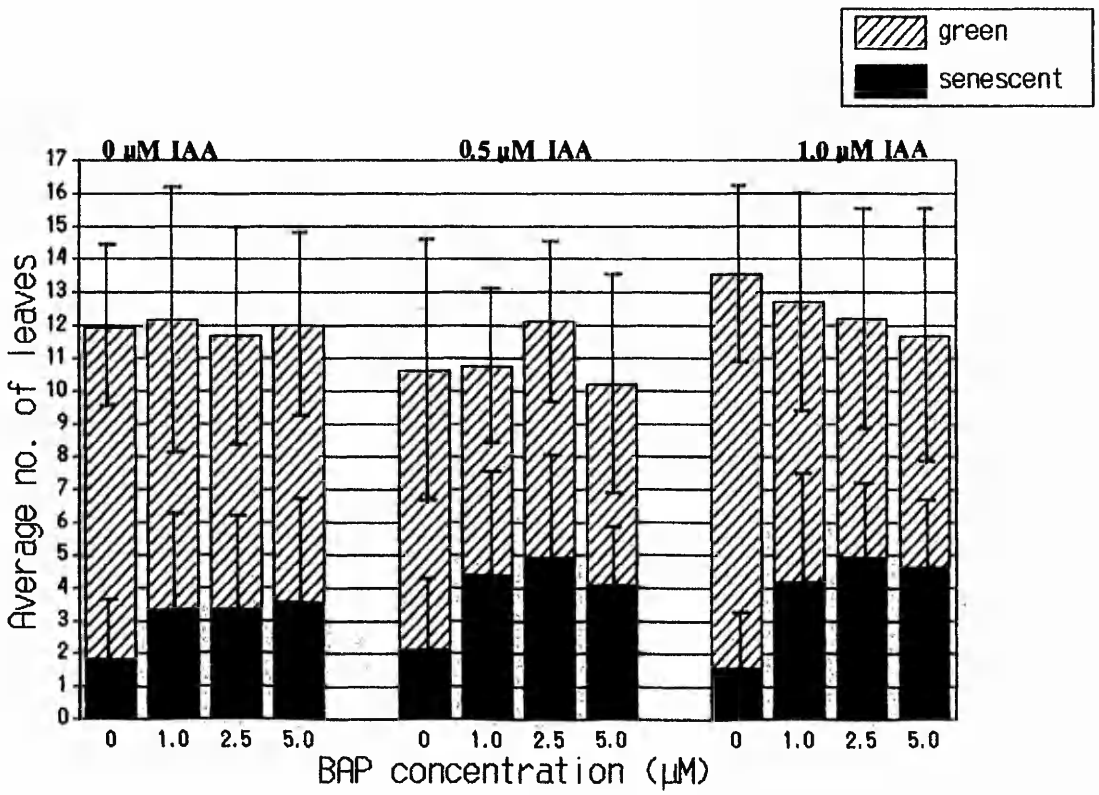


Figure 3.6: The effect of BAP and IAA on leaf production and leaf senescence of Breeding Line E (mean values ± SD).

Table 3.4: The influence of BAP and IAA on the percentage of plants of Breeding Line E producing axillary shoots.

BAP (µM)	0	1.0	2.5	5.0
IAA(µM)				
0	56.25	37.50	18.75	11.11
0.5	33.33	20.00	0.00	0.00
1.0	77.77 *	11.76	0.00	0.00

* = P(0.05)

Table 3.5: The influence of BAP and IAA on the percentage of plants of Breeding Line E producing inflorescences.

BAP (µM)	0	1.0	2.5	5.0
IAA (µM)				
0	75.00	68.75	50.00	38.80
0.5	80.00	53.33	31.25	22.22
1.0	100.00 *	88.20 *	62.50	37.50

* = P(0.05)

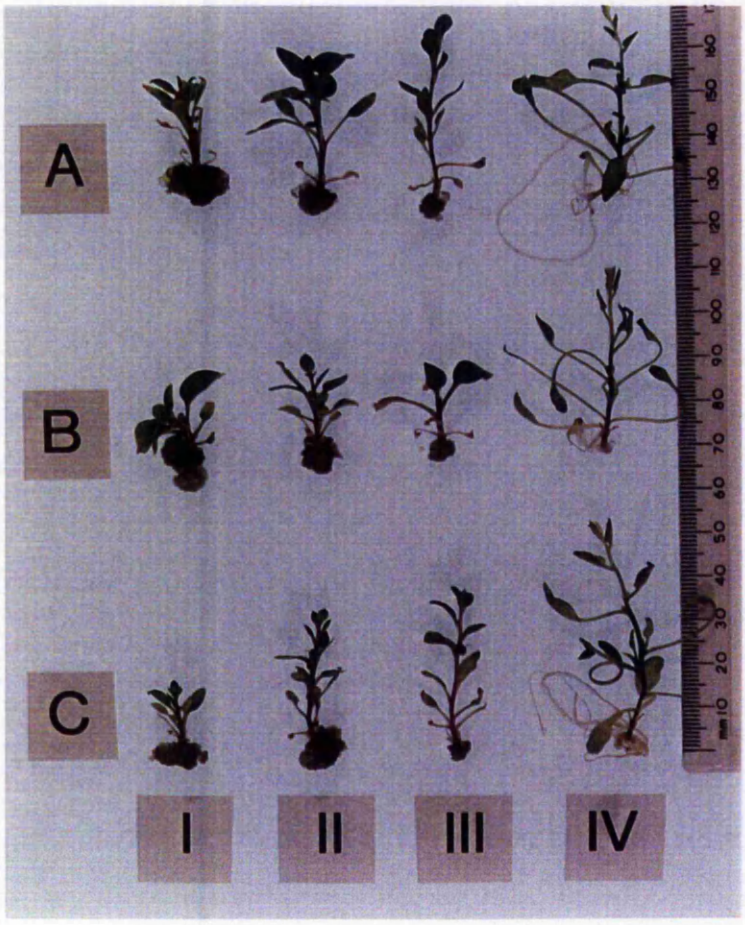


Plate 3.3: The influence of BAP and IAA on the development of Breeding Line E.

Key to treatments: A = 0 µM IAA
B = 0.5 µM IAA
C = 1.0 µM IAA

I = 5.0 µM BAP
II = 2.5 µM BAP
III = 1.0 µM BAP
IV = 0 µM BAP

iii. BAP in combination with NAA

The influence of BAP and NAA on plant development of cultured explants of Breeding Line A followed typically that found in the previous experiment combining BAP and IAA. Plant height decreased with increasing BAP levels and also with increasing BAP the amount of stem base callus was enlarged.

With regard to hyperhydricity, Breeding Line A seemed more susceptible to BAP than Breeding Line E. The percentage of plants showing symptoms of hyperhydricity increased the more BAP was included into the culture medium (Table 3.6); 5.0 µM BAP caused significantly the highest percentage of hyperhydric plants.

The greatest amount of axillary shoots were produced on culture medium containing 0.5 or 1.0 µM NAA or 1.0 µM NAA and 1.0 µM BAP. The growth of axillary shoots was suppressed at the high levels of BAP (Table 3.7).

Table 3.6: Percentage of plants showing symptoms of hyperhydricity.

BAP (µM)	0	1.0	2.5	5.0
NAA (µM)				
0	0.00	10.00	14.30	33.30 *
0.5	0.00	0.00	18.20	88.80 **
1.0	0.00	8.30	14.30	35.70 *

* = P(0.05); ** = P(0.01)

Table 3.7: The influence of NAA and BAP on the percentage of plants of Breeding Line A producing axillary shoots.

BAP (µM)	0	1.0	2.5	5.0
NAA (µM)				
0	7.10	0.00	0.00	0.00
0.5	30.80 *	9.10	0.00	0.00
1.0	44.40 *	30.00 *	12.50	0.00

* = P(0.05)

3.3.3 The influence of 2iP and NAA on the axillary shoot production of Breeding Line E

Plants grown on medium devoid of NAA and supplemented with 2iP attained a height between 2.0 and 2.5 cm. Plants grown on 1.0 µM NAA, or on 1.0 µM NAA combined with 1.0 µM 2iP, were very significantly larger than the plants grown on the other hormone combinations (Figure 3.7).

Other characteristics observed, e.g. axillary shoot production, formation of inflorescences or hyperhydricity, are shown in Table 3.8. A significantly higher percentage of plants produced axillary shoots and formed inflorescences when cultured on medium without plant growth regulators or containing only 1.0 µM NAA. Increasing the amount of 2iP in the culture medium resulted in plants become more hyperhydric and encouraged the production of large amounts of stem base callus (Plate 3.4).

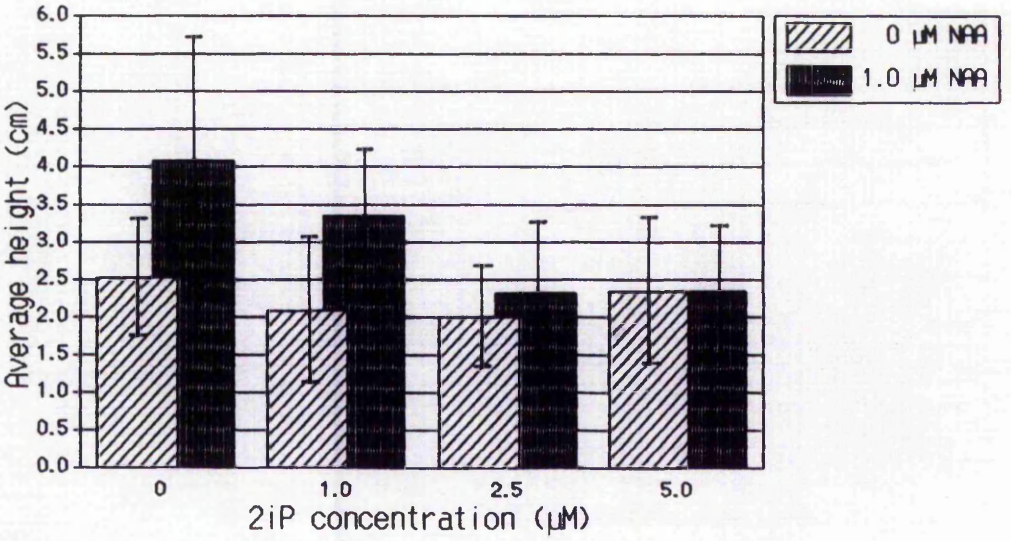


Figure 3.7: The influence of 2iP and NAA on the plant height of Breeding Line E (mean values ± SD).

Table 3.8: The influence of 2iP and NAA on the percentage of plants producing axillary shoots, inflorescences and showing symptoms of hyperhydricity.

2iP (µM) + NAA (µM)		Axillary shoots (%)	Inflorescences (%)	Hyperhydricity (%)
0	0	33.30 *	72.20 *	0.00
1.0	0	25.00	35.00	15.00
2.5	0	27.30	18.20	13.60
5.0	0	10.00	10.00	25.00
0	1.0	47.60 **	66.60 *	0.00
1.0	1.0	27.70	61.10	11.10
2.5	1.0	27.70	38.80	27.30
5.0	1.0	9.10	22.70	38.80

* = P(0.05); ** = P(0.01)

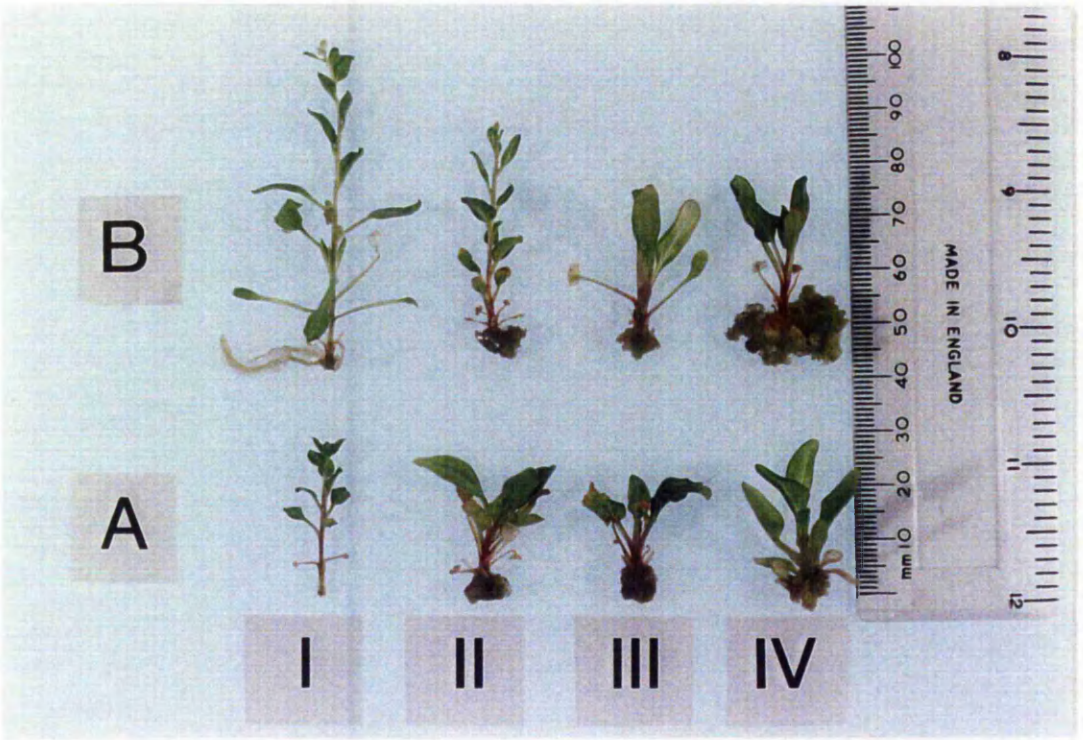


Plate 3.4: The influence of 2iP in combination with NAA on the development of Breeding Line E.

Key to treatments: A = 0 µM NAA
B = 1.0 µM NAA
I = 0 µM 2iP
II = 1.0 µM 2iP
III = 2.5 µM 2iP
IV = 5.0 µM 2iP

3.3.4 The influence of NAA on the growth of Breeding Lines A and E

I. Results for Breeding Line A

The plant height of Breeding Line A was significantly reduced in comparison to the other treatments, when plant growth regulators were omitted from the medium, or when 5.0 µM or 10.0 µM NAA were added to the culture medium (Figure 3.8).

The percentage of cultured plants of Breeding Line A producing axillary shoots and the mean number of lateral shoots produced are shown in Table 3.9. More plants (80 - 90.9 %) produced axillary shoots when 0.5 µM or 1.0 µM NAA were included in the growth medium. The number of axillary shoots per plant was largely the same when the applied NAA concentrations were between 0 and 2.5 µM, but significantly decreased at the higher concentrations of 5.0 or 10.0 µM NAA.

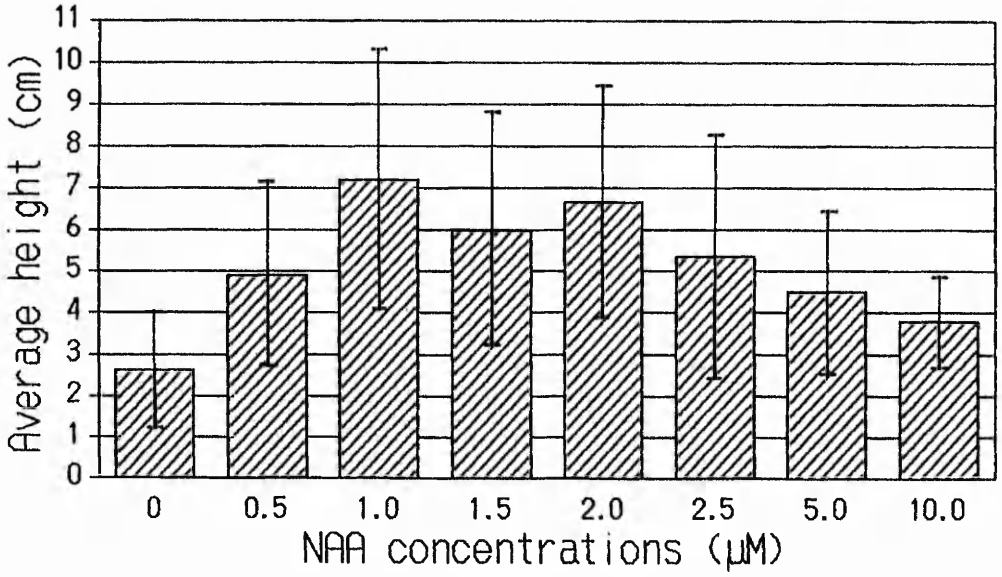


Figure 3.8: Effect of NAA on the plant height of Breeding Line A (mean values ± SD).

Table 3.9: Percentage of plants producing axillary shoots and average number of lateral shoots per plant.

NAA (µM)	Axillary shoot production (%)	Mean no. of ax. shoots/plant (mean values ± SD)
0	56.25	2.13 ± 0.64
0.5	80.00 **	2.00 ± 0.73
1.0	90.90 **	2.30 ± 1.25
1.5	54.55	2.83 ± 1.17
2.0	60.00	2.56 ± 0.73
2.5	66.67	2.25 ± 0.75
5.0	63.36	1.71 ± 0.95
10.0	11.11	1.00 ± 0.00

** = P(0.01)

Inflorescences were formed by 50 % of the plants, regardless of the NAA concentration. Treatments with 10 µM NAA, however, caused 75 % of the plants to flower. Between 75 % and 92 % of all plants developed roots, but no significant differences regarding the root formation were detected between the individual NAA treatments. Plate 3.5 illustrates plant development under the influence of the various NAA concentrations.

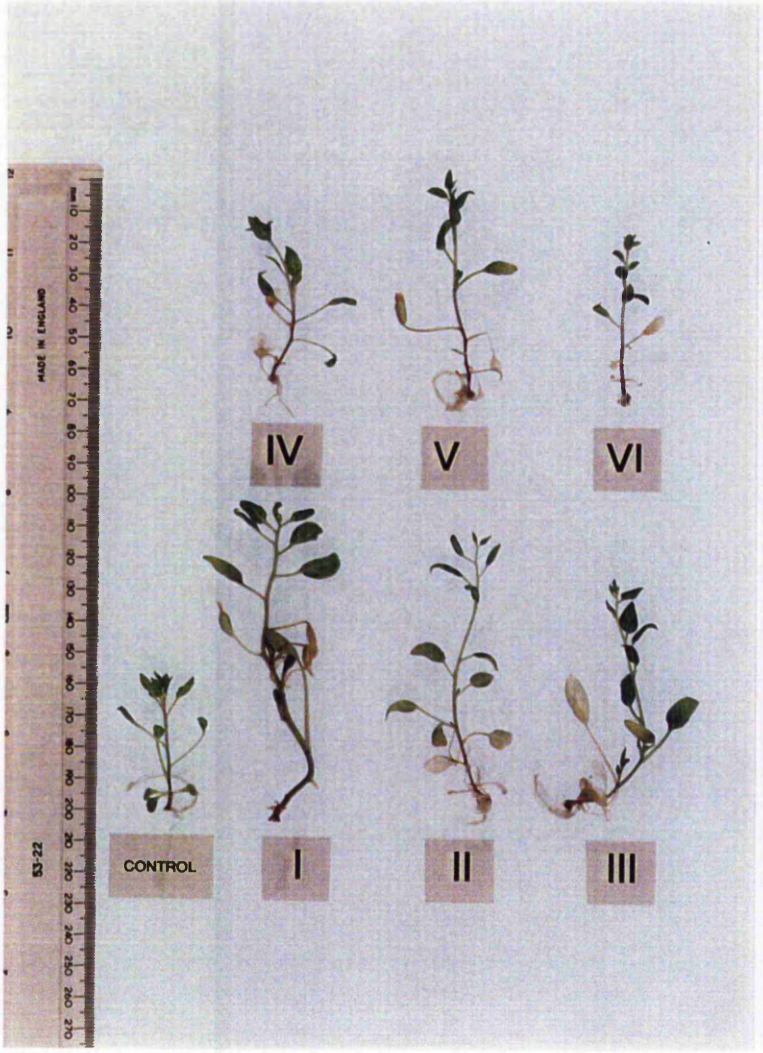


Plate 3.5: The influence of NAA on the development of Breeding Line A.

Key to treatments: I = 1.0 µM NAA
II = 1.5 µM NAA
III = 2.0 µM NAA
IV = 2.5 µM NAA
V = 5.0 µM NAA
VI = 10.0 µM NAA

Axillary shoots were harvested from cultures and within 8 weeks grown to maturity on MS-based culture medium supplemented with 1.0 µM NAA. The plants grown previously on medium without any auxin or on medium supplemented with 0.5 µM or 1.0 µM NAA exhibited the greatest plant height and most proliferation of axillary shoots. These 3 treatments also gave rise to significantly more plants setting seed than any of the other treatments. Significantly more seed set was achieved by plants cultured previously on medium containing 0.5 µM NAA or 1.0 µM NAA, than by plants grown on medium without plant growth regulators or on medium previously containing 5.0 µM NAA. Table 3.10 presents the percentage of plants producing axillary shoots, inflorescences and the average number of produced seeds per plant. Plate 3.7 A shows a plant of Breeding Line A producing seeds. This second generation of plants developed only 1 - 2 shoots, when axillary shoot production occurred.

Table 3.10: Percentage of 2nd generation plants of Breeding Line A producing axillary shoots, inflorescences and average number of set seeds per plant.

Previous NAA-conc. (µM)	Ax. shoots(%)		Inflorescences (%)		Mean no. of seeds/plant (mean values ± SD)
0	53.85	*	100.00	*	2.56 ± 1.24
0.5	71.43	*	85.71	*	4.75 ± 3.30
1.0	55.57	*	78.57	*	5.13 ± 3.52
1.5	28.57		42.86		3.33 ± 0.58
2.0	31.25		43.75		3.00 ± 2.00
2.5	26.09		56.52		3.27 ± 2.10
5.0	33.33		58.33		2.00 ± 1.09

* = P(0.05)

II. Results for Breeding Line E

The influence of NAA on the plant height of Breeding Line E is demonstrated in Figure 3.9. Plants grown on medium containing 0.75 or 1.0 µM NAA were significantly higher than the plants cultured on medium lacking plant growth regulators or on medium supplemented with 0.25, 0.5 or 10.0 µM NAA, respectively.

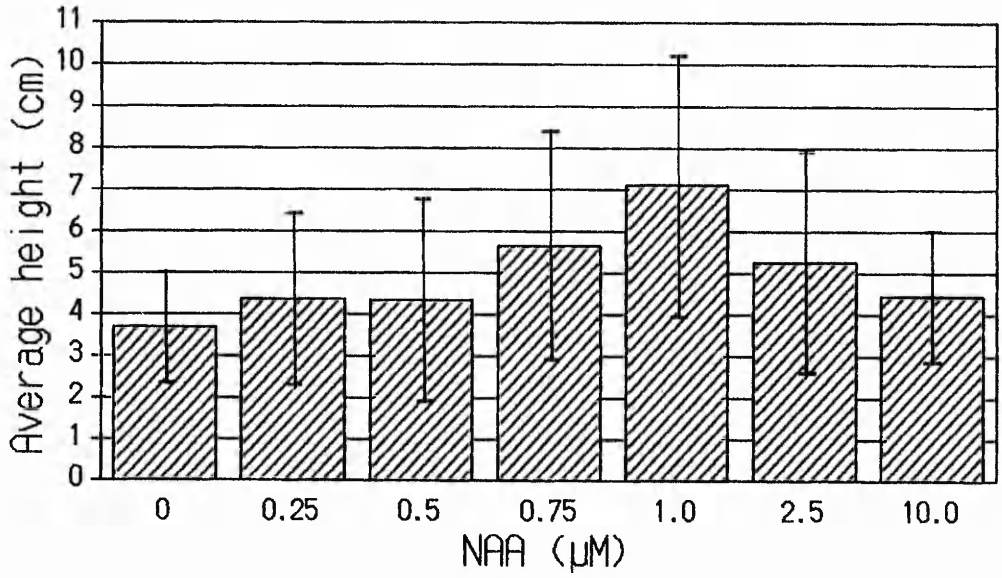


Figure 3.9: Effect of NAA on the plant height of Breeding Line E (mean values ± SD).

No significant differences were obtained for axillary shoot production from shoots cultured on medium containing various amounts of NAA, except for the 10.0 µM NAA treatment, in which significantly less plants formed lateral shoots. The same result was found with respect to root growth. Plants grown on medium containing 1.0 µM NAA expressed the highest percentage of axillary shoot production (70.59 %) and root development (76.47 %).

Although 53.85 % of the plants grown on medium without NAA produced axillary shoots, the average number of axillary shoots per plant of 1.43 was significantly less than the average number achieved by any other treatment. No statistical differences between the other treatments could be detected (Table 3.11).

At the time of assessment, approximately 50 % of the plants in each treatment started to produce inflorescences. Plate 3.6 shows plants of Breeding Line E developing under the influence of various NAA concentrations.

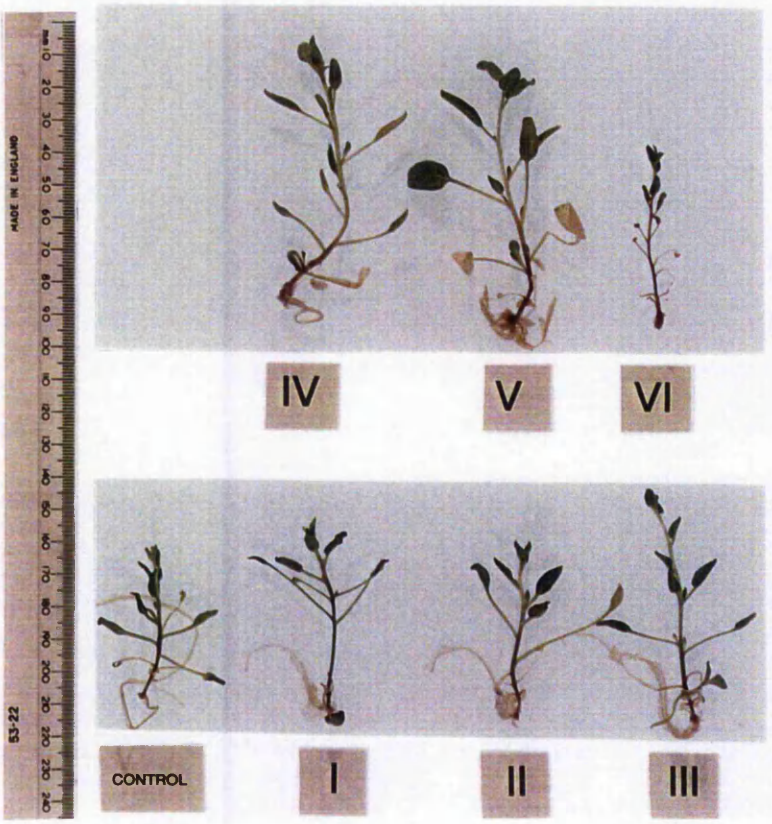


Plate 3.6: The influence of NAA on the development of Breeding Line E.

Key to treatments: I = 0.25 µM NAA

II = 0.5 µM NAA

III = 0.75 µM NAA

IV = 1.0 µM NAA

V = 2.5 µM NAA

VI = 10.0 µM NAA

Table 3.11: Percentage of plants producing axillary shoots and roots and average number of lateral shoots per plant of Breeding Line E.

NAA (µM)	Ax. shoot production (%)	Mean no. ax. shoots/plant (mean values ± SD)	Root prod. (%)
0	53.85	1.43 ± 0.79	53.85
0.25	43.75	3.00 ± 1.73	56.25
0.5	45.29	3.00 ± 2.19	49.76
0.75	64.71	2.73 ± 1.00	70.59
1.0	70.59	2.25 ± 1.22	76.47
2.5	62.50	2.30 ± 1.25	68.75
10.0	16.67	2.33 ± 0.56	0.00

Excised axillary shoots of Breeding Line E were grown to maturity within 8 weeks on MS-based culture medium containing 1.0 µM NAA. The plants grown previously on medium without auxin or containing 10.0 µM NAA were not used for further culture purposes because of dissatisfactory plant quality. This second generation of Breeding Line E plants produced less axillary shoots than the first generation. The least number of axillary shoots were developed by plants previously cultured with 2.5 µM NAA (Table 3.12).

Table 3.12: Percentage of second generation plants of Breeding Line E producing axillary shoots, inflorescences and average number of set seeds per plant.

Previous NAA-conc. (µM)	Ax. shoots(%)	Inflorescences (%)	Mean no. of seeds/plant (mean values ± SD)
0.25	45.00	70.00	4.88 ± 4.12
0.5	33.33	77.78	2.40 ± 1.52
0.75	55.56	55.56	3.00 ± 1.83
1.0	33.33	77.78	4.25 ± 1.71
2.5	9.09	77.27	5.07 ± 3.25

A high percentage of plants entered their reproductive cycle during the culture period. After 8 weeks of culture, seed set had taken place. The percentage of plants forming inflorescences and the mean number of produced seeds per plant seemed to be independent of the previously applied NAA concentrations. Figure 3.7 B shows a plant of Breeding Line E with seeds, which spontaneously germinated on the plant after further 2 weeks of culture.

Plate 3.7: (A) *In vitro* seed production of Breeding Line A.

(B) Spontaneous seed germination of Breeding Line E.

3.3.5 The Influence of IAA on the development of Breeding Line A

IAA was found to have a similar effect on the plant height of Breeding Line A as reported for NAA (3.3.4). Axillary shoot production and the formation of flowers were similar in each treatment and no statistical differences were found (Table 3.13).

Table 3.13: The influence of IAA on the percentage of plants producing axillary shoots and inflorescences.

	Ax. shoots (%)	Inflorescences (%)
IAA (µM)		
0	21.70	78.30
0.5	23.80	100.00
0.75	25.20	100.00
1.0	20.70	95.00

3.3.6 The influence of temperature on the development of *in vitro* grown spinach

'Longstanding' and Breeding Line E reacted in a very similar way to temperature. The development of plant height over a culture period of 6 weeks resulted in highly significantly taller plants at 15°C and 25°C in comparison to the plants grown at 5°C. Although the plants were taller at 15°C than at 25°C, no significant differences could be obtained (Figures 3.10 and 3.11).

The number of leaves produced was strongly influenced by temperature. The differences between each temperature regime were highly significant for both the breeding line and the commercial variety (Figures 3.12 and 3.13). In addition, the leaves at 15°C had longer petioles in comparison to plants from the 5°C or 25°C treatments. Standard deviations calculated for plant height and number of leaves for both varieties are listed in Table 3.14.

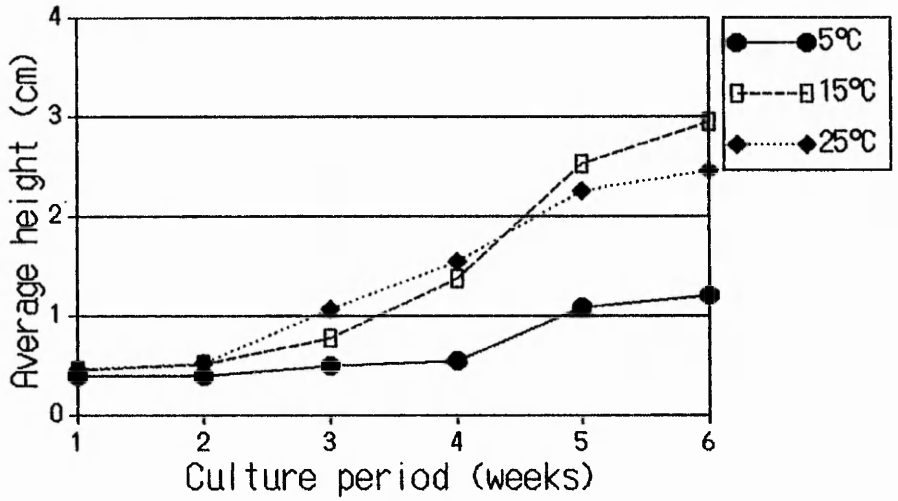


Figure 3.10: Plant height of 'Longstanding' over a culture period of 6 weeks at 5°C, 15°C or 25°C.

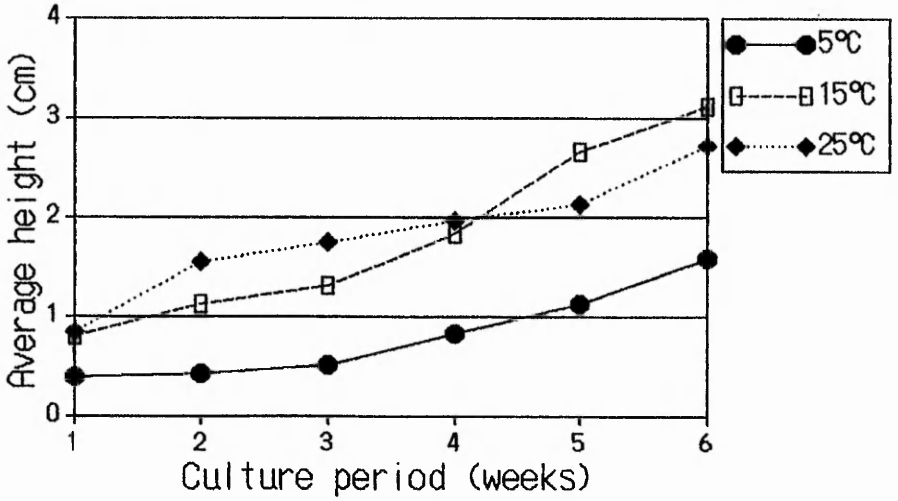


Figure 3.11: Plant height of Breeding Line E over a culture period of 6 weeks at 5°C, 15°C or 25°C.

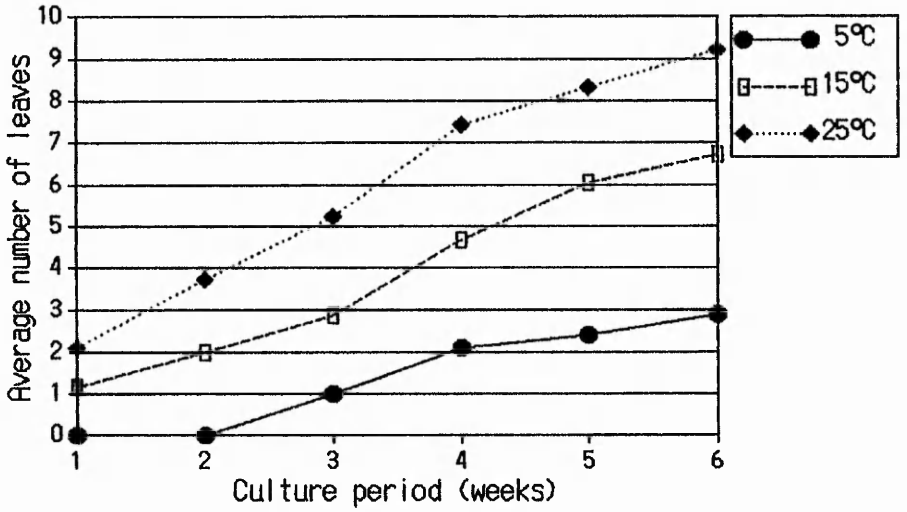


Figure 3.12: Number of leaves of 'Longstanding' developed over a period of 6 weeks at 5°C, 15°C or 25°C.

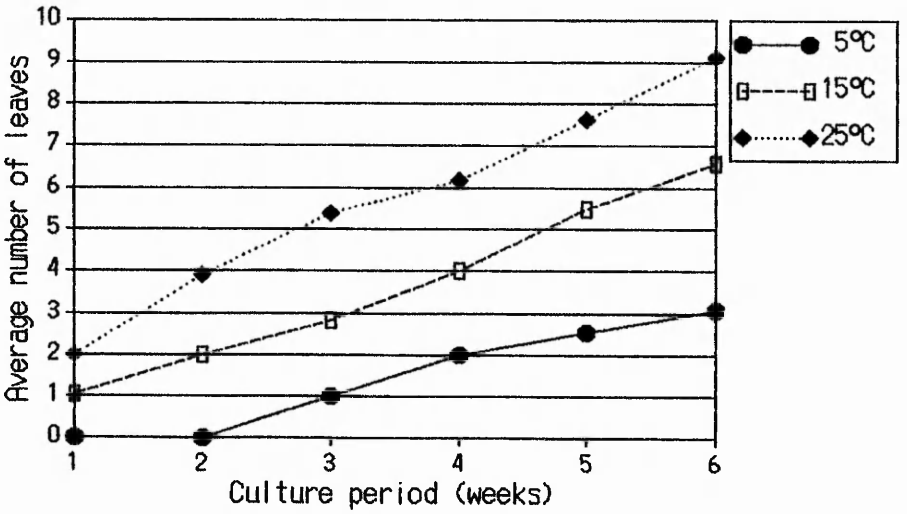


Figure 3.13: Number of leaves of Breeding Line E developed over a period of 6 weeks at 5°C, 15°C or 25°C.

Table 3.14: Standard deviations for the data obtained for height and leaf development for 'Longstanding' and Breeding Line E at 5°C, 15°C or 25°C.

LONGSTANDING

Culture week	1	2	3	4	5	6
Height (cm)						
5°C	0.00	0.04	0.13	0.19	0.21	0.21
15°C	0.03	0.20	0.26	0.40	0.57	0.66
25°C	0.05	0.23	0.32	0.58	0.53	0.68
No. of leaves						
5°C	0.00	0.00	0.19	0.40	0.43	0.52
15°C	0.90	0.03	0.70	1.20	0.66	0.71
25°C	0.10	0.30	1.01	1.09	1.72	2.35

BREEDING LINE E

Culture week	1	2	3	4	5	6
Height (cm)						
5°C	0.00	0.00	0.06	0.20	0.21	0.50
15°C	0.03	0.53	0.18	0.20	0.34	1.10
25°C	0.05	0.26	0.37	0.56	0.83	1.71
No. of leaves						
5°C	0.00	0.00	0.40	0.58	0.46	0.50
15°C	1.02	0.00	0.78	0.80	0.85	1.10
25°C	0.00	0.90	0.87	1.10	1.42	1.71

Regarding the fresh weights achieved (Figure 3.14 A) and the leaf area (Figure 3.14 B) no differences occurred between the 15°C and 25°C treatments, but the 5°C treatment generated highly significantly smaller values.

Determination of the chlorophyll content revealed no differences between the 5°C and 15°C treatment for 'Longstanding', but both treatments contained highly significantly more chlorophyll than the plants grown at 25°C. Plants of Breeding Line E grown at 5°C contained significantly more chlorophyll than plants cultured at 15°C and both temperature regimes produced a highly significant larger chlorophyll content than the 25°C treatment (Figure 3.14 C).

The number of axillary shoots was not affected by temperature and corresponded with all other results previously obtained. The growth of axillary shoots, however, was delayed with the lowering of the temperature.

Table 3.15 shows that no significant differences occurred between root formation at 15°C or 25°C. Breeding Line E, however, was more inclined to produce roots than the commercial cultivar 'Longstanding'. With regard to flower formation, a significantly higher proportion of plants produced flowers at 25°C than at 15°C. A temperature of 5°C suppressed both root and flower formation. Plates 3.8 A and 3.8 B illustrate the plant quality obtained under the 3 culture temperatures.

Due to the very slow plant development at 5°C, spinach cultures could be kept for 16 weeks between subcultures. Plate 3.9 shows the plants lacking inflorescences, after a culture period of 16 weeks.

Table 3.15: The influence of three different temperatures on the percentage of plants of 'Longstanding' and Breeding Line E forming roots and inflorescences.

	Root production (%)			Flower formation (%)		
	5°C	15°C	25°C	5°C	15°C	25°C
'Longstanding'	0	31.60	54.74	0	53.32	71.45 *
Breeding Line E	0	83.29	72.90	0	27.43	55.50 *

* = P(0.05)

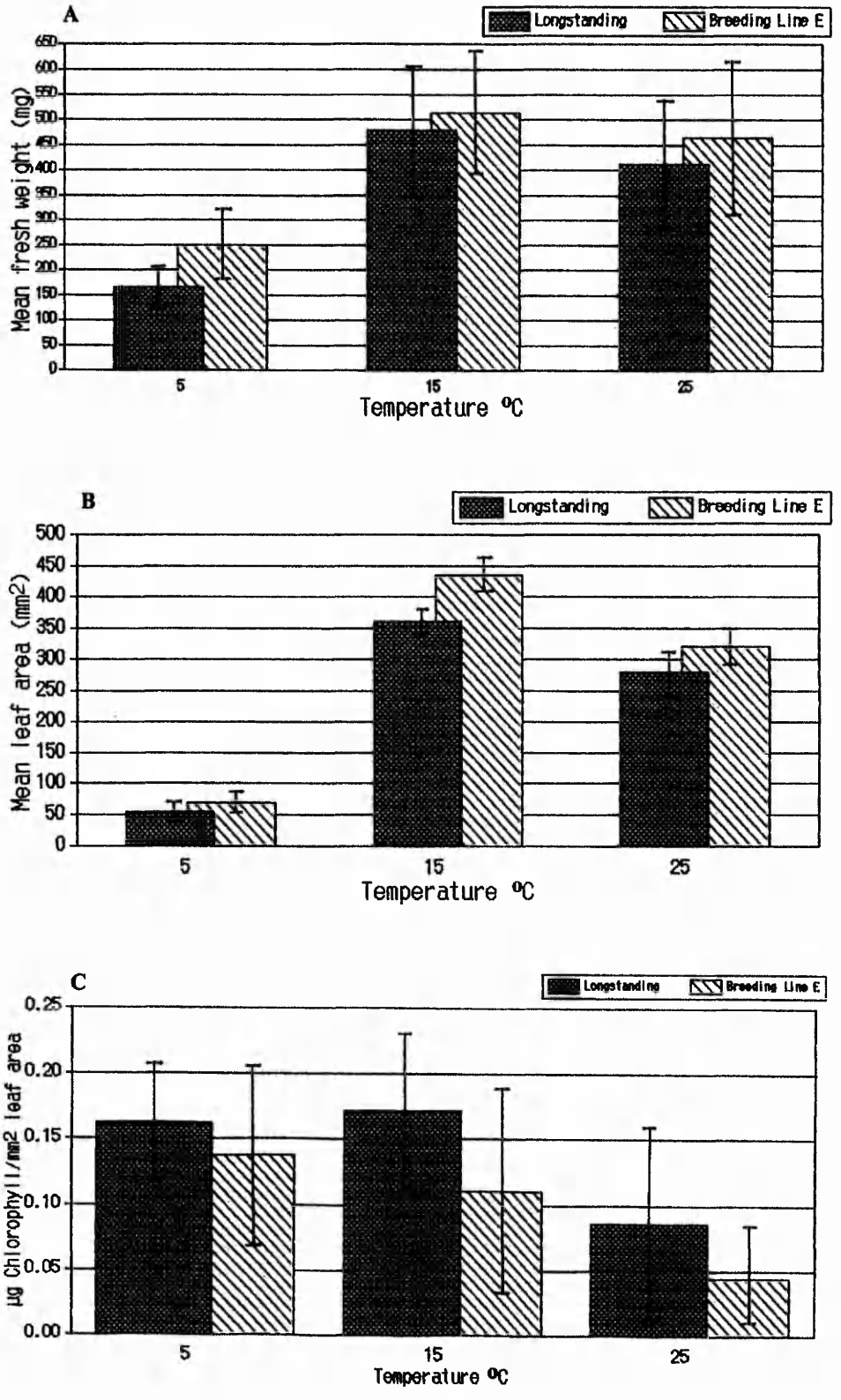


Figure 3.14: (A) Fresh weight, (B) leaf area and (C) chlorophyll content per mm^2 leaf area achieved by 'Longstanding' and Breeding Line E after a culture period for 6 weeks at 5°C, 15°C or 25°C (mean values ± SD).

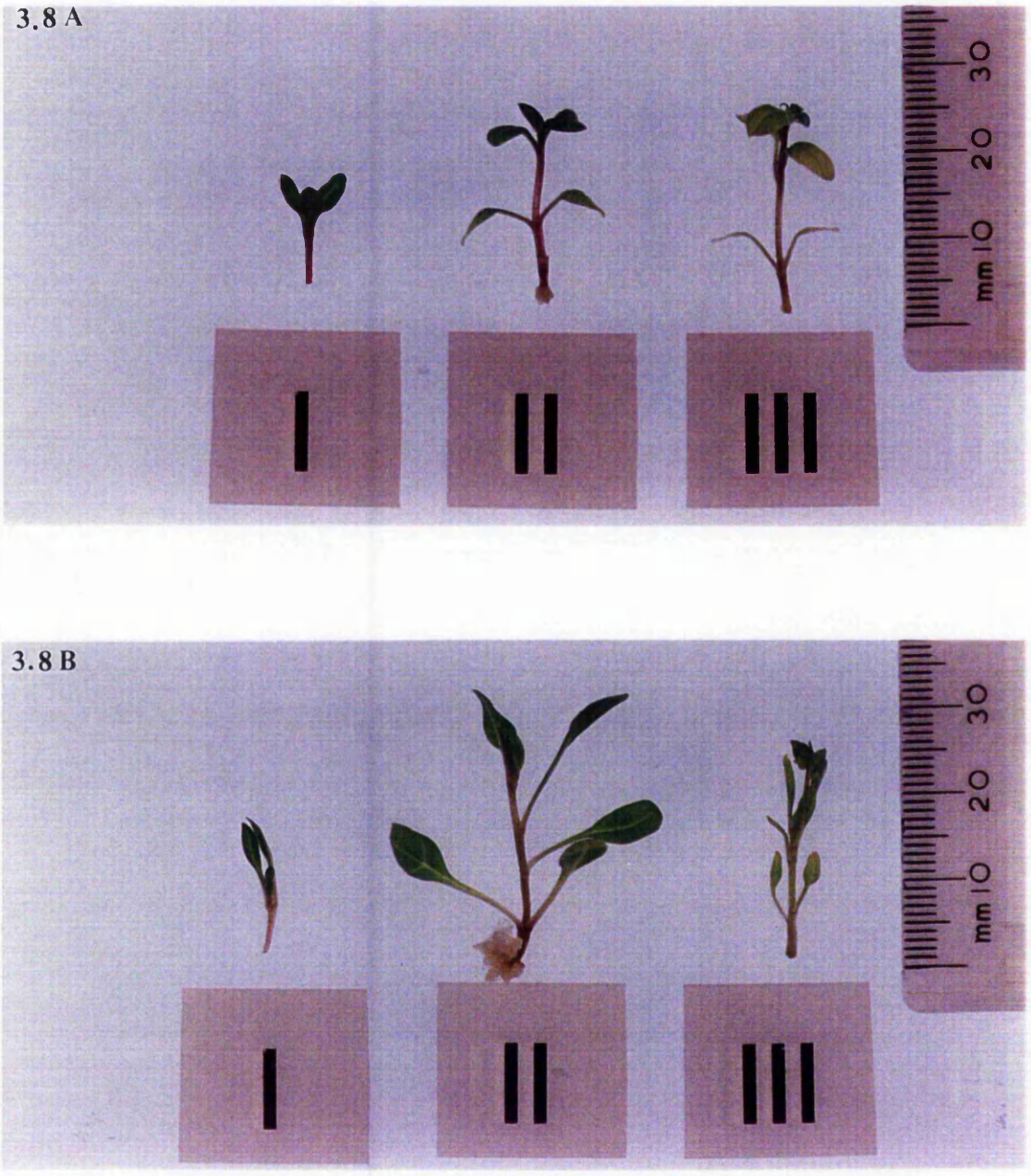


Plate 3.8: The influence of different culture temperatures on the development of (A) 'Longstanding' and (B) Breeding Line E.

Key to treatments: I = 5°C

II = 15°C

III = 25°C

3.9

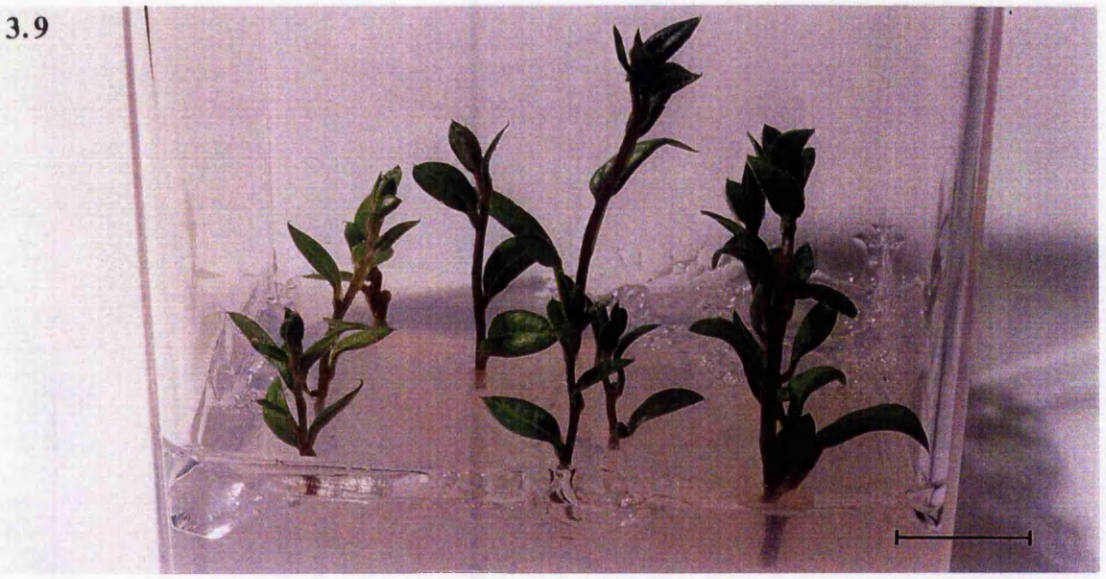


Plate 3.9: Shoots of Breeding Line E after a culture period of 16 weeks at 5°C (bar = 1 cm).

3.3.7 The influence of light on the development of *in vitro* grown spinach

Breeding Line A and the commercial variety 'Virkade' reacted in a similar way to variation of the photoperiod *in vitro*. No significant differences could be detected between the number of leaves or the fresh weight under photoperiods of 12 h, 14 h or 16 h per day. A light treatment of 16 h per day produced significantly the largest plants in comparison to the other two light regimes (Figure 3.15).

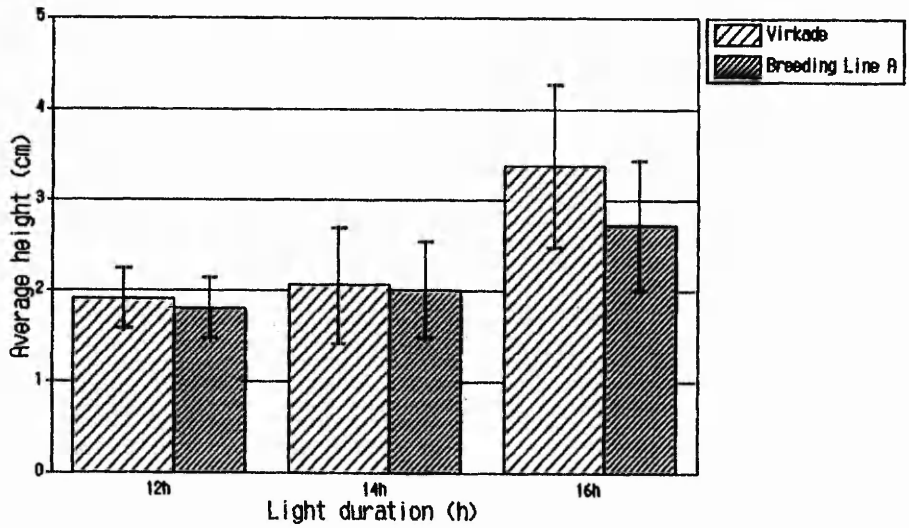


Figure 3.15: Average plant height achieved by Breeding Line A and 'Virkade' under light treatments of 12, 14 or 16 h per day (mean values ± SD).

Analysis of axillary shoot production (Table 3.16) showed, that light treatments of 14 h or 16 h per day led to a greater production of axillary shoots in Breeding Line A than a 12 h photoperiod, resulting in significantly more plants producing axillary shoots than the commercial variety 'Virkade'.

The effect of light treatments on the formation of inflorescences became most obvious in the commercial variety. Flowering was suppressed under a photoperiod of 12 h and slightly reduced under 14 h per day compared to the 16 h per day treatment. The overall plant quality of both varieties appeared best under a light period of 16 h per day; most stem callus developed under this light regime (Plate 3.10).

Table 3.16: The effect of light on axillary shoot growth and formation of inflorescences of Breeding Line A and 'Virkade'.

	Breeding Line A			'Virkade'		
Light duration	**12 h**	**14 h**	**16 h**	**12 h**	**14 h**	**16 h**
Axillary shoots (%)	4.16	14.29 **	15.38 **	18.18	30.00 *	33.30 *
Inflorescences (%)	4.16	3.57	7.69	23.30	40.00 *	50.00 *

* = P(0.05), ** = P(0.01)

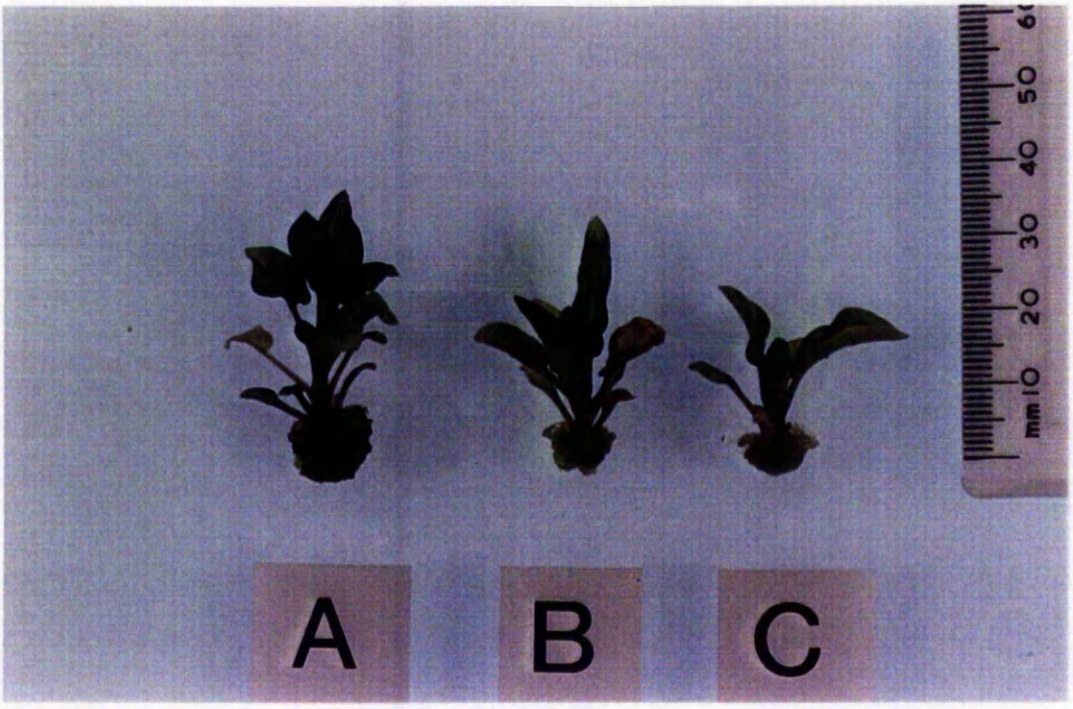


Plate 3.10: The effect of photoperiod at durations of 16 h (A), 14 h (B) and 12 h (C) on the development of Breeding Line A.

3.4 Discussion

3.4.1 The effect of plant growth regulators on tissue cultures of spinach

To date, there are few reports on the establishment of a micropropagation system for spinach from cultured meristems. Culafic (1973) and Neskovic and Culafic (1988) described the culture of apical buds on culture medium without growth regulators, giving rise to long leafy shoots, but their attempts to propagate spinach by multiple branching despite the use of high cytokinin concentration was unsuccessful.

In the present study on spinach, similar observations were made. The plants, regardless of the cultivar, grew long and spindly on culture medium without plant growth regulators. In order to break apical dominance, cytokinins were added to the culture medium, thereby inducing axillary shoot production. The cytokinins kinetin, BAP and 2iP were tested. In this order, with increasing strength of the cytokinins, the plant shape changed from an elongate plant to a short rosette type. Cytokinins did not encouraged multiple axillary shoot formation. On the contrary, the percentage of plants producing axillary shoots was reduced or axillary shoot production was inhibited with increasing amounts of cytokinins. Cytokinins also inhibited root growth at the stem base, but initiated the development of stem base callus. Concentrations of 2.5 µM and 5.0 µM of kinetin or BAP produced the largest amounts of stem base callus.

The common phenomenon of hyperhydricity also occurred with the higher concentrations of BAP and 2iP. Breeding Line A proved to be more susceptible to hyperhydricity than the other breeding lines. Debergh (1983) suggested that cytokinins could be the cause of hyperhydricity, and Kevers (1984) stated that hyperhydricity results from a burst of ethylene caused by stress, like excess cytokinin or auxin. The inclusion of an anti-auxin has been suggested to overcome hyperhydricity by changing cell wall plasticity, but the incorporation of TIBA into the culture medium did not improve the tissue cultures of globe artichoke (Debergh, 1981). This observation is confirmed by the present study on spinach tissue cultures. When the anti-auxin TIBA was included in the culture medium, hyperhydricity was enhanced and led eventually to plant death.

For other members of the *Chenopodiaceae* different hormonal requirements have been observed. *Beta vulgaris* multiplies well on a medium containing only cytokinin (Ritchie *et al.*, 1989). In contrast, cultures of *Artiplex canescens* require the presence of auxin, cytokinin and gibberellic acid (Wochok and Sluis, 1980), while *Chenopodium quinoa* was successfully multiplied when both benzyladenine and NAA were added to the culture medium (Burnouf-Radosevich and Pauparding, 1985). These results indicate that within the *Chenopodiacea*, shoot multiplication is encouraged on culture media containing low concentrations of cytokinin and, in some cases, the presence of auxins is required. The results obtained from the experiments conducted on various spinach cultivars confirms this assumption. Moreover, cytokinins were found not to be essential for shoot cultures and, on the contrary, cytokinins at higher concentrations inhibited the development of axillary shoots.

The inclusion of auxin in the culture medium proved to be most beneficial for the production of axillary shoots and also for the overall plant condition. Although the average number of axillary shoots per plant did not exceed 1-3 shoots, the percentage of plants producing axillary shoots was highest when the shoot tips were cultured on auxin-containing medium. The optimum combinations for stimulating axillary shoots were 1.0 µM IAA, 1.0 µM IAA with 1.0 µM kinetin, 0.5 µM NAA, 0.5 µM NAA with 1.0 µM kinetin, 1.0 µM NAA or 1.0 µM NAA with 1.0 µM kinetin.

Plant development was similar on culture medium containing NAA or IAA only. However, more plants grew axillary shoots under the influence of NAA, and 1.0 µM NAA performed slightly better than 0.5 µM NAA. Kinetin was not essential for the culture of spinach.

High concentrations of auxin caused decreasing numbers of axillary shoots due to their phytotoxic effect. Low concentrations, in contrast, produced satisfactory plant quality and also proved to be adequate for culture maintenance.

3.4.2 The influence of temperature on the development of *in vitro* grown spinach

Tissue cultures of spinach are usually grown at a temperature of 20°C, whereas under field conditions spinach is acknowledged as a cold temperature crop plant. Most studies concerning the culture temperature of spinach are connected with the occurrence of photoinhibition at low temperatures. Gray *et al.* (1994) found that plants grown at 16°C exhibited hardly any resistance to photoinhibition at low

temperatures in comparison to plants grown at 5°C. In an earlier study, Guy *et al.* (1987) showed that spinach seedlings grown in soil or *in vitro* can cold acclimate at very early stages of growth. Plants cultured *in vitro* retained the capacity to cold acclimate, but *in vitro* plants were slightly less hardy than soil grown plants.

In the present study, distinct differences could be detected between the growth pattern of spinach plants under the three temperature regimes tested. Plant height and leaf development at 5°C were significantly slower than at 15°C or 25°C. Although height, fresh weight and leaf area were larger at 15°C than at 25°C, the differences were not significant for either of the tested varieties. Visually, however, the plants grown at 15°C appeared stronger and healthier than the plants grown at 25°C for the same period of time.

An interesting result could be achieved with regard to the chlorophyll content of the leaves. 'Longstanding' produced similar amounts of chlorophyll per leaf area at 5°C and at 15°C, but very significantly less chlorophyll per leaf area was present at 25°C. Breeding Line E had considerably the highest chlorophyll content when cultured at 5°C. Plants grown at 25°C contained the least chlorophyll. Somersalo and Krause (1990) reported an altered pigment composition of leaves, with lowered chlorophyll content, during cold acclimating conditions. A study conducted by Boese and Huner (1990) was based on the developmental stage of plants. The second leaf pair of leaves of *in vivo* grown spinach plants had developed after 32 d at 16°C or after 92 d at 5°C. Culture at 5°C resulted in increased leaf area, dry weight and leaf thickness, but pigment content was not affected. In addition, cold was found to inhibit flower production, which was also observed in the present study.

The possibility of low temperature preservation of sugar beet, another member of the *Chenopodiaceae*, was investigated by Miedema (1982). The normal culture temperature for sugar beet is 20°C. Rooted shoots could be kept for one year at 5°C-10°C, whilst showing a slow leaf extension. A similar observation could be made for spinach by keeping unrooted shoots at 5°C for 16 weeks before the next subculture.

This study has determined, that a culture temperature of 15°C is most suitable for the growth *in vitro* of spinach. A temperature of 5°C proved to be appropriate for the storage of shoots without applying any stress factors, while a temperature of 25°C can be used, if progression to the reproductive life cycle, followed by seed set, is required.

3.4.3 The influence of light duration on the development of *in vitro* grown spinach

Spinach readily produces flowers *in vitro* cultures under long day conditions of 16 h. Depending on the cultivar, the change from the vegetative phase to the generative phase occurs after 4 - 6 weeks in culture. The maintenance of the vegetative phase, however, is desirable in order to facilitate an ongoing clonal propagation of the plants.

Culafic and Neskovic (1980) prevented flower formation by applying a photoperiod of 8 h per day. With a light duration of 16 h per day flower formation was generated in 74 % of the treated plants. A decrease of flowering under long day conditions was achieved by the addition of kinetin or the growth retardant 'Amo 1618' to the culture medium.

In the present study, it was found, that the readiness of spinach cultures to flower increased by extending the photoperiod. The commercial variety, in particular, exhibited a sensitive response to the three different photoperiods used. The inclusion of BAP in the culture medium can be considered responsible for greatly reducing the flower production in the breeding line and reducing the flower production in the commercial variety.

Light treatment had a considerable impact on plant height. Under a photoperiod of 12 h per day, the plants maintained a rosette shape and hardly elongated. An explanation for this observation is offered by a study of Zeevaart (1974), who found that stem elongation in spinach does not occur unless the plants had been exposed to long day conditions (8 h high intensity illumination followed by 16 h low intensity illumination) for at least one week.

Considering that the spinach cultures were grown under unfavourable conditions regarding the cytokinin supplement in the culture medium and a culture temperature of 25°C, qualitatively the best plants were produced under a photoperiod of 16 h. Drawing on the previous experience that flowering of spinach can also be controlled by temperature, a photoperiod of 16 h per day would seem to be optimal for spinach tissue cultures in order to optimize plant quality.

CHAPTER 4

The application of a double phase culture system to shoot cultures of spinach cultivars (*Spinacia oleracea* L.)

4.1 Introduction

Axenic shoots are usually cultured on nutrient media semi-solidified with agar. However, depending on the culture method, liquid media only may also be used. A higher propagation rate of *Guara scolymus* was achieved when shoot explants were cultured in liquid medium on cotton wool, compared to their culture on semi-solidified medium (Debergh, 1981). Nodal stem segments of chrysanthemums exhibited high growth rates when cultured on cellulose rods in liquid nutrient medium (Short, 1986). Rafts floating on liquid culture medium were used by Watad *et al.* (1994) to improve the propagation rate of *Aconitum napellus*.

Some work has been undertaken using a double phase culture system employing semi-solid and liquid media. The semi-solid phase of the culture medium was overlayed with the liquid phase of the same medium. Viseur (1987) and Rodriguez *et al.* (1991) achieved a 50 % increase of the shoot numbers per explant of *Pyrus communis* cultivars and improved the shoot quality significantly when cultured in a double phase system. This culture method has also been used with *Beta vulgaris* L., another member of the *Chenopodiaceae*, and resulted in multiple axillary shoot production (Short and Davey, 1990).

The aim of this study was to investigate the possibility of enhanced axillary shoot production of spinach by the use of the double phase culture system. The most suitable volume of liquid medium, the length of preculture before adding the liquid medium and the period of time in double phase culture were determined.

Preliminary experiments had shown that the explants should have developed six to eight leaves before the application of the liquid phase. At this stage of development, the plants are strong enough to remain fixed in the semi-solidified culture medium when the liquid medium is added, thereby preventing the explant floating in the medium. An applied volume of 20 ml and above resulted in hyperhydricity. Studies with spinach revealed that the period of time in the double phase culture should not exceed 14 days. Prolonged culture results in the plants becoming hyperhydric and malformed, regardless of the volume of the liquid medium employed.

The experimental framework was outlined for the following experiments within these limiting factors.

4.2 Materials and Methods

4.2.1 The establishment of axenic cultures for the use in the double phase system and culture conditions

Shoot tips of spinach seedlings, 10 mm in length, were cultured on MS-based culture medium (3.2.1). In all experiments, the culture containers used were 100 ml capacity screw-capped polystyrene containers (Sterilin), each filled with 15 ml of the semi-solid medium. The plant growth regulator supplements to the culture medium differed in the individual experiments. Three explants were initially cultured in one container.

After 8 - 10 d in culture, when the plants had developed 4 - 6 leaves, the shoots were transferred onto the same basic culture medium after the cotyledons were removed from the stem base. Two shoots were cultured in a 100 ml capacity culture container.

The plants were cultured for 7 d, before the liquid culture medium was added. The liquid medium contained the same constituents as the semi-solid medium, but was devoid of the agar component. The amount of liquid medium added and the period of time before the liquid medium was decanted, varied in the different experiments. A volume of 5 ml of the liquid medium occupied a height of approximately 4 mm in the culture vessels used. Figure 4.1 illustrates the arrangement of layers of the semi-solid and liquid medium in each culture container.

All cultures were grown under a 16 h photoperiod using 36 W White Pluslux 3500 fluorescent tubes giving 42 ± 14 $\mu E\ m^{-2}s^{-1}$ of irradiance at a temperature of 25°C.

The plants were typically harvested and assessed after a culture period of 42 d. Data were collected for the number of leaves, plant height, leaf area, fresh weight, dry weight, axillary shoot production and flowering. Plants were screened for symptoms of hyperhydricity. In each experiment, the treatments were arranged in a latin square design and the data were statistically processed as described in 3.2.9.

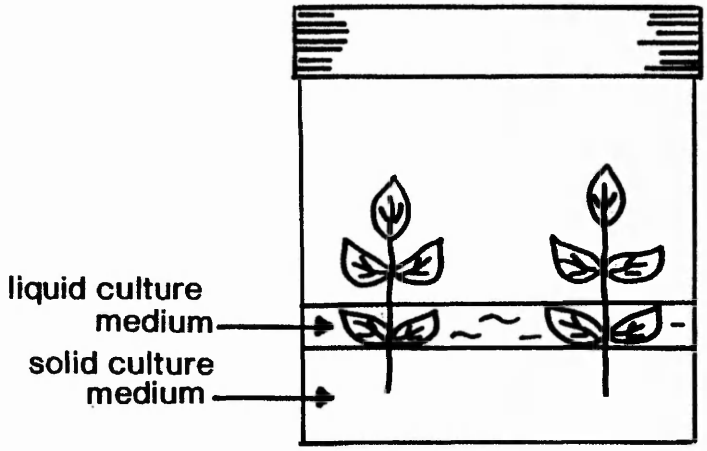


Figure 4.1: Illustration of the double phase culture system used for spinach shoot explants. Semi-solidified medium is overlayed by the liquid culture phase.

4.2.2 The influence of varying volumes of liquid medium, in a double phase culture system, on the development of spinach cultivars

Breeding Lines A, B, D, E and the commercial varieties 'Medania' and 'Virkade' were established on MS-based culture medium supplemented with 1.0 µM kinetin and 1.0 µM IAA. The liquid medium, containing the same plant growth regulators, was applied in volumes of 0 ml, 5 ml, 10 ml and 15 ml. After 7 d the liquid medium was removed and the plants were maintained in culture for another 21 d, before being assessed.

The number of plants used in each treatment was: Breeding Lines A: n = 24, B: n = 11, D: n = 7, E: n = 14; 'Medania': n = 10, 'Virkade': n = 11.

4.2.3 The effect of different culture periods in double phase systems on the development of Breeding Lines A and E

Breeding Lines A and E were established on MS-based culture medium containing 1.0 µM IAA. The liquid medium used contained the same concentrations of plant growth regulator as the semi-solid medium. The volumes of liquid medium used were 5 ml and 10 ml for Breeding Line A and 5 ml for Breeding Line E. The culture period in the double phase culture was either 7 or 14 d. After this time, the plant material was cultured on semi-solid medium for the remainder of the experimental period. After a total culture period of 35 d, the plants were harvested and assessed.

The number of replicates per treatment were for Breeding Line A: n = 24 and for Breeding Line E: n = 17.

4.2.4 The application of a double phase system to established spinach cultures

Apical explants from seedlings of Breeding Line E were established on MS-based culture medium supplemented with 1.0 µM NAA. After 28 d the stem bases were excised, leaving shoots of 1.5 cm in height. The shoots were transferred to the same medium and after 7 d of culture, 10 ml of the liquid MS-based medium containing 1.0 µM NAA was added. The liquid medium was decanted after another 7 d and the plants were assessed for their plant height and the production of axillary shoots, inflorescences, seeds and roots after further 21 d in culture.

The number of replicates was for control plants n = 37, for treated plants n = 105.

4.2.5 The application of a double phase culture system to plants of Breeding Line E grown under a low temperature regime

Plants of Breeding Line E were grown in incubators at 15°C under a 16 h photoperiod using 36 W Hybec White fluorescent tubes, giving an irradiation of 45 ± 10 µE $m^{-2}s^{-1}$ until they had reached the 6 leaf stage. The liquid medium was then applied in volumes of 0, 5 and 10 ml and removed after 10 d. Both the semi-solid and liquid MS-based culture medium contained 1.0 µM NAA. The plants were assessed after another 21 d of culture.

The number of replicates for each treatment was n = 18.

4.3 Results

4.3.1 The influence of different volumes of liquid medium on the development of spinach cultivars

The only parameter which was not influenced by the use of liquid medium was the number of leaves produced by cultured shoots.

The average plant height increased in all treatments in comparison to that of the control plants (Figure 4.2). Significant differences in height were obtained for Breeding Line A, where the control plants were very significantly smaller than the treated plants. Plants of Breeding Line D treated with 15 ml of liquid medium and plants of Breeding Line E with 10 ml of liquid medium were very significantly larger than the untreated control.

The leaf area was distinctly enlarged in all varieties, when the plants were grown in the double phase culture system (Figure 4.3). An enlargement of the leaf area was caused mainly by the application of liquid medium at volumes of 10 ml or 15 ml.

The difference in plant growth between conventional and double phase culture methods was demonstrated by the data obtained for fresh and dry weights. Fresh and dry weights increased with higher volumes of liquid medium. The fresh weight reached by all varieties in double phase culture were at least significantly larger than the fresh weights obtained for the control plants. However, this was not found to be the case in Breeding Line D, where only 15 ml of liquid medium achieved very significantly larger fresh and dry weights compared to all other treatments.

With regard to the dry weights, the treatment with 15 ml of liquid medium produced significantly larger dry weights than the control plants or the 5 ml treatments. No significant differences were obtained between the control and the 5 ml treatments (Figure 4.4).

The ratio between fresh and dry weights can be related to the amount of water taken up in the different treatments. The ratio between fresh and dry weight widened with increasing volumes of liquid medium. All varieties generated very similar ratios, with the exception of Breeding Line E, which produced more constant ratios throughout the treatments (Table 4.1).

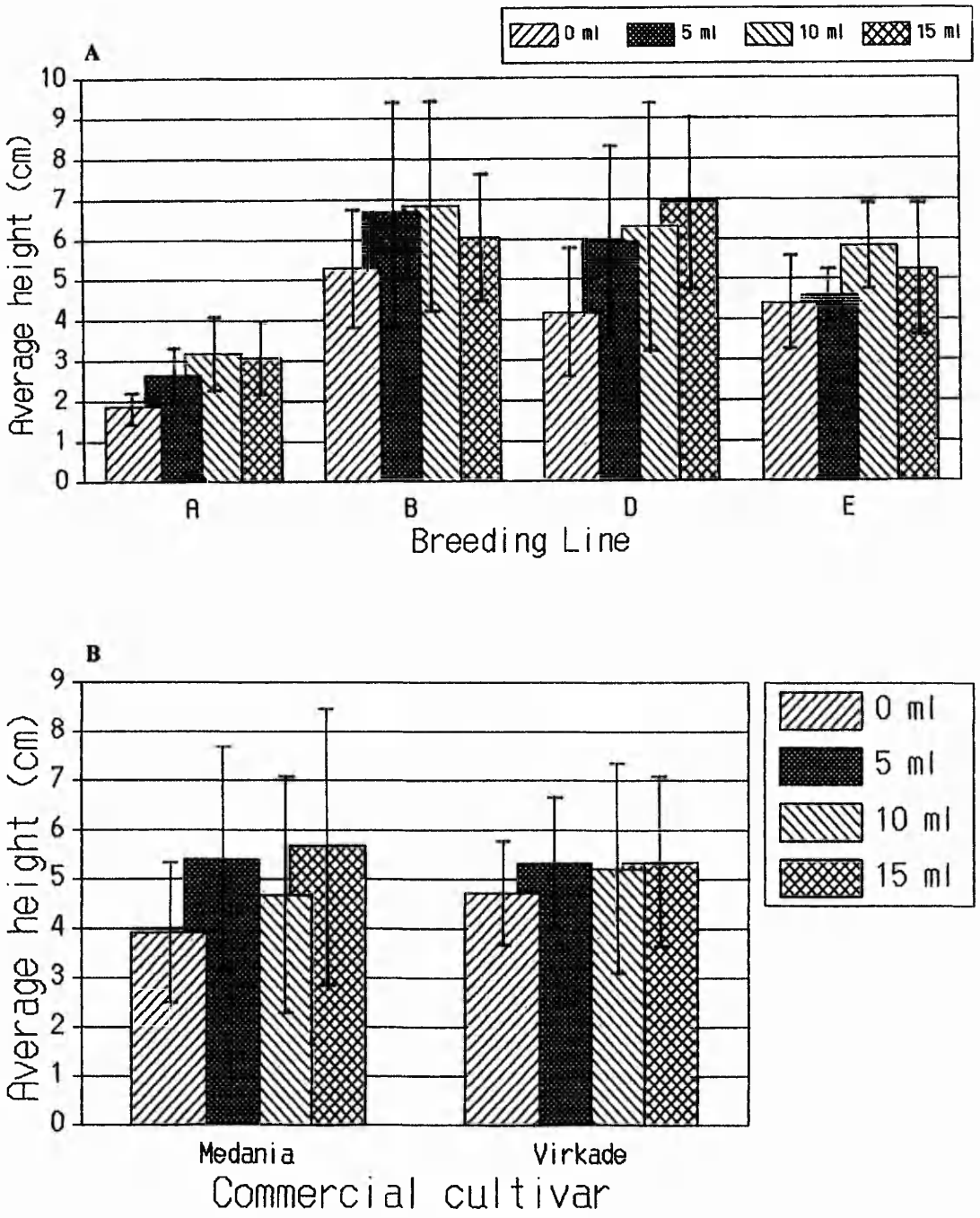


Figure 4.2: The influence of different volumes of liquid medium, in a double phase culture system, on the average height of (A) spinach breeding lines and (B) commercial spinach cultivars (mean values ± SD).

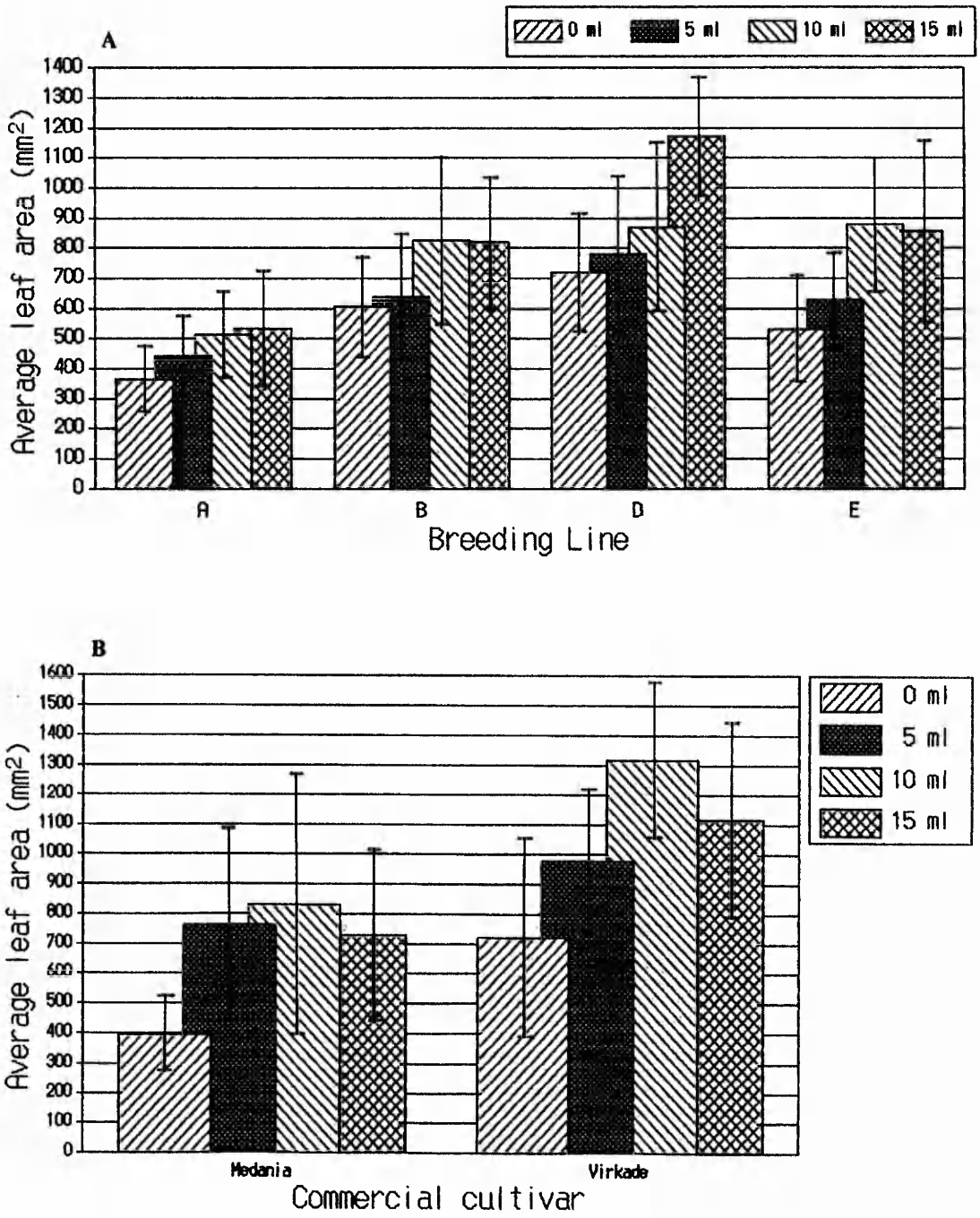


Figure 4.3: The influence of different volumes of liquid medium, in a double phase culture system, on the leaf area of (A) spinach breeding lines and (B) commercial spinach cultivars (mean values + SD).

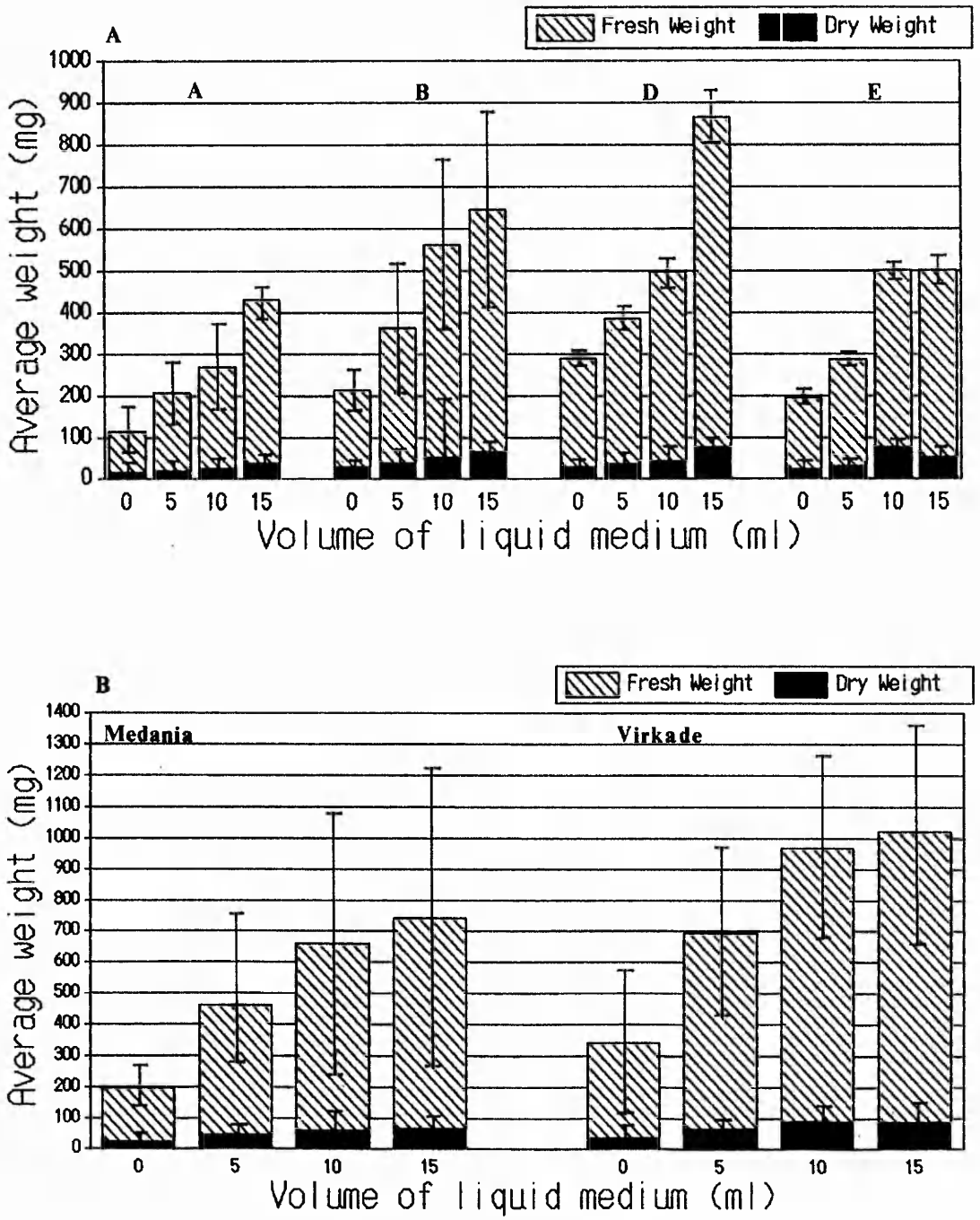


Figure 4.4: The influence of different volumes of liquid medium, in a double phase culture system, on the fresh and dry weights of (A) spinach breeding lines and (B) commercial cultivars (mean values ± SD).

Table 4.1: The influence of different volumes of liquid medium, in a double phase culture system, on the ratio of fresh weight : dry weight of breeding lines and commercial cultivars of spinach.

	Fresh weight : Dry weight			
Liquid medium (ml)	0	5	10	15
Cultivar				
A	7.30:1	10.36:1	10.73:1	11.27:1
B	7.49:1	9.63:1	10.82:1	11.20:1
D	10.37:1	10.61:1	11.73:1	12.71:1
E	8.83:1	9.61:1	9.13:1	9.80:1
'Medania'	8.10:1	10.13:1	11.16:1	11.43:1
'Virkade'	9.68:1	10.80:1	10.87:1	11.84:1

The percentage of plants producing axillary shoots in all varieties is high in each treatment. However, the amount of liquid medium added to the cultures did not stimulate plants to produce more axillary shoots than the control treatment. Similarly, an increase was not found in the average number of axillary shoots produced per shoot (Table 4.2).

Table 4.2: The influence of different volumes of liquid medium, in a double phase culture system, on the percentage of plants producing axillary shoots (%) and the average number of side shoots produced per cultured explant (Ø).

Liquid medium (ml)		0	5	10	15
Cultivar					
A	%	62.50	75.00	87.54	73.93
	Ø	1.47 ± 0.64	3.82 ± 1.56	2.76 ± 2.72	3.29 ± 1.16
B	%	100.00	90.90	100.00	100.00
	Ø	4.10 ± 3.34	2.40 ± 1.08	2.00 ± 1.18	2.36 ± 1.21
D	%	71.43	100.00	87.50	85.72
	Ø	4.2 ± 1.30	3.21 ± 1.78	2.86 ± 1.77	4.17 ± 1.47
E	%	84.62	100.00	85.71	84.62
	Ø	1.55 ± 0.60	2.93 ± 1.34	2.58 ± 1.24	2.67 ± 1.37
'Medania'	%	77.78	70.00	90.90	100.00
	Ø	2.00 ± 0.58	2.43 ± 1.13	3.40 ± 2.37	3.00 ± 1.29
'Virkade'	%	90.00	100.00	100.00	100.00
	Ø	3.44 ± 2.19	2.91 ± 1.04	3.40 ± 0.84	3.64 ± 1.43

Ø = mean values ± SD

The change in plant morphology caused by the application of the liquid medium is shown in Plates 4.1 and 4.2. The plants grown in a double phase culture system appeared stronger due to the thickened main stem than the plants cultured on semi-solid medium only. However, plants derived from the 15 ml treatments showed symptoms of hyperhydricity with enlarged and glassy leaf laminae. Table 4.3 demonstrates that with higher amounts of liquid medium the percentage of hyperhydric plants was also increased. The 15 ml treatments produced significantly more hyperhydric plants than any other treatment. The commercial variety 'Virkade' was more sensitive to the application of the liquid medium than the other varieties, with every volume causing significantly more hyperhydricity than culture without liquid medium.

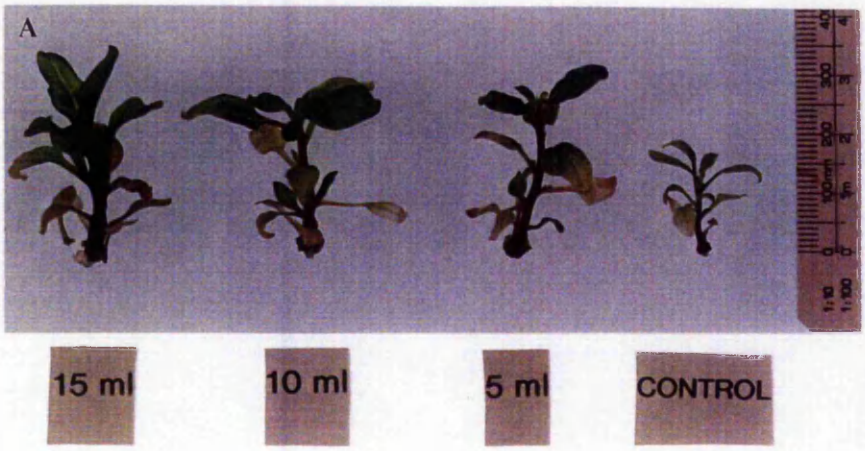
The flowering of the plants was not influenced by liquid medium. Both, the change to the regeneration cycle and also the percentage of plants forming inflorescences, corresponded with those of the control plants.

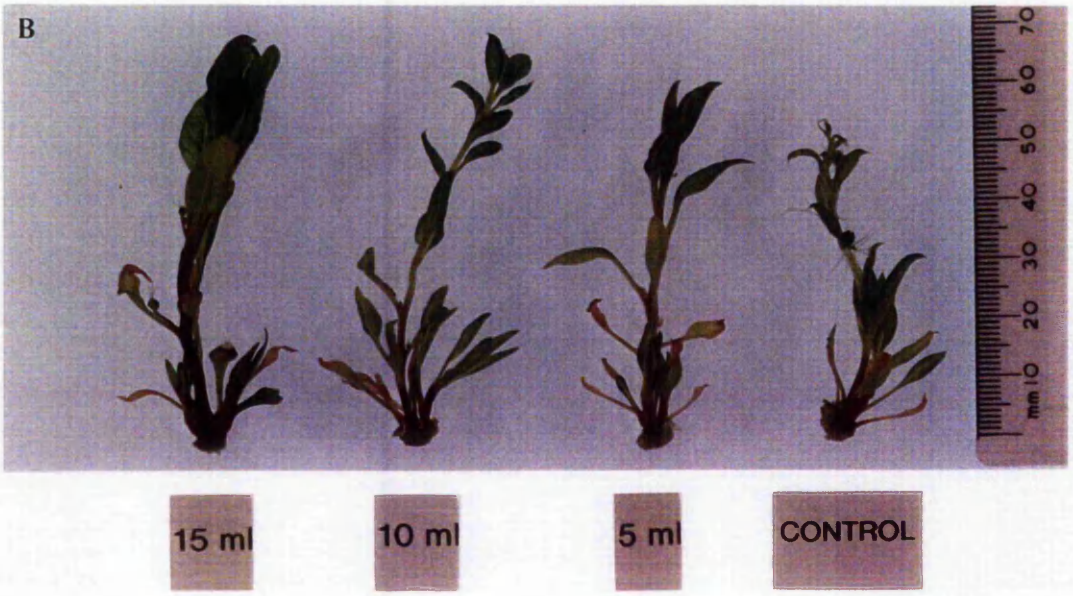
Table 4.3: Percentage of spinach plants showing symptoms of hyperhydricity in double phase cultures.

Liquid medium (ml)	0	5	10	15
Cultivar				
A	16.66	25.00	33.33	65.22 *
B	0.00	0.00	27.27	54.54 *
D	14.37	28.57	28.57	85.72 **
E	0.00	6.67	14.28	38.46 *
'Medania'	0.00	10.00	18.18	42.86 *
'Virkade'	0.00	36.36 *	36.36 *	45.45 *

* = P(0.05), ** = P(0.01)

Plate 4.1: The influence of different volumes of liquid medium on the development of (A) Breeding Line A, (B) Breeding Line B and (C) Breeding Line D. Plants were grown in double phase culture for 7 d, followed by 21 d culture without liquid medium.


A
15 ml
10 ml
5 ml
CONTROL


B
15 ml
10 ml
5 ml
CONTROL

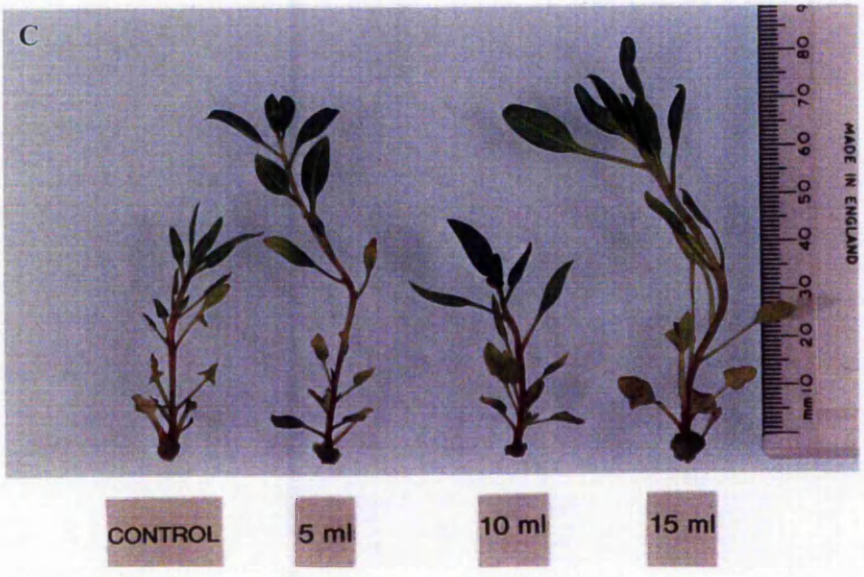
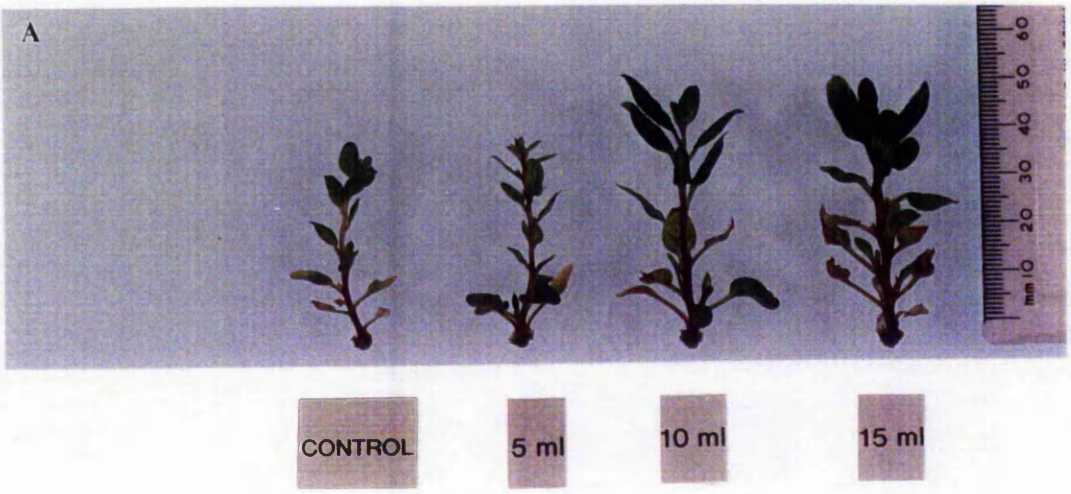
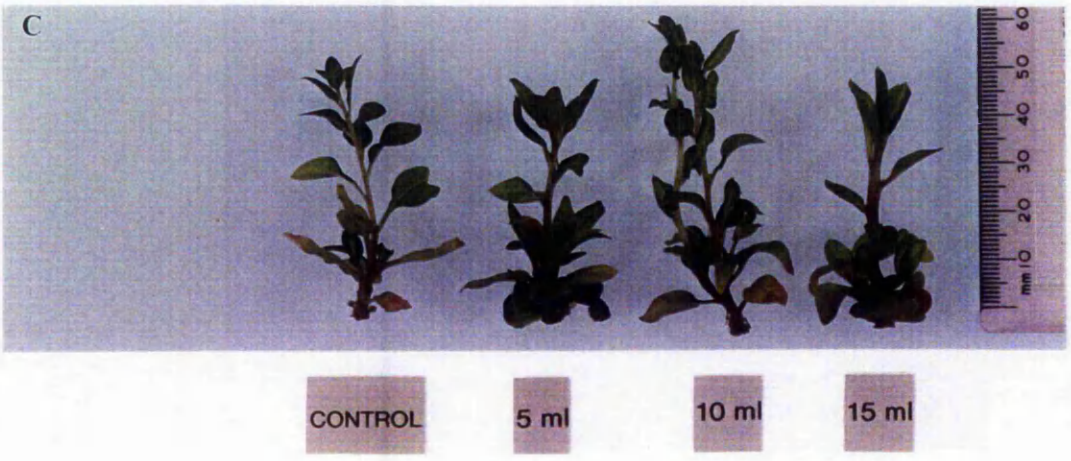

C
MADE IN ENGLAND
CONTROL
5 ml
10 ml
15 ml

Plate 4.2: The influence of different volumes of liquid medium on the development of (A) Breeding Line E, (B) 'Medania' and (C) 'Virkade'. Plants were grown in double phase culture for 7 d, followed by 21 d culture without liquid medium.


A
CONTROL
5 ml
10 ml
15 ml


B
CONTROL
5 ml
10 ml
15 ml


C
CONTROL
5 ml
10 ml
15 ml

4.3.2 The effect of different time intervals in double phase culture on the development of Breeding Lines A and E

The average plant height of Breeding Line A was influenced by both the period of time in double phase culture and the volume of liquid medium employed. Within the culture period of 7 d the 10 ml treatment produced very significantly higher plants than the 5 ml treatment or the control. The plant height achieved by 5 ml and 10 ml treatments during the culture period of 14 d did not differ. These treatments, however, produced very significantly higher plants than the control or the 5 ml treatment within a culture period of 7 d (Figure 4.5 A). No significant differences were generated for the leaf area or for the fresh weight, apart from the plants having been cultured for 14 d with 10 ml of liquid medium. These plants produced highly significant larger leaf areas and fresh weights in comparison to any other treatment (Figure 4.5 B,C). Plate 4.3 illustrates the development of Breeding Line A in the double phase culture with liquid medium for 7 or 14 d.

Table 4.4 shows that neither the length of the culture period nor the volume of liquid medium influenced the axillary shoot production. The proportion of hyperhydric plants increased in relation to the larger volume of liquid medium and with longer periods in the double phase culture regime. Significantly more plants became hyperhydric when cultured for 14 d with the liquid medium in the double phase system.

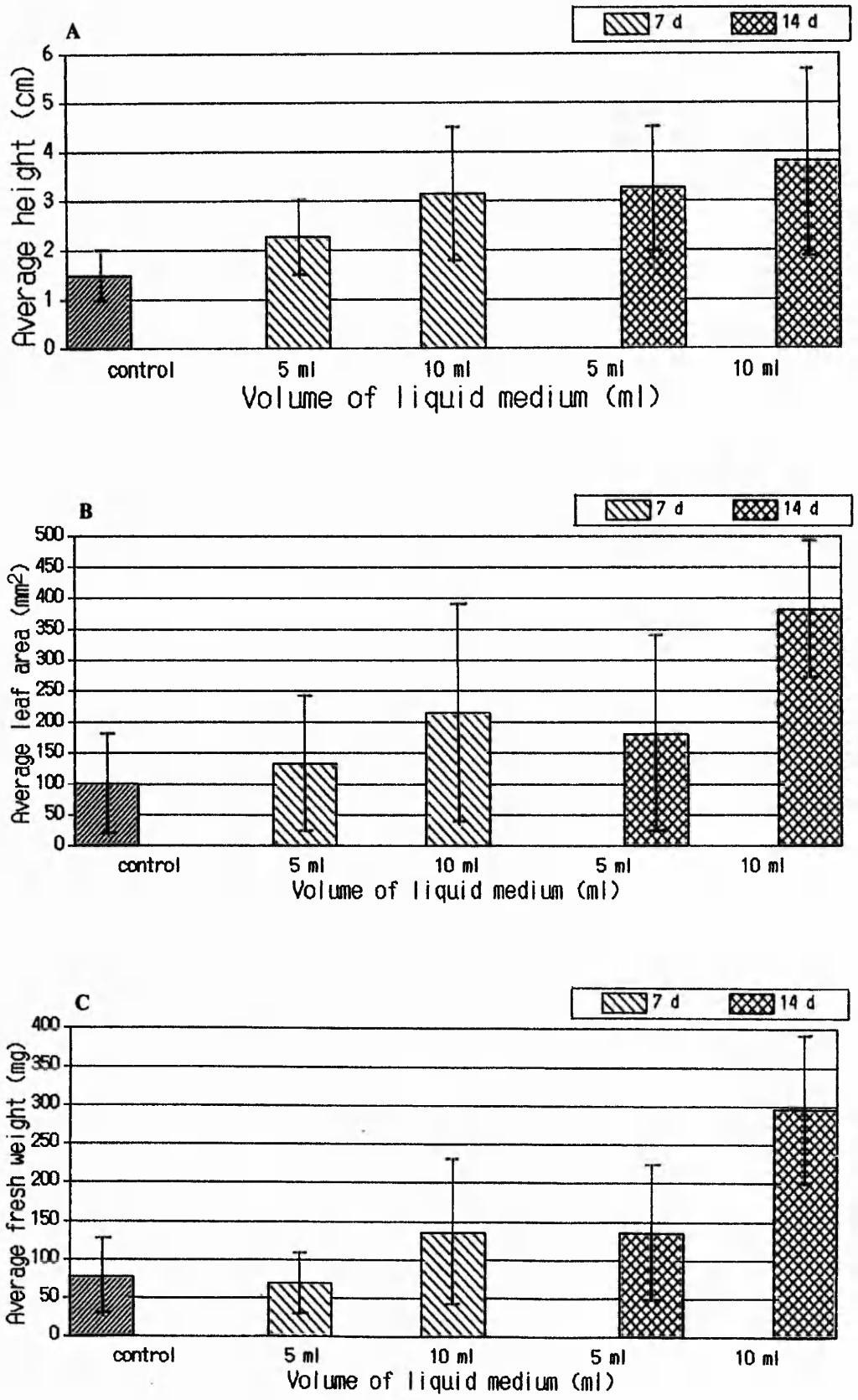


Figure 4.5: (A) Average plant height, (B) leaf area and (C) fresh weight of Breeding Line A after growth in a double phase culture system with 5 ml or 10 ml of liquid medium for 7 or 14 d (mean values ± SD).

Table 4.4: The influence of the culture period in double phase culture and different volumes of liquid medium on axillary shoot production and occurrence of hyperhydricity in spinach Breeding Line A.

	Axillary shoot production (%)		
Culture period (d)	ctrl.	7	14
Liquid medium (ml)			
0	38.88		
5		50.00	58.33
10		45.45	60.87

	Hyperhydricity (%)		
Culture period (d)	ctrl.	7	14
Liquid medium (ml)			
0	12.5		
5		8.33	9.91
10		33.33 *	37.50 *

* = P(0.05)

Plate 4.3: Plant development of Breeding Line A in double phase culture with volumes of liquid medium of 5 or 10 ml for 7 or 14 d.

The plant height of Breeding Line E was significantly larger when the plants were cultured for 14 d with 5 ml of liquid medium in comparison to the control, or to plants cultured for 7 d with liquid medium (Figure 4.6 A). The leaf area was not influenced by the length of time in the double phase culture system. The fresh weight, however, significantly increased during 7 or 14 d in the double phase culture system compared to the controls, but differences were not detected between the culture period of 7 or 14 d (Figures 4.6 B, C).

Table 4.5 demonstrates that axillary shoot production was not improved by culture with liquid medium. Only 1 or 2 axillary shoots developed per plant. However, with the extension of the culture period to 14 d, there was a significantly higher percentage of hyperhydric plants than without the liquid medium.

Table 4.5: The influence of the culture period in a double phase culture regime, using 5 ml of liquid medium, on the axillary shoot production and the occurrence of hyperhydricity in spinach Breeding Line E.

Culture period (d)	ctrl.	7	14
Axillary shoot production (%)	27.20	28.57	29.40
Hyperhydricity (%)	5.88	29.41	41.18 *

* = P(0.05)

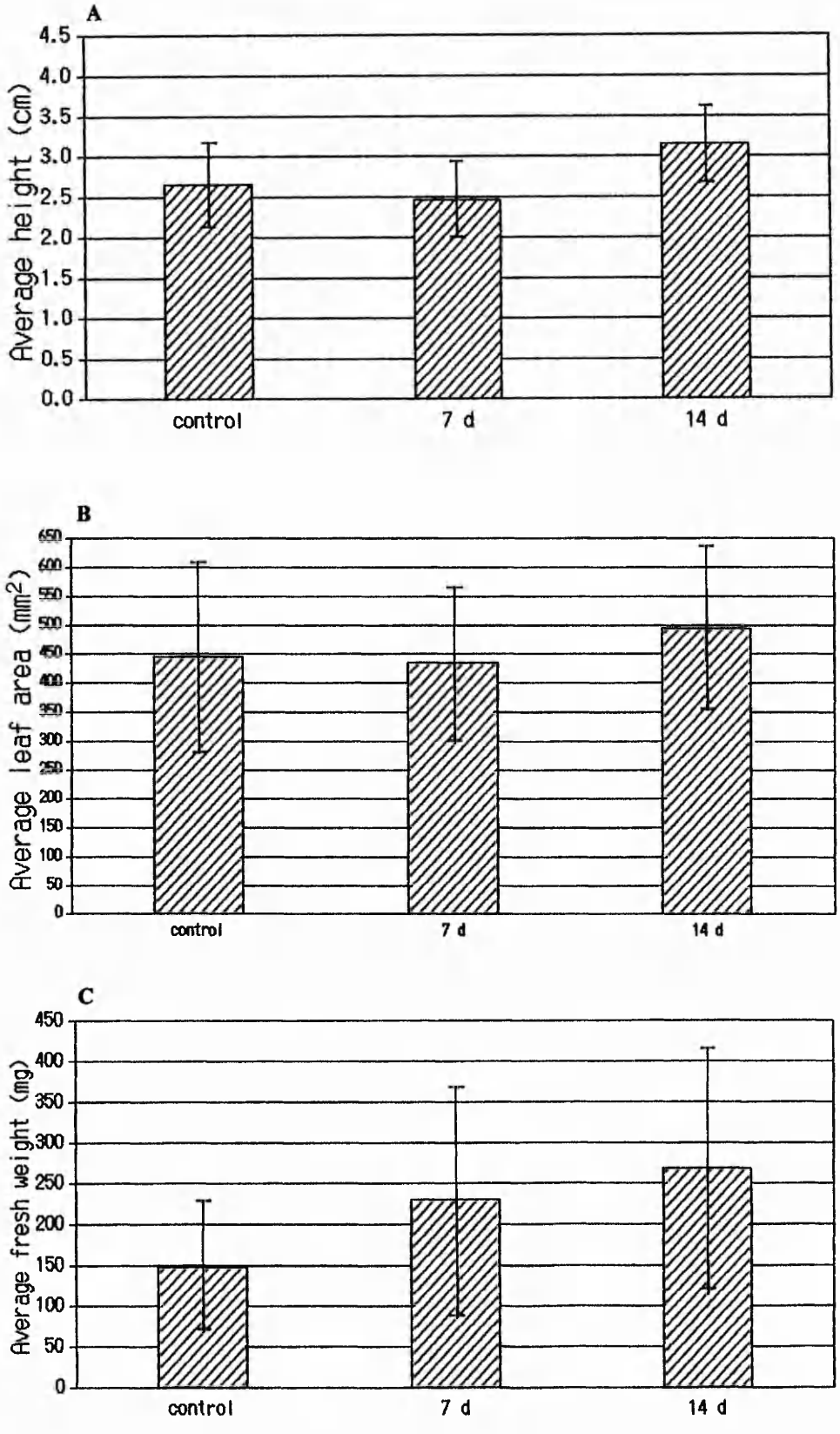


Figure 4.6: (A) Average plant height, (B) leaf area, and (C) fresh weight achieved by Breeding Line E after growth in a double phase culture regime with 5 ml liquid medium for 7 or 14 d (mean values ± SD).

4.3.3 The application of the double phase culture system to established plants of Breeding Line E

The results obtained after a culture period of 63 d are illustrated in Table 4.6. No significant differences were detected concerning any of the key growth parameters height, axillary shoot production, flower production, seed set and root production. The percentage of plants producing axillary shoots was slightly increased when cultured in double phase culture intermittently for 7 d. The number of axillary shoots was between 1-3 shoots per shoot-forming plant. Flower production and subsequent seed set were not influenced by either culture method. Root production appeared to be little reduced in the double phase culture regime. Although 9.5 % of the plants became hyperhydric under the influence of the liquid medium, this percentage was lower than those observed on younger spinach plants in the previous experiments.

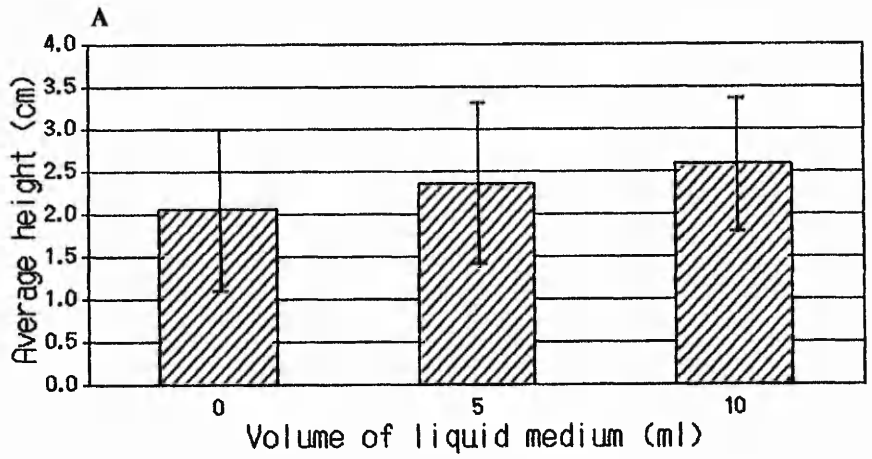
Table 4.6: Comparison of growth characteristics of plants of Breeding Line E grown in conventional culture or in a double phase culture system for 7 d following 28 d of preculture on semi-solid medium.

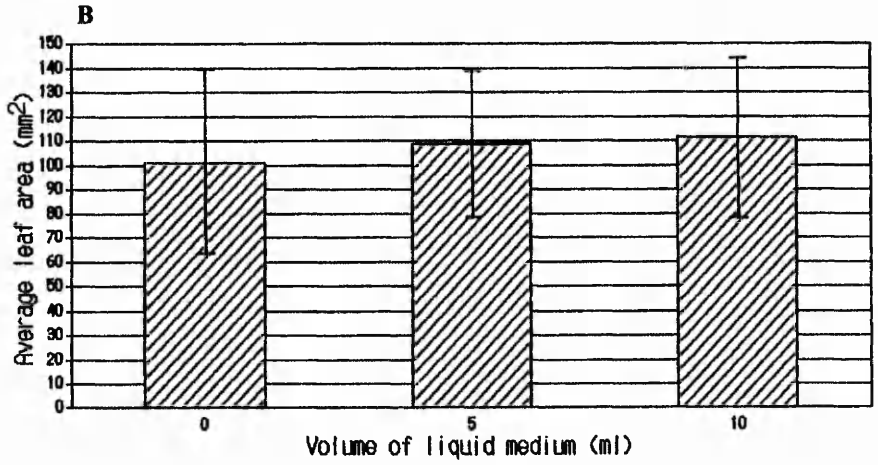
Growth characteristics	Control plants	Plants derived from double phase culture
Plant height (cm)	4.42 ± 2.63	5.38 ± 3.43
Axillary shoot production (%)	40.54	52.30
Root production (%)	29.73	23.81
Flower production (%)	94.59	95.24
Number of produced seeds	3.00 ± 2.45	3.14 ± 2.23
Seed set (%)	40.54	40.95
Hyperhydricity (%)	0.00	9.50

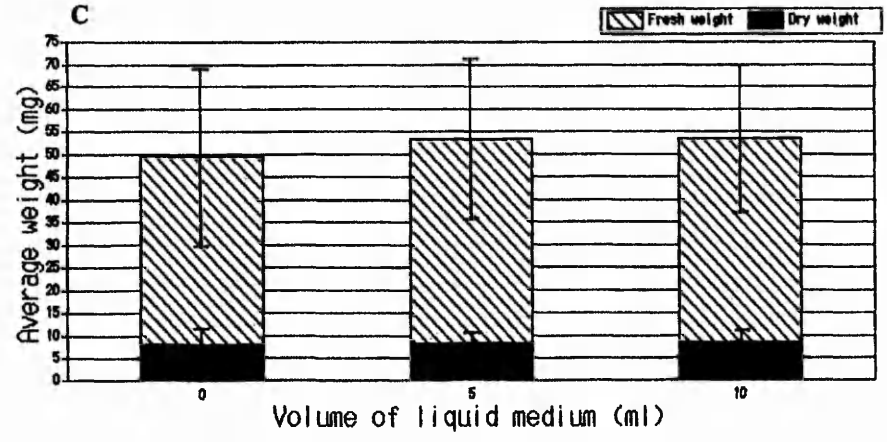
mean values ± SD

4.3.4 The application of liquid medium to cultures of Breeding Line E grown under a low temperature regime

The effect of culturing spinach explants in a double phase culture system was reduced by a growth temperature of 15°C. The monitored growth parameters of plant height, leaf area, fresh and dry weight did not increase significantly with larger volumes of the liquid medium (Figure 4.7). Although the overall plant quality was improved under the lower temperature regime in comparison to shoots cultured at 25°C in the previous experiments, axillary shoot production was not enhanced. The formation of inflorescences was also reduced to approximately 50 % in all treatments. However, none of the cultures grown at 15°C showed symptoms of hyperhydricity.







(Ratio fresh weight : dry weight: 0 ml - 6.59:1, 5 ml - 6.08:1, 10 ml - 6.35:1)

Figure 4.7: The influence of liquid medium on (A) plant height, (B) leaf area and (C) fresh and dry weight of Breeding Line E cultured at 15°C (mean values ± SD).

4.4 Discussion

Double phase culture systems, involving the addition of liquid medium to explants cultured on semi-solidified medium, have been employed to improve the performance of difficult species *in vitro*. Debergh (1994) has used this culture method for species which are prone to browning in culture. This system has the advantage that the liquid medium can be frequently exchanged and the accumulation of toxic substances in the liquid culture medium is thereby minimized. In addition, the control of systematic infection in tissue culture explants is possible by the addition of antibiotics to the liquid phase.

In this study, an objective of the work was to stimulate and to enhance axillary shoot production of spinach and a double phase culture regime has been tested. Nutrients and plant growth regulators are readily available to the explant in the liquid medium and absorption of nutrients is facilitated over the entire surface of the cultured plant material. Such a treatment may trigger the development of axillary shoots.

The different volumes of liquid medium employed caused an increase in plant height, an enlargement of leaf area and, in particular, enhanced fresh and dry weights compared to the control plants. None of the tested treatments, however, gave rise to an enhancement in axillary shoot production. The tested volume of 15 ml of liquid medium produced the best growth response, but also caused the highest percentage of plants to become hyperhydric. This tends to confirm the view of Kevers *et al.* (1984) that hyperhydricity may be a morphological response to waterlogging. The increased ratios between fresh weight and dry weight support this statement, indicating that larger amounts of water had been taken up with increased levels of liquid medium.

The exposure of plant material to the double phase culture treatment became more critical when 10 ml rather than 5 ml liquid medium were used. Over a treatment period of 14 days, plant height, leaf area and fresh weight were found to be significantly larger than those produced over a period of 7 days. The extension of the culture period in the double phase system, however, did not stimulate axillary shoot production. An extension of the preculture treatment period before the use of the liquid medium overlayer did not lead to enhanced axillary shoot production. However, these plants seemed to be less susceptible to hyperhydricity.

The reduction of the culture temperature to 15°C did not bring any additional advantages to the double phase culture system. None of the monitored growth parameters was affected, and control and treatments were found to produce similar results. The ratios of fresh weight to dry weight showed that at the lower temperature excessive water uptake was prevented and, therefore, the occurrence of hyperhydricity was eliminated.

Although no obvious benefit concerning the improvement of the multiplication rate of spinach was found with the use of a double phase culture system, the quality of spinach plants cultured with a liquid overlayer was enhanced over the standard treatment. The application of an overlayer of liquid medium was possible at a volume of 5 ml for 7 or 14 days or at a volume of 10 ml for 7 days without any detrimental effects to the plants. This information may be of value for the resolution of specific cultural problems. For example, Maene and Debergh (1985a), in order to stimulate root induction, applied liquid medium containing root inducing hormones to *Magnolia solangeana* cultures before transfer to *in vivo* conditions. In a different study, the addition of liquid nutrient medium to exhausted cultures of *Philodendron* proved to be feasible in order to avoid expensive routine manual transfer (Maene and Debergh, 1985b). However, considering the susceptibility of spinach to hyperhydricity in culture with liquid medium, the newly developed system by Teisson and Alvard (1994) allowing temporary immersion of the cultures in liquid medium, might offer an approach for the improvement of spinach cultures.

CHAPTER 5

The development of a regeneration system for spinach (*Spinacia oleracea* L.)

5.1 Introduction

Adventitious meristems are '*de novo*' shoot meristems, which arise from somatic cells in a fully developed organ. Almost every plant organ has been used for the initiation of adventitious meristems and most explant types have been excised from stem, roots and leaves (Hussey, 1974).

Preliminary experiments used various explant sources in attempts to stimulate adventitious shoot production in spinach. Cuttings were taken either from axenic seedlings (cotyledons, hypocotyls and radicles) or from *in vitro* grown spinach plants (leaves, stems, inflorescences and roots). Initially, the response of the explants was screened by applying the following protocols for shoot regeneration.

Adventitious shoot production from petiole explants of *Beta vulgaris* L. cultivars (*Chenopodiaceae*), was achieved after incubation on MS-based medium supplemented with 5.0 µM BAP (Ritchie *et al.* 1986). In contrast, high cytokinin additions to the incubation medium for spinach explants promoted intensive callus growth, and organogenesis did not occur. Earlier publications reported that organogenesis in spinach is a rare event and that the induction of organ formation does not follow the conventional pattern of auxin to cytokinin ratios (Neskovic and Radojevic, 1973). Sasaki (1989) induced adventitious shoots from hypocotyl explants cultured on Murashige and Skoog (1962) medium supplemented with 15 mg l^{-1} (85.62 µM) IAA and various concentrations of GA_3, depending on the spinach cultivar. A similar approach was taken by Xiao and Branchard (1993), leading to somatic embryogenesis from hypocotyl explants. Mii *et al.* (1992) found that incubation of hypocotyl segments for 20 days on Nitsch and Nitsch (1969) medium containing 10 - 15 mg l^{-1} (40.97 µM - 61.45 µM) of the synthetic auxin 5,6-dichloro-indole-3-acetic acid, followed by transfer to growth regulator-free medium, resulted in 80 % of cultured explants forming shoots.

Shoot regeneration has also been reported from leaf explant-derived callus. Al-Khayri *et al.* (1991a) achieved a regeneration frequency of 16.7 % when callus was incubated on MS-based medium supplemented with 2.0 mg l^{-1} (9.3 µM) kinetin, 0.01 mg l^{-1} (0.05 µM) 2,4-D and 1.0 mg l^{-1} (2.89 µM) GA_3. In a subsequent study, Al-Khayri *et al.* (1992a) regenerated shoots from callus derived from cultured seeds.

By following these protocols, no immediate success was obtained for shoot regeneration of spinach cultivars tested in the present study.

The objective of this study was an attempt to develop a reliable regeneration system for spinach and to analyse regenerants, following acclimatisation, for trueness to type. Flow cytometric analysis was employed to study the ploidy levels of regenerated plants and their progenies.

5.2 Materials and Methods

5.2.1 Media and conditions for the establishment of axenic shoot cultures

MS-based medium supplemented with 3.0 % (w/v) sucrose and 0.75 % (w/v) Oxoid agar was used to establish axenic cultures of the spinach Breeding Line E and the commercial cultivar 'Longstanding'. The pH of the medium was adjusted to 6.2 prior to autoclaving (121°C for 15 min).

Stem apices, 10 mm in length, were excised from germinated seedlings and transferred to MS-based medium supplemented with 1.0 µM NAA in 100 ml capacity screw-capped polystyrene containers (Sterilin) with 15 ml of the culture medium per vessel. Two shoot explants were cultured in each container.

All cultures were incubated under a 16 h photoperiod using 36 W White Pluslux 3500 fluorescent tubes, giving 42 ± 14 µE m^{-2} s^{-1} of irradiance at 25°C.

After 42 d, the roots which developed on the cultured shoots were used for studies of organogenesis (Plate 5.1 A).

5.2.2 Adventitious shoot production from cultured root explants derived from axenic shoots

Root explants were excised from the lateral roots of *in vitro* grown plants. Ten mm long sections from the tips, middle and basal region of the root explants were incubated on 20 ml aliquots of Nitsch and Nitsch (1969) medium containing 3.0 % (w/v) sucrose, 0.75 % (w/v) Oxoid agar (pH 6.2) in 60 ml capacity screw-capped Sterilin polystyrene containers. Growth regulator supplements for Breeding Line E were 20 µM NAA, 5.0 µM GA_3 and kinetin at concentrations of 0, 20, 60 and 100 µM. For 'Longstanding', the kinetin supplements were at concentrations of 0, 10, 20, 40 and 60 µM. Three explants were cultured in each container.

Root explants of both cultivars were incubated under the same culture conditions as those used to establish axenic shoot cultures. In two sets of replicates, 6 root explants from each section were assessed in each treatment for shoot regeneration after 63 d of culture.

In a second experiment, using Breeding Line E, root explants were incubated on culture medium containing 0, 10 or 20 µM kinetin only. After 35 d of incubation, the explants were transferred to the same culture medium and shoot regeneration was scored after 63 d of culture. The number (n) of replicates per treatment was 20.

5.2.3 Adventitious shoot production from explants of roots induced on hypocotyls

Hypocotyls, 15 mm in length, were excised from germinated seedlings of Breeding Line E and the commercial cultivar 'Longstanding'. The hypocotyl explants were incubated on 20 ml aliquots of Nitsch and Nitsch (1969) culture medium containing 3.0 % (w/v) sucrose, 0.75 % (w/v) Oxoid agar (pH 6.2) in 9 cm Petri dishes with 5 explants per dish. The growth regulator additions were 20 µM NAA and 0, 10, 20, 40 or 60 µM kinetin. Twenty five hypocotyl explants were allocated to each treatment.

After 21 d, the roots developing on the hypocotyls were excised and sectioned into 10 mm long segments. Tip, middle and basal root sections of 'Longstanding' were incubated on Nitsch and Nitsch (1969) culture medium containing 20 µM NAA, 5.0 µM GA_3 and 0, 20, 60 or 100 µM kinetin. In two sets of replicates, 5 root explants from each section were scored after 63 d in culture.

Root sections of Breeding Line E were incubated for 63 d on Nitsch and Nitsch (1969) medium supplemented with 20 µM NAA and 0, 1.0, 5.0, 10, 20 or 40 µM GA_3. The number (n) of replicates per treatment was 25.

The culture conditions for hypocotyl and root explants were the same as those described in 5.2.2, with the root explants being cultured in 60 ml capacity screw-capped polystyrene containers (Sterilin).

5.2.4 Light microscopy studies on cultured root explants

In order to identify the loci of adventitious shoot formation, specimens were prepared as described by Constabel (1982) and embedded in 'Historesin' (LKB, Bromma, Sweden). The protocol for the preparation of the specimens was used in the following sequence:

a. Fixation of the specimens in 3.0 % (v/v) glutaraldehyde in phosphate buffer (pH 7.0) for 16 h at 22°C after vacuum infiltration for 10 min.

b. After fixation, dehydration of the specimens in successive washes of 30 %, 60 % and 95 % (v/v) ethanol for 10 min each.

c. Soaking in half-strength infiltration solution ('Historesin') for 4 - 6 h at 4°C.

d. Soaking in full-strength infiltration solution overnight at 4°C.

e. Embedding in 'Historesin'.

Sections, 4.0 µm in thickness, were cut on metal knives, collected on glass slides and stained with 1.0 % (w/v) aqueous toluidine blue (BDH) for 60 s at 20°C prior to light microscopy.

5.2.5 Culture conditions for the maintenance of regenerating tissues of Breeding Line E and 'Longstanding'

Cubes (5 mm x 5 mm) of regenerating tissue, comprising a firm network of roots with associated callus and induced shoot buds, were excised and transferred to Nitsch and Nitsch (1969) culture medium containing 3.0 % (w/v) sucrose and 0.75 % (w/v) Oxoid agar supplemented with 20 µM NAA and 5.0 µM GA_3 in 60 ml screw-capped polystyrene containers (Sterilin). The regenerating plant material was incubated under a 16 h photoperiod using 36 W White Pluslux 3500 fluorescent tubes giving 42 ± 14 µE m^{-2} s^{-1} of irradiance at temperatures of either 15°C or 25°C.

In two sets of replicates, 30 explants were allocated to each treatment with 3 explants cultured in each container. Shoot development was assessed after 28 d in culture.

The maintenance of the shoot regenerative capacity of the culture was monitored over a period of 5 successive months. Regenerating plant material of Breeding Line E and 'Longstanding', consisting of roots, callus and shoot buds, was subcultured in cubes (5 mm x 5 mm) every 28 d. The culture conditions were the same as described before (5.2.5) and the temperature was 25°C. Shoots (> 4 mm) regenerated from 30 explants of both spinach varieties were scored and harvested every 28 d, before the next subculture.

5.2.6 The production of *in vitro* plantlets from regenerated shoots

Regenerated shoots of Breeding Line E, each with a minimum height of 4 mm, were transferred to MS-based medium, either devoid of plant growth regulators, or containing 1.0 µM NAA, or 4.9 µM IBA in Geneco containers (Fisons, Loughborough, UK) or alternatively in Magenta GA_7 (Sigma) containers. In two sets of replicates, 17 regenerated shoots were allocated to each treatment.

The shoots were grown under a 16 h photoperiod using 36 W Hybec White fluorescent tubes giving 45 ± 10 µE m^{-2} s^{-1} of irradiance at 15°C. After 28 d, the plants were subcultured onto the same growth medium and cultured for another 28 d, before they were assessed and transferred to glasshouse conditions.

5.2.7 The transfer of regenerated plants to the glasshouse

Plants of Breeding Line E were transferred to glasshouse with minimum night and maximum day temperatures of 10°C and 28°C, respectively, under natural daylight supplemented with a 16 h photoperiod. The additional light was provided by sodium high pressure lamps (Philips SON-T-AGRO 400), giving an irradiation of 307 ± 25 µE m^{-2} s^{-1}.

Agar residues were removed from the plant roots by washing with water, before transfer to a 3:1 (v/v) compost (J. Arthur Bower's seed and potting compost, Lincoln, UK) / perlite mixture. Trays were covered with a translucent plastic lid with vents in order to create a high humidity micro-climate. Seven days after transfer, the vents were opened and 14 d later the lids were removed. After an additional 14 d, the plants were transferred into individual plastic containers and left to mature. Regenerated plants were transferred to glasshouse conditions in 4 sets of 15 replicates.

5.2.8 Seed viability tests

Harvested seeds of regenerated plants of Breeding Line E that had grown to maturity were sown into soil (J.Arthur Bower's seed and potting compost, Lincoln, UK) in seed trays and germinated under the same glasshouse conditions as described in 5.2.7. After germination, 3 seedlings were planted into 11 cm plastic containers and the plants were allowed to grow to maturity. Harvested seeds from this generation were also tested for viability and compared with the germination rate of original seeds of Breeding Line E. Seeds of each generation were tested in 4 sets of replicates, with 20 seeds germinating in each seed tray.

All data obtained from each experiment were statistically processed as described in section 3.2.9.

5.2.9 Flow cytometric analysis

i. Plant material

Seeds of Breeding Line E, seeds collected from regenerated plants of Breeding Line E (R_1-generation) and from their first progeny (R_2-generation) were sown into compost as described in section 5.2.8. The first two fully expanded leaves after germination were used for flow cytometric analysis.

Leaves of *in vitro* grown shoots of Breeding Line E were used when the plants had reached the 4-6 leaf stage after germination. Regenerated shoots derived from root explants were cultured for six weeks (5.2.5) before analysis by flow cytometry. Five plants from each population were chosen as replicates.

ii. Flow cytometry

Nuclei were isolated from fully expanded leaves by chopping 0.5 g fresh weight of tissue in 2.5 ml of staining buffer (15 mM Tris, 2mM EDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 0.1 % (v/v) Triton X-100, 50 µg ml^{-1} ethidium bromide, pH 7.5; Dolezel *et al.*, 1989). The isolated nuclei were separated from the chopped leaves by passing the solution through a 30 µm nylon sieve. Fifty µl of a solution of fluorescently labelled 'Immuno-Check' beads (Coulter Electronics Ltd., Luton, UK) were added to the filtrate as an internal fluorescence standard.

Fluorescence measurements were performed using a Coulter EPICS 541 flow cytometer. The argon laser was tuned to produce 100 mW output at a wavelength of 488 nm. Ten thousand particles were analyzed per sample; single parameter green fluorescence histograms (log scale) were acquired and stored on disk. The histograms were transferred to an IBM PC computer and the relative mean linear fluorescence values of the G_0/G_1 nuclei were determined using programmes written by N.W. Blackhall (Plant Genetic Manipulation Group, University of Nottingham). Adjustments to these values were made to compensate for variations in the values obtained for the fluorescent beads. Fluorescence measurements were conducted by N.W. Blackhall.

5.3 Results

5.3.1 Adventitious shoot production from root explants of axenic shoots

Lateral roots developed on the primary root explants after 21 d of incubation on kinetin-free medium accompanied by callus production at the cut ends of the explants (Plate 5.1 B). Increasing levels of kinetin suppressed root development on the cultured explants and also inhibited shoot formation in Breeding Line E. 'Longstanding' was less sensitive to kinetin supplements in the culture medium and shoot regeneration still occurred at levels of 60 µM of kinetin. Shoot regeneration in both varieties became visible after 42 d of culture (Plate 5.2 A, B).

Tip and middle sections of roots displayed the highest shoot regeneration capacity. The regeneration of middle sections of Breeding Line E was significantly higher than that of the root tips (Table 5.1). Root tip sections of 'Longstanding', however, showed a significantly higher regeneration capacity than the middle or basal sections (Table 5.2). The cultured root basal explants showed limited organogenic potential.

Subculture of the root explants of Breeding Line E after 35 d incubation stimulated 50 % more root tip and basal sections to regenerate shoots in comparison to incubation without subculture. The average number of shoots per regenerating explant, however, was reduced (Table 5.3).

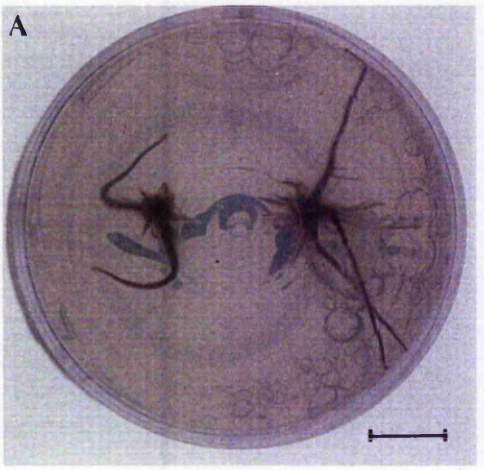


Plate 5.1: (A) Roots developed on cultured shoots of spinach, which were used for studies of organogenesis (bar = 0.7 cm).
(B) Lateral root growth on middle root sections after 21 d of incubation on kinetin-free culture medium (bar = 1.05 cm).

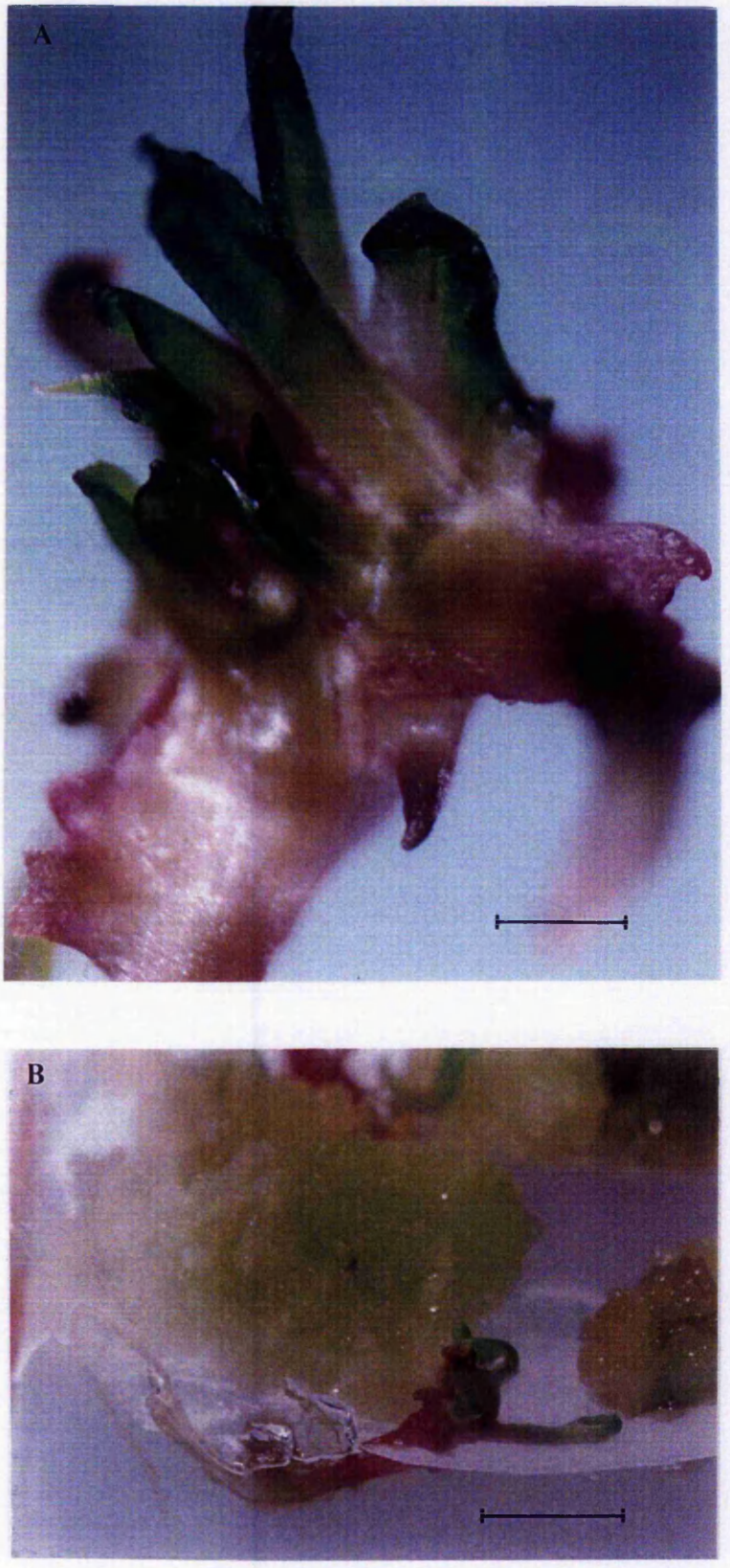


Plate 5.2: (A) Shoot buds induced on a middle root section of Breeding Line E and (B) a root tip section of 'Longstanding' after incubation of the explants for 42 d on Nitsch and Nitsch (1969) medium supplemented with 20 µM NAA and 5.0 µM GA_3 (A: bar = 0.25 cm, B: bar = 0.6 cm).

Table 5.1: Percentage of cultured spinach root explants producing adventitious shoots (Breeding Line E).

Root tip explants

Kinetin (µM)	Shoot regeneration (%)	Mean no. of shoots/reg. explant (mean values ± SD)
0	22.22 **	12.00 ± 7.07 *
20	0.00	0
60	0.00	0
100	0.00	0

Middle root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	33.33 **	9.33 ± 1.16 ***
20	0.00	0
60	0.00	0
100	0.00	0

Basal root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	6.67	1.67 ± 1.16
20	0.00	0
60	0.00	0
100	0.00	0

* = P(0.05) ** = P(0.01) *** = P(0.001)

Table 5.2: Percentage of cultured spinach root explants producing adventitious shoots ('Longstanding').

Kinetin (µM)	Shoot regeneration (%)	Mean no. of shoots/reg. explant (mean values ± SD)
Root tip explants		
0	63.63 ***	1.86 ± 1.22
10	8.33	2.00 ± 0.00
20	0.00	0
40	22.22 *	1.50 ± 0.71
60	60.00 ***	1.67 ± 1.21
Middle root explants		
0	33.33 *	1.67 ± 0.58
10	25.00 *	1.00 ± 0.00
20	0.00	0
40	36.36 *	2.00 ± 2.00
60	11.11	2.00 ± 1.41
Basal root explants		
0	8.33	1.50 ± 0.71
10	8.33	1.50 ± 0.71
20	0.00	0
40	0.00	0
60	25.00 *	1.50 ± 0.71

* = P(0.05) ** = P(0.01) *** = P(0.001)

Table 5.3: Percentage of cultured spinach root explants of Breeding Line E producing adventitious shoots after one subculture.

Root tip explants

Kinetin (µM)	Shoot regeneration (%)	Mean no. of shoots/reg. explant (mean values ± SD)
0	40.00 **	2.88 ± 2.48 *
10	0.00	0
20	0.00	0

Middle root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	61.11 ***	2.14 ± 1.42 *
10	0.00	0
20	0.00	0

Basal root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	20.00	1.00 ± 0.00
10	0.00	0
20	0.00	0

* = P(0.05) ** = P(0.01) *** = P(0.001)

5.3.2 Adventitious shoot production from root explants derived from hypocotyls of Breeding Line E and 'Longstanding'

Lateral roots developed on the hypocotyl explants after 21 d of incubation (Plate 5.3). The highest percentage of hypocotyl explants producing roots was achieved on kinetin-free medium and root production was inhibited at kinetin levels of 40 or 60 µM. Table 5.4 summarises the induction of roots on hypocotyl explants of both tested varieties.

Table 5.4: Lateral root production from cultured hypocotyl explants of Breeding Line E and 'Longstanding'.

	Root production (%)	
	Breeding Line E	'Longstanding'
Kinetin (µM)		
0	80.00 ***	96.00 ***
10	44.00	48.00
20	28.00	36.00
40	0.00	0.00

*** = P(0.001)

Roots derived from hypocotyls on kinetin-free medium of 'Longstanding' were sectioned and allocated to different kinetin treatments. Shoot regeneration occurred after 42 d of incubation. Root tip, middle and basal sections proved to be organogenic, with the root tip sections showing the highest percentage of regeneration on kinetin-free medium. The inclusion of kinetin in the culture medium only slightly reduced the number of regenerated shoots per explant and only a concentration of 100 µM kinetin prevented adventitious shoot production from all root explants (Table 5.5). No statistical differences were obtained for the average number of regenerated shoots per explant.

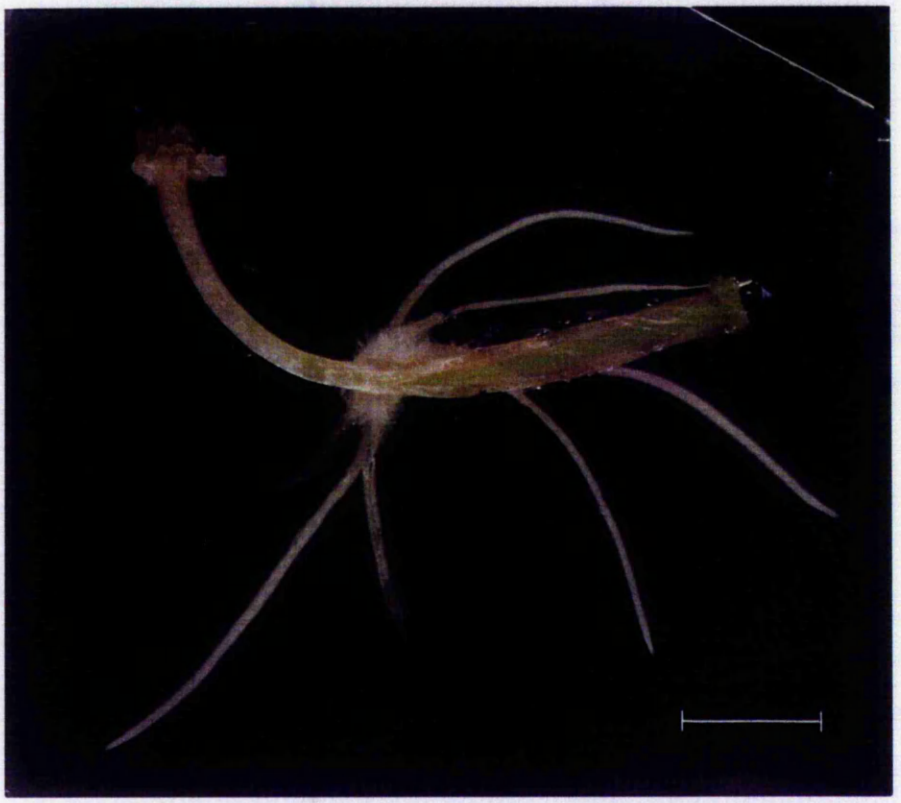


Plate 5.3: Lateral root growth on a hypocotyl explant of 'Longstanding' after 21 d incubation on Nitsch and Nitsch (1969) culture medium supplemented with 20 µM NAA (bar = 0.9 cm).

Table 5.5: Adventitous shoot production of cultured spinach root explants derived from hypocotyls of 'Longstanding'.

Root tip explants

Kinetin (µM)	Shoot regeneration (%)	Mean no. of shoots/reg. explant (mean values ± SD)
0	40.00 *	5.00 ± 2.45
20	20.00	3.00 ± 1.41
60	20.00	2.50 ± 0.71
100	0.00	0

Middle root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	20.00	5.50 ± 3.54
20	30.00	3.25 ± 0.96
60	0.00	0
100	0.00	0

Basal root explants

	Shoot regeneration (%)	Mean no. of shoots/reg. explant
0	30.00	2.14 ± 1.41
20	30.00	3.00 ± 1.00
60	30.00	1.50 ± 0.71
100	0.00	0

* = P(0.05)

Tip and middle root sections derived from hypocotyls of Breeding Line E were incubated on kinetin-free Nitsch and Nitsch (1969) medium supplemented with 20.0 µM NAA and various concentrations of GA_3. Table 5.6 indicates that a gibberellic acid concentration of 5.0 µM was the most advantageous for adventitious shoot production from root explants of spinach. The percentage of the incubated root explants responding to 5.0 µM GA_3 was highly significantly greater in comparison to the other treatments. The average number of shoots produced by the regenerating explants, however, do not differ statistically.

Table 5.6: Adventitious shoot production from cultured root explants derived from hypocotyls of Breeding Line E.

GA_3 (µM)	Shoot regeneration (%)	Mean no. of shoots/reg. root explant (mean values ± SD)
0	0.00	0
1.0	12.00	2.67 ± 1.53
5.0	56.00 ***	2.86 ± 1.46
10.0	8.00	2.00 ± 0.00
20.0	0.00	0
40.0	0.00	0

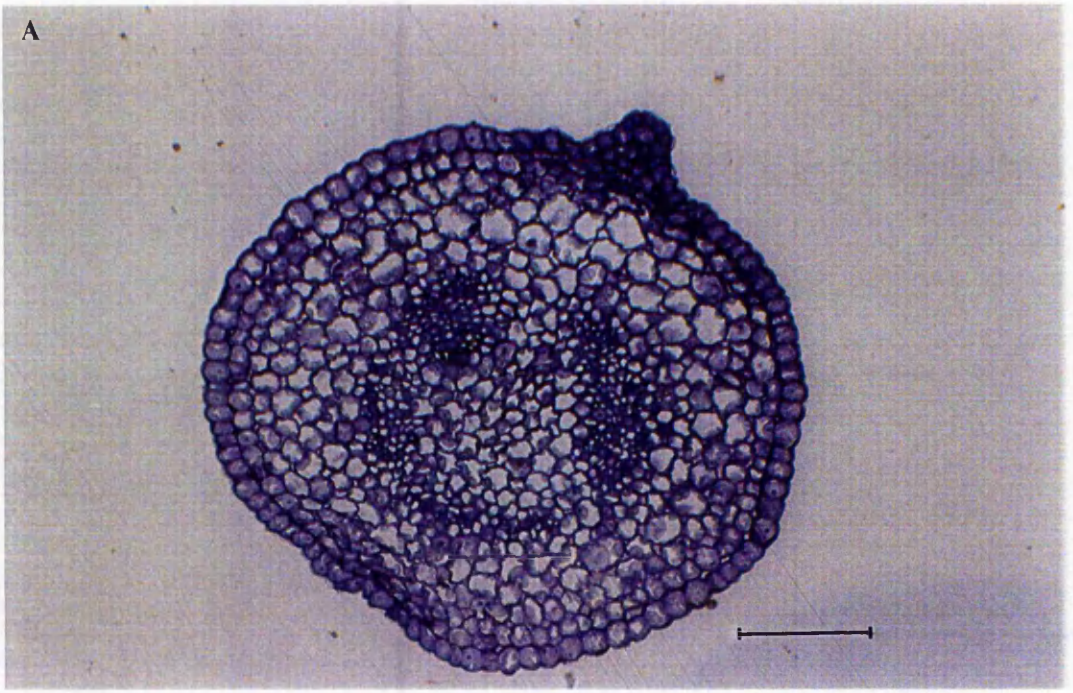
*** = P(0.001)

5.3.3 Light microscopical studies on regenerating root explants

Light microscopy confirmed that the origin of adventitious shoots was from root epidermal and subepidermal cells and not from callus tissue. A transverse section through a root explant, after 14 d incubation, showed actively dividing cells beneath the exodermis, indicating the initiation of meristematic activity (Plate 5.4 A).

Shoot primordia became visible after an additional 10 d of culture. A longitudinal section through a middle root segment showed unequivocally that the regenerating shoot originated directly from the root explant (Plate 5.4 B).

The sequence of shoot regeneration from spinach root explants is described schematically in Figure 5.1.



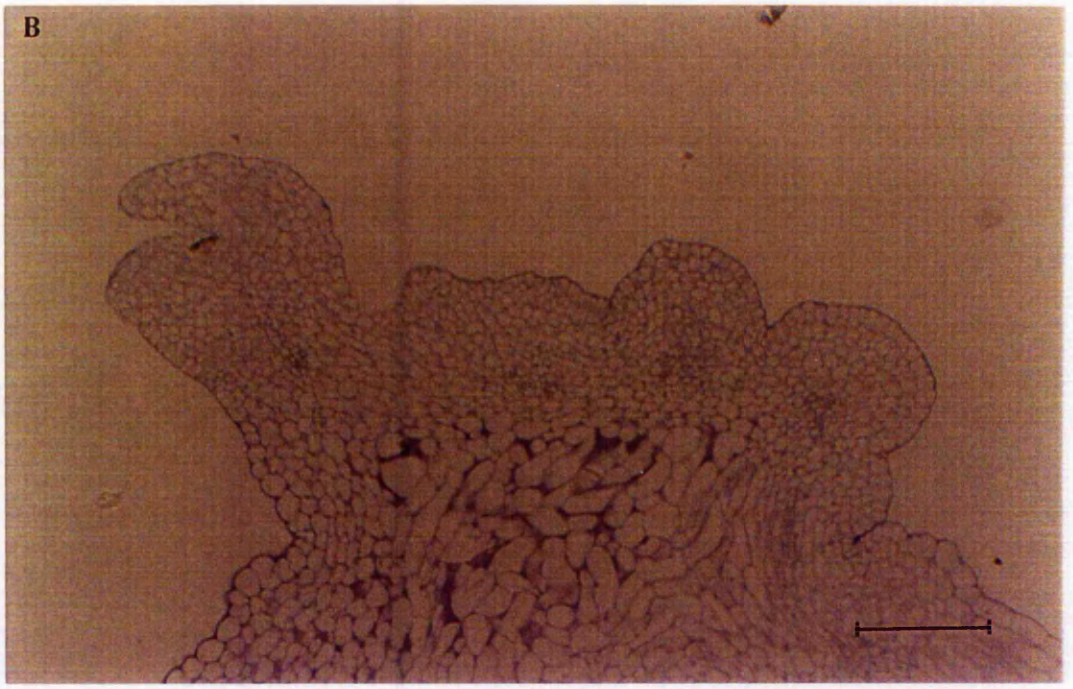


Plate 5.4: (A) Transverse section through a root explant after 14 d of culture showing meistematic regions developing in and below the epidermis (bar = 250 µm).

(B) Longitudinal section through middle root explant after 24 d incubation showing shot primordia originating from the explant (bar = 250 µm).

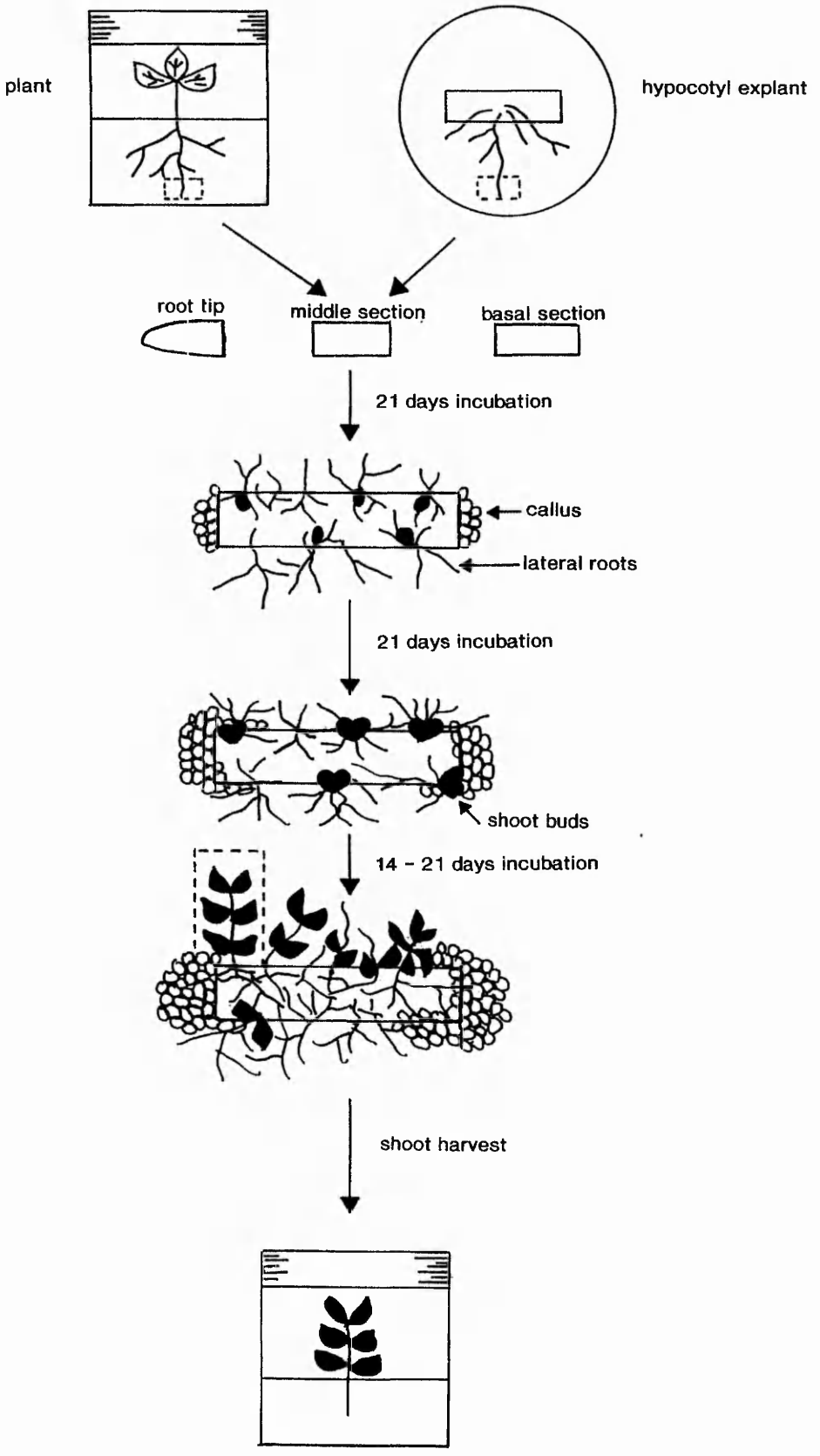


Figure 5.1: Schematic representation of the events during shoot regeneration from root explants derived from axenic shoots or hypocotyls.

5.3.4 The maintenance of shoot regenerating tissues of Breeding Line E and 'Longstanding'

Subcultured explants of Breeding Line E and 80 % of the cultured explants of the commercial cultivar 'Longstanding' continued to develop and to regenerate adventitious shoots throughout a culture period of 4 weeks. A reduction of the culture temperature from 25°C to 15°C was not advantageous with regard to plant quality or shoot production. The number of shoots produced from both spinach varieties decreased significantly following culture at 15°C (Table 5.7).

The height of the regenerated shoots of Breeding Line E and 'Longstanding' varied between 2 mm and 14 mm. The majority of the adventitious shoots regenerated at 25°C were less than 8 mm in height and most shoots regenerated at 15°C attained a height of less than 6 mm. Figure 5.2 illustrates the frequency distribution of the plant heights of regenerated shoots after 4 weeks of culture.

Table 5.7: The influence of temperature on shoot production of Breeding Line E and 'Longstanding'.

	Average number of shoots/reg.explant	
	Breeding Line E	'Longstanding'
Temperature		
15°C	1.97 ± 1.36	1.94 ± 1.32
25°C	3.14 ± 2.26 **	3.61 ± 2.97 **

Data obtained from first subculture explants;
** = P(0.01), mean values ± SD

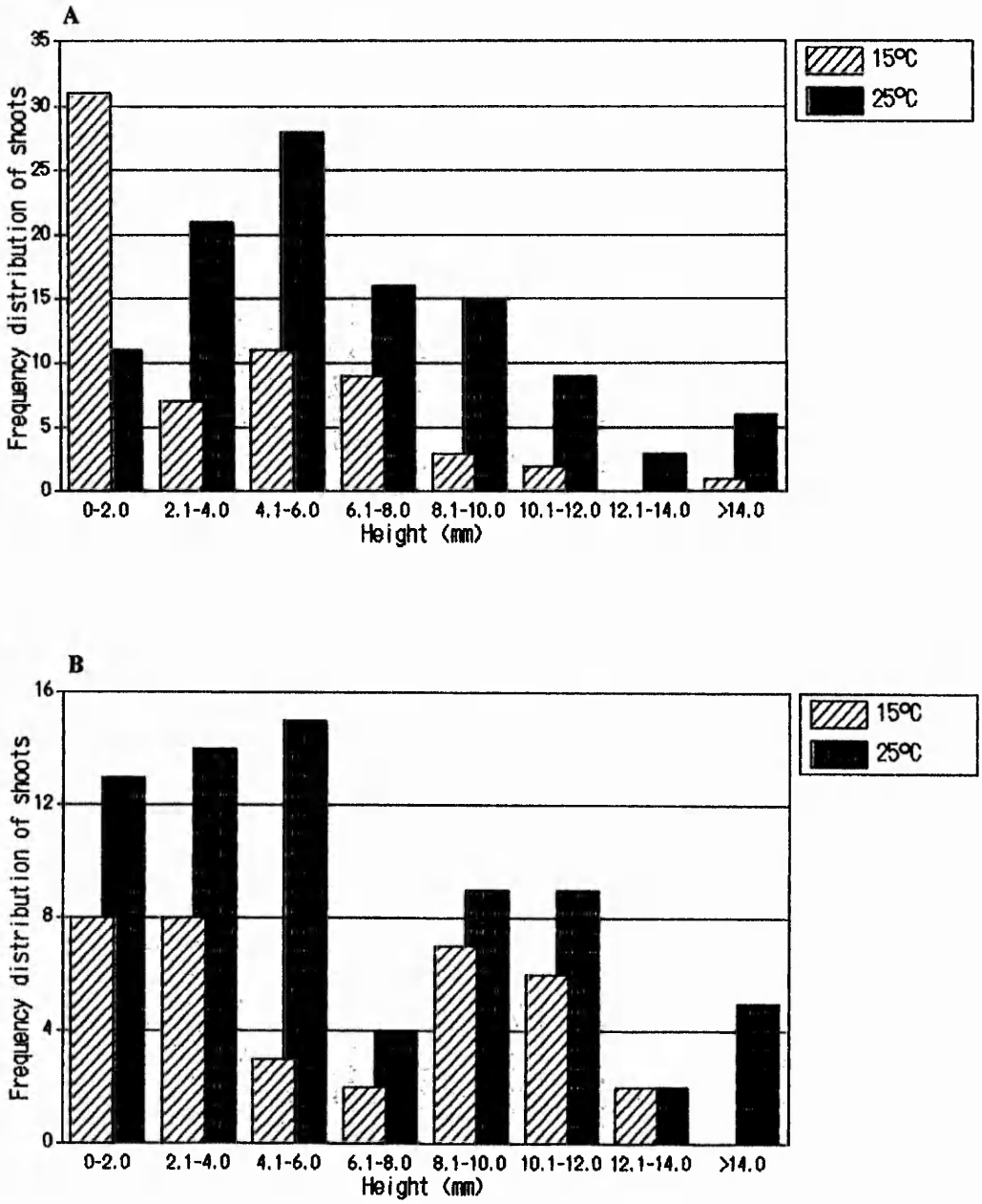


Figure 5.2: Frequency distribution of heights of regenerated shoots of (A) Breeding Line E and (B) 'Longstanding' obtained after 4 weeks of culture of regenerating plant material at 15°C or 25°C.

The regenerative capacity of both spinach varieties was monitored over a period of five successive months. Shoots of 4 mm in height and above were harvested and the remaining plant material was subcultured every 4 weeks onto Nitsch and Nitsch (1969) culture medium. Table 5.8 illustrates the ongoing adventitious shoot production of Breeding Line E and 'Longstanding'. A high percentage of the explants maintained their ability to regenerate shoots. The number of shoots produced per explant of Breeding Line E remained constant throughout successive subcultures, whereas the number of shoots regenerated by 'Longstanding' was more varied, but not significantly different. Plate 5.5 shows regenerated shoots with associated callus and roots after the first 4 weeks of subculture.

Table 5.8: Shoot production from explants of Breeding Line E ('E') and 'Longstanding' ('LS').

Culture period	% explants producing shoots		Mean no. of shoots/explant (mean values ± SD)	
	'E'	'LS'	'E'	'LS'
4 weeks	76.67	86.67	1.68 ± 0.67	1.57 ± 0.79
8 weeks	93.33	90.00	1.27 ± 0.47	2.75 ± 1.71
12 weeks	96.67	93.33	2.00 ± 0.77	1.63 ± 1.41
16 weeks	90.00	90.00	2.06 ± 0.97	3.22 ± 1.95
20 weeks	80.00	83.33	2.30 ± 1.16	3.82 ± 2.19

Shoot production was recorded at 4 week intervals over a 20 week period.

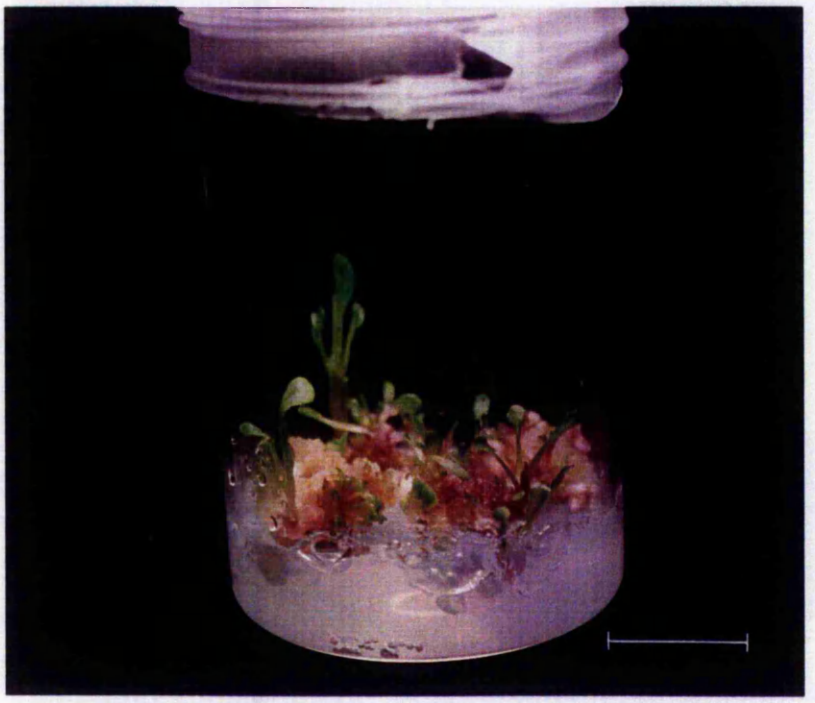


Plate 5.5: Adventitious shoot production of (A) Breeding Line E and (B) 'Longstanding' 4 weeks after subculture on Nitsch and Nitsch (1969) culture medium supplemented with 20 µM NAA and 5.0 µM GA_3 (bars = (A) 1.2 cm, (B) 0.8 cm).

5.3.5 The production of *in vitro* plantlets from Breeding Line E

Individual, adventitious shoots were cultured for 28 d on MS-based medium devoid of plant growth regulators or supplemented with 1.0 µM NAA or 4.9 µM IBA. Subsequently, the material was subcultured onto the same media for another 28 d. At this time, the shoots had developed into plantlets and were ready for the transfer to the glasshouse.

The highest percentage of plants which rooted in compost were those derived from culture medium containing NAA. Culture media without growth regulators or containing IBA did not promote root development (Table 5.9). However, those shoots that had developed at least 8 leaves, but no roots, were still considered suitable for the transfer to glasshouse conditions. At this time, approximately 75 % of the plantlets started to flower. Plate 5.6 A shows the growth of plantlets after 2 weeks in culture, while Plate 5.6 B demonstrates plant development after 7 weeks in culture and 1 week before transfer to the glasshouse.

Table 5.9: The influence of auxins on plant height, root formation and transfer to compost of regenerated shoots of Breeding Line E.

Plant growth regulator	Height (cm) (mean values ± SD)	Root formation (%)		Transfer to *ex vitro* conditions (%)
0	1.91 ± 0.52	20.00		82.86
NAA (1.0 µM)	2.26 ± 0.48	74.29	***	85.71
IBA (4.9 µM)	1.92 ± 0.41	22.86		82.86

*** = P(0.001)

Plate 5.6: The development of adventitious shoots of Breeding Line E in plantlets on MS-based culture medium supplemented with 1.0 μM NAA.

(A) Plantlets after 2 weeks in culture (bar = 1.0 cm)

(B) Plantlets after 7 weeks in culture and 1 week before transfer to the glasshouse (bar = 1.0 cm).

5.3.6 The acclimatisation of regenerated shoots of Breeding Line E to the glasshouse environment

Studies demonstrated that plants could be acclimatized to glasshouse conditions within 28 d of transfer from culture (Plate 5.7).

Fifty percent of the regenerated plants survived transfer to the glasshouse, but only 30 % of the plants continued to grow to maturity. Plate 5.8 A shows a regenerated plant 35 d after its transfer to glasshouse conditions.

All plants surviving transfer to glasshouse conditions flowered within 56 days and set seeds (Plate 5.8 B). After a total of 14 weeks of culture after transfer to the glasshouse, 35-40 clusters of mature seeds were harvested from each acclimatized plant. The seed clusters consisted of 5 - 12 seeds. The seeds were stored desiccated at 4°C in darkness until they were used for viability tests.

Plate 5.7: Variable growth response of acclimatized plants of Breeding Line E after 28 d of transfer to the glasshouse (bar = 5.08 cm).

A

B

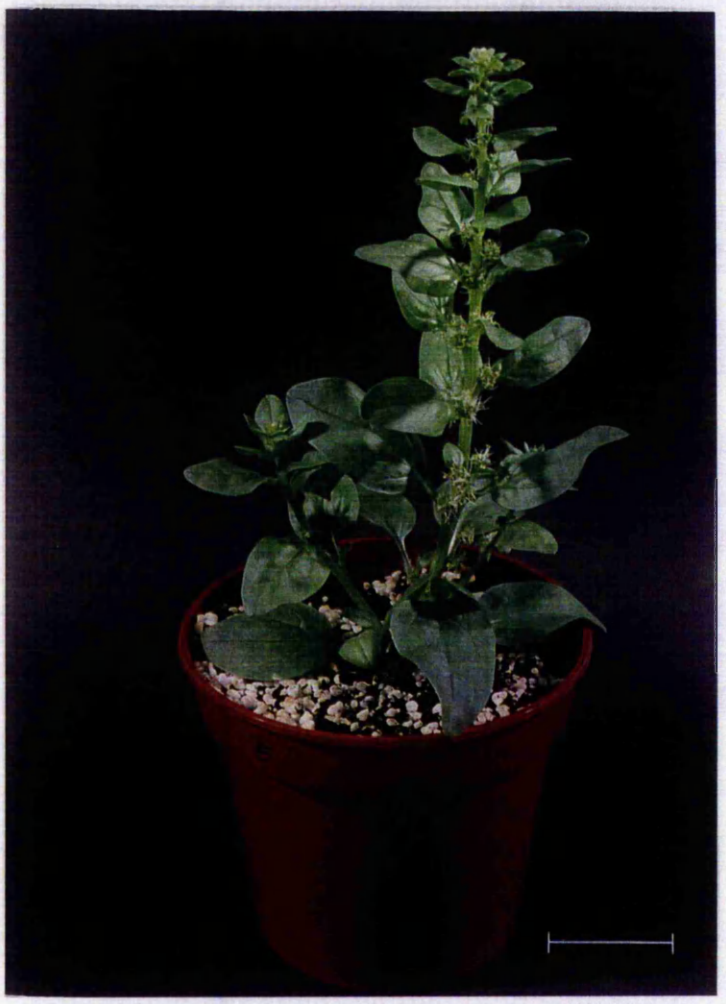


Plate 5.8: Regenerated plants of Breeding Line E

(A) 35 d after transfer to glasshouse conditions (bar = 5.0 cm)

(B) reaching maturation after additional 21 d (bar = 5.0 cm).

5.3.7 Seed viability of regenerated spinach plants and development of seed generations

Seeds collected from regenerated plants of Breeding Line E germinated 7 d after sowing in compost (Plate 5.9) Three plants at the two-leaf stage were planted into plastic containers. These plants were designated R_1-generation being the first seed propagated generation following shoot regeneration from explants. Plate 5.10 A shows 4-week old plants shortly before entering the flowering stage. The average plant height at this stage was 19.27 ± 6.90 cm. Nine weeks after seed germination, the R_1-plants flowered and began to set seed (Plate 5.10 B). Seeds were collected 4 weeks after seed set and also tested for viability, producing the second seed propagated (R_2-) generation.

Table 5.10 shows a comparison of germination rates of seed of the original Breeding Line E and of the R_1- and R_2-generations derived from regenerated plants. The lowest germination rate, which was still 82 %, was obtained by the R_2-seeds, but no statistical difference compared to the other generations was obtained. Plate 5.11 illustrates the development of plants of Breeding Line E and of R_2-plants 4 weeks after seed sowing. The number of leaves, leaf area and fresh and dry weights were determined at this stage of development (Figure 5.3). The fresh weight achieved by seed-derived Breeding Line E was significantly higher than that of the R_1- or R_2-generations. However, no significant differences could be detected between the growth parameters of number of leaves and leaf area.

Table 5.10: Germination rates of seeds of Breeding Line E and of R_1- and R_2-generations.

	Germination rate (%)
Breeding line E	92.50
R_1-Generation	95.08
R_2-Generation	82.50

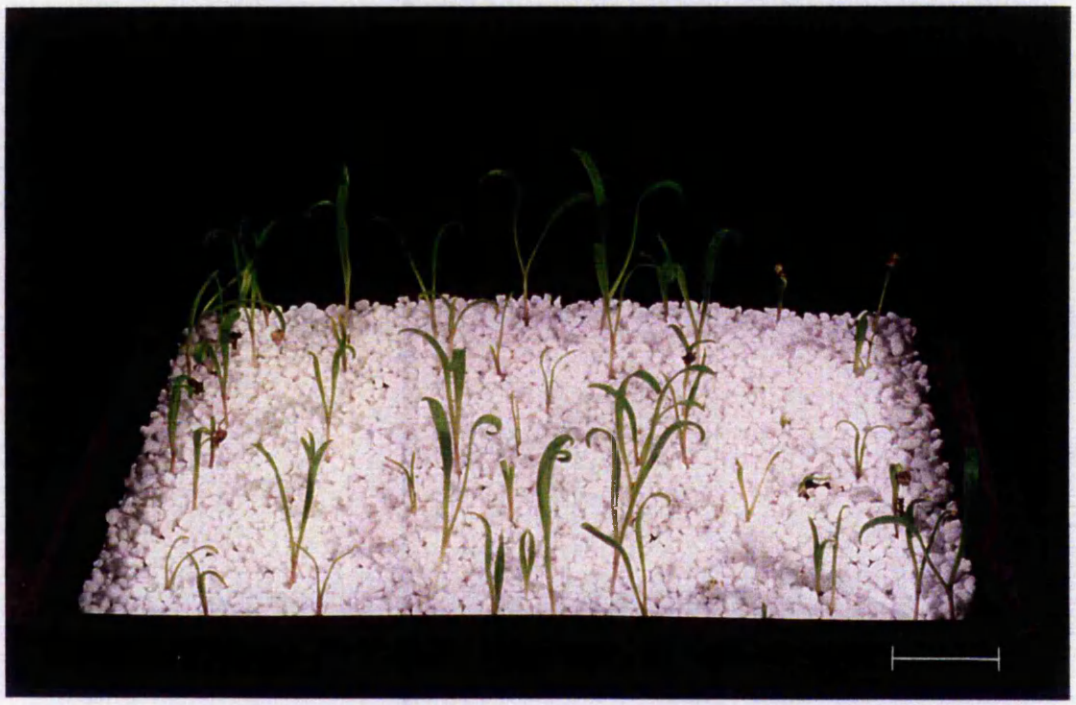


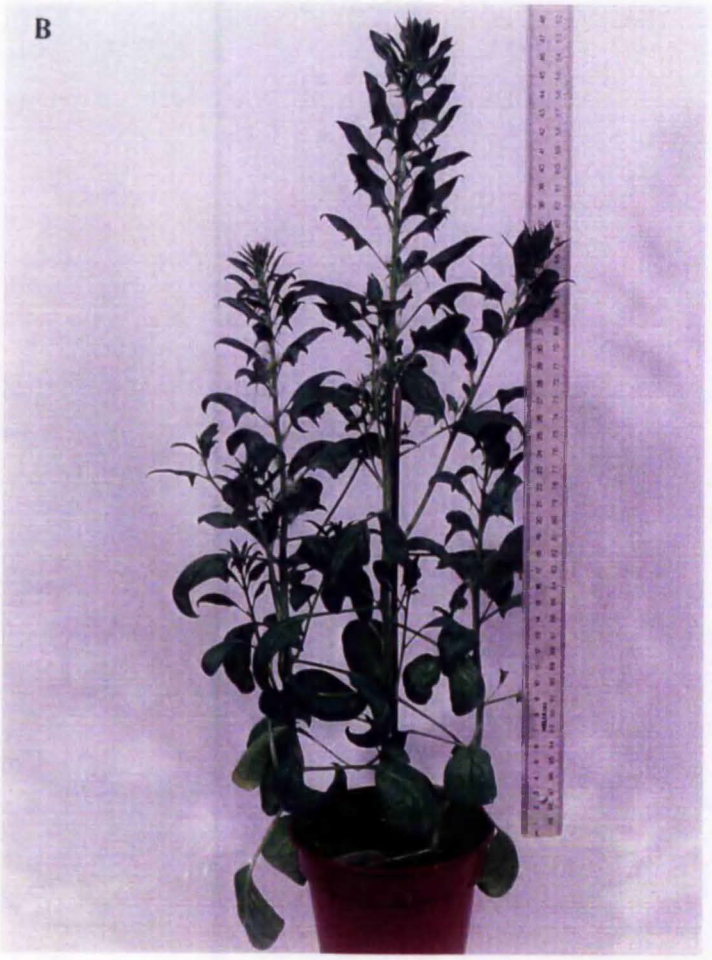
Plate 5.9: Germinated seeds (R_1-generation) from regenerated plants of Breeding Line E 7 d after sowing in compost (bar = 4.65 cm).

Plate 5.10: Development of R_1-generation plants of Breeding Line E

(A) 4 week-old plants

(B) 9 week-old plants flowering and setting seeds.


A


B

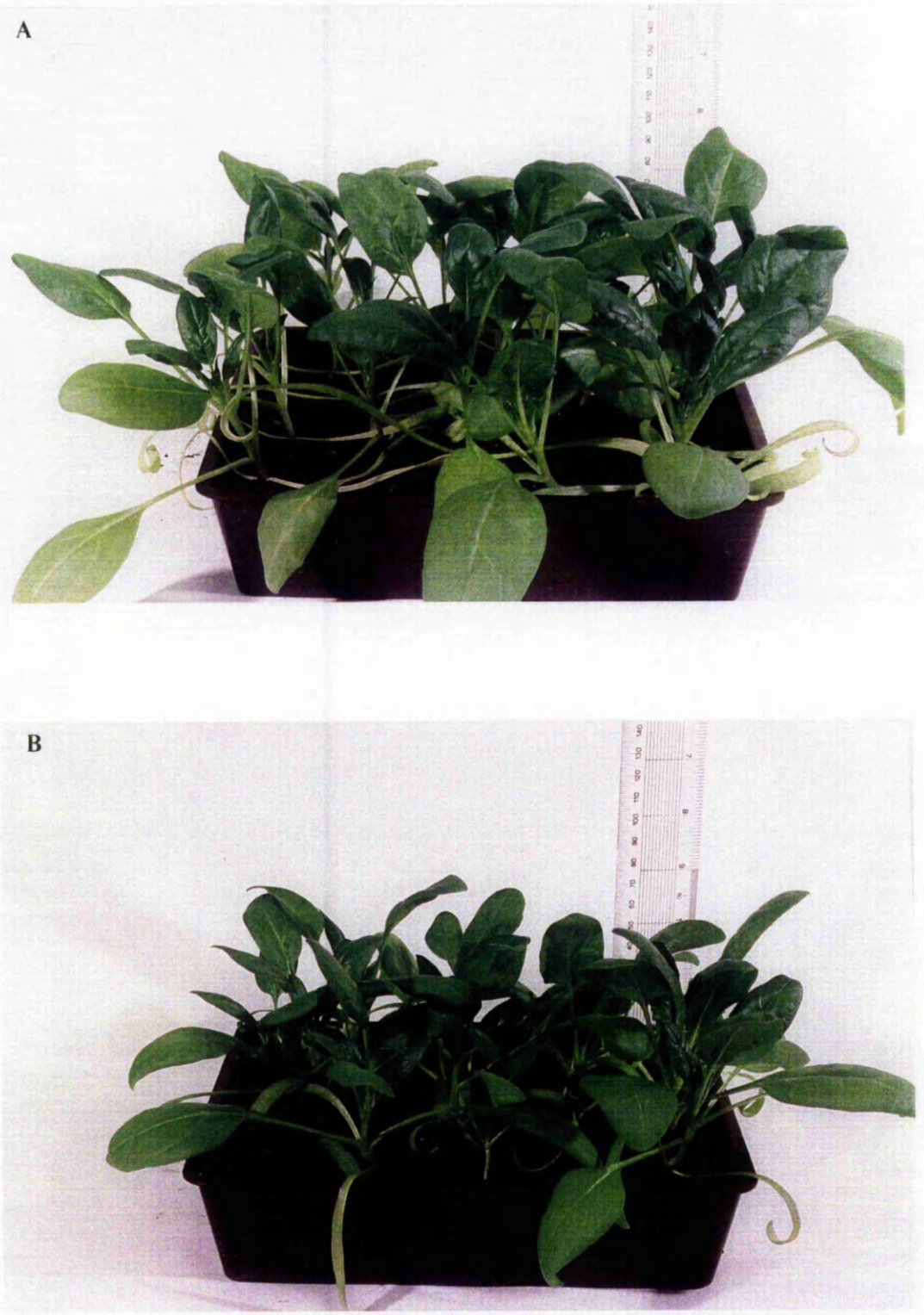


Plate 5.11: Plants of (A) original Breeding Line E and of (B) R_2-generation of Breeding Line E, 4 weeks after seed sowing.

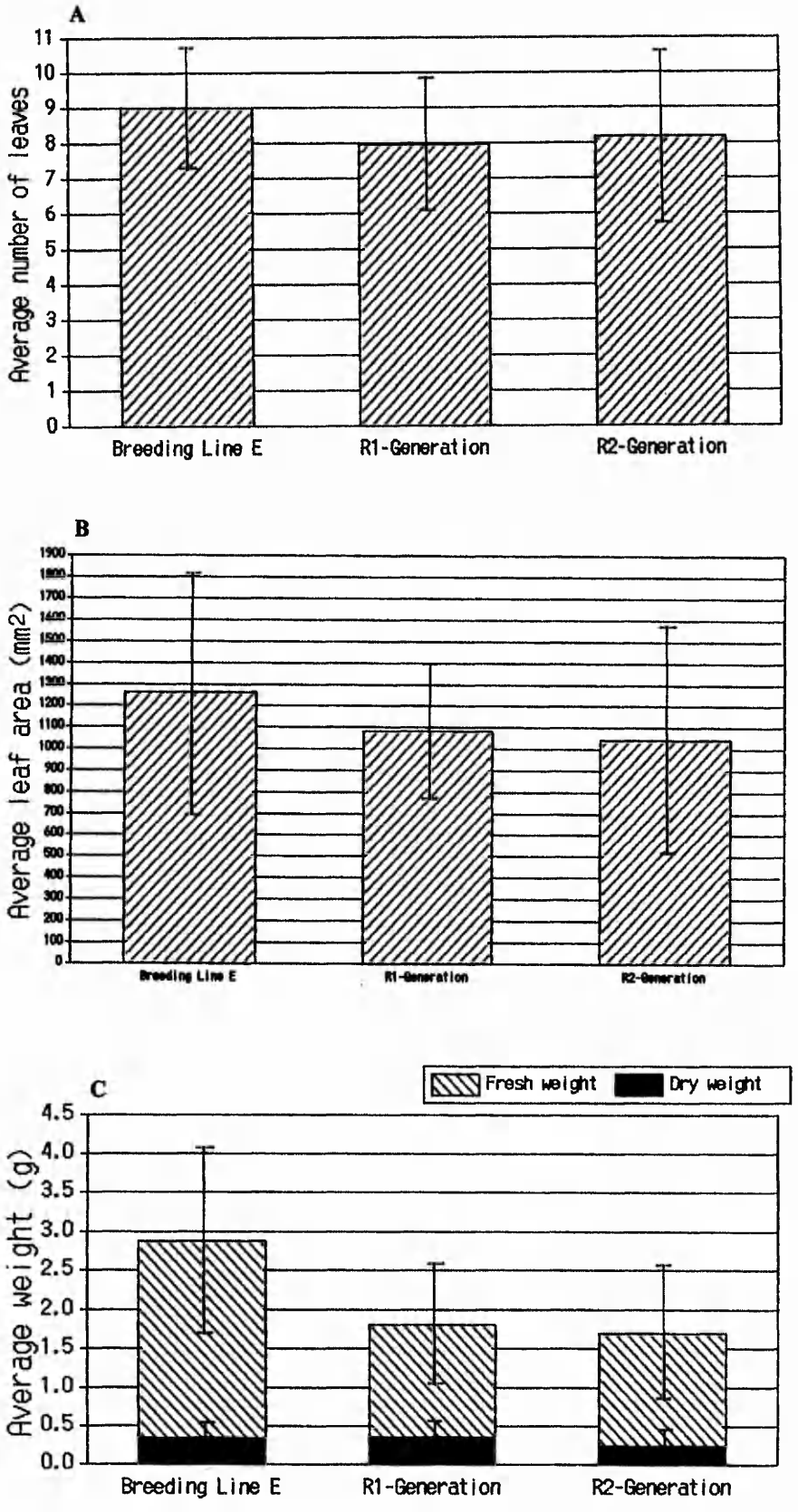


Figure 5.3: Comparison of the (A) average number of leaves, (B) leaf area and (C) fresh and dry weights achieved by 4-week old spinach plants from three different generations of Breeding Line E.

5.3.8 Flow cytometric analysis of *in vitro* and glasshouse-grown plant material of Breeding Line E

Flow cytometric analysis for different generations of Breeding Line E revealed that an even proportion of cells in leaf tissue were arrested in the G_0/G_1 and G_2/M phases and the ratio between G_0/G_1 cells and G_2/M cells was uniform for each generation tested (Figure 5.4). The uniformity between the ratios of the two peaks indicated that, in spinach leaf material, an equal number of cells were present with either resting nuclei or passing through mitosis. The G_2/M peaks, however, could also have been a result of tetraploidy.

Seed propagated plants of the original Breeding Line E, both glasshouse-grown and those maintained *in vitro*, and the glasshouse grown R_1- and R_2-generations derived from regenerated plant material produced similar fluorescence peaks and, statistically, differences were not obtained for the DNA content. Regenerated plants from root explants, however, appeared to be different to the seed propagated plants. Smaller fluorescence values were obtained, which suggested a loss of DNA.

The difference of DNA content between regenerated plants and seed propagated plants was statistically secured at a probability of 95 % (Blackhall, 1995).

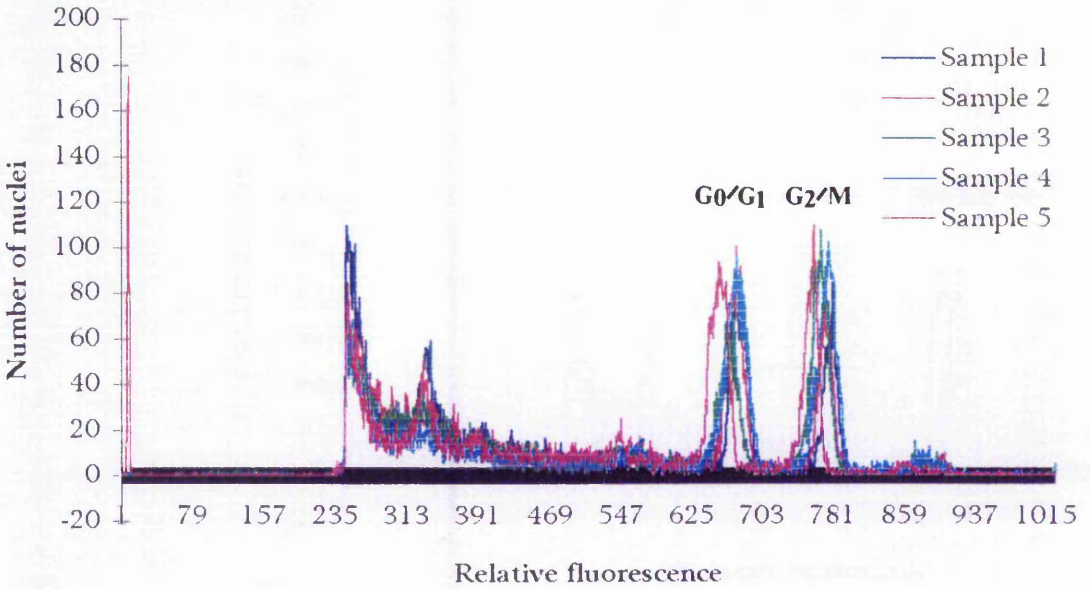


Figure 5.4 : Fluorescence histogram of spinach leaf nuclei.

Key to legend: Sample 1: R_1-generation, glasshouse-grown

Sample 2: Original Breeding Line E, glasshouse-grown

Sample 3: R_2-generation, glasshouse-grown

Sample 4: Original Breeding Line E, *in vitro*

Sample 5: R_0-generation, *in vitro*

5.4 Discussion

In previous research on shoot regeneration of spinach, Sasaki (1989) and Al-Khayri *et al.* (1991a,b, 1992a) were able to induce shoot regeneration on hypocotyl segments and on callus derived from leaves and mature seeds. Shoot regeneration was stimulated by 10 mg l^{-1} (57.09 µM) IAA (Sasaki, 1989), and by 15 mg l^{-1} (85.62 µM) IAA or lower concentrations of the synthetic auxin 5,6-Cl_2-IAA (Mii *et al.*, 1992). Studies by Al-Khayri employed a growth regulator combination of 0.045 µM 2,4-D and 9.3 µM kinetin, which resulted in shoot regeneration. Attempts to repeat their work with spinach varieties used in the present study were unsuccessful. In particular, treatment of hypocotyl explants of Breeding Line E and 'Longstanding' with 5,6-Cl_2-IAA at concentrations of 10 and 20 mg l^{-1} resulted in necrosis of the explants.

In the present study, shoot formation was obtained only from root explants. This was achieved by a hormone treatment of 20 µM NAA and 5.0 µM GA_3. Root tip explants and middle root explants of Breeding Line E and 'Longstanding' exhibited the highest shoot regeneration capacity. Shoot regeneration of Breeding Line E was suppressed on culture medium containing kinetin. While the commercial cultivar tolerated kinetin supplements in the regeneration medium, an improvement of the regeneration rate was not achieved.

The previously successful studies on plant regeneration in spinach reported that root formation also occurred in addition to shoot regeneration. Al-Khayri *et al.* (1991a,b, 1992a) observed proliferation of roots on leaf explant-derived callus, whilst Sasaki (1989) observed root formation on hypocotyl segments of spinach. Mii *et al.* (1992) found that callus growth occurred on hypocotyl explants after 10 days of culture. Roots were formed with the callus and shoot regeneration occurred after an additional 28 days. In the present study, the use of hypocotyl segments of Breeding Line E and 'Longstanding' resulted in callus and root formation along the entire length of the explants, but shoot formation could not be achieved. Adventitious shoot production, however, could be initiated when the adventitious roots were excised from the hypocotyl explant and incubated on Nitsch and Nitsch (1969) medium supplemented with 20 µM NAA and 5.0 µM GA_3.

Histological studies on regenerating root explants showed unequivocally that shoots were induced directly from root explants, without passing through a callus phase.

Gibberellic acid has been used in all previous investigations on shoot regeneration in spinach. For example, Al-Khayri (1992c) found that 3.0 µM GA_3 not only stimulated adventitious shoot production, but was beneficial for shoot regeneration from callus. In the present study, GA_3 was found to be essential in stimulating shoot initiation and elongation when adventitious shoots were induced on root explants. A gibberellic acid supplement of 5.0 µM encouraged 56 % of the root explants of Breeding Line E to regenerate shoots, whereas the omission of GA_3 did not induce shoot regeneration. Zeevaart *et al.* (1993) confirmed that gibberellic acid was required for stem elongation in spinach.

Once shoot regeneration had been initiated, the regenerative capacity of the explants continued, with the production of shoots and roots. This source material could be maintained over a period of several months without any noticeable decline in regenerative capacity. A continuous harvest of shoots was therefore possible, which could be exploited for the production of rooted, acclimatized plantlets.

One limiting factor for the successful acclimatisation of plantlets was the change from an environment of high humidiy to one of low humidity. Ritchie *et al.* (1991) used the anti-gibberellin 'Paclobutrazol' in their rooting medium to induce *in vitro* hardening of chrysanthemum and sugar beet plants. Spinach, however, showed a high sensitivity to 'Paclobutrazol' treatment, which inhibited plant growth. Before transfer to the glasshouse, Al-Khayri *et al.* (1991 a,b) rooted regenerated shoots on a culture medium containing 1.0 $mg\,l^{-1}$ (4.9 µM) IBA. Sixty seven percent of the plants formed roots and a survival rate of 40 - 70 % in the glasshouse was achieved. In the present study, the inclusion of 4.9 µM IBA in the culture medium resulted in only 22 % of the shoots rooting. However, shoots were successfully rooted on MS-based culture medium containing 1.0 µM NAA. About 74 % of the regenerated shoots were rooted in this way and approximately 50 % of the plants survived upon the transfer to glasshouse conditions. Surviving plants showed a varying growth response, while those that grew to maturity were similar in appearance and general morphology.

Acclimatized plants could be grown to maturity and were fertile. Seeds collected from these plants were fully viable and produced a homogeneous R_1-generation. Plants of the R_1-generation also grew into mature plants, completing their natural life cycle by producing viable seeds for the following progeny.

Flow cytometric analysis was used to determine the stability of ploidy levels of regenerated plants of Breeding Line E and their progenies. Flow cytometry has a wide field of application, being used to analyse the cell cycle (Galbraith *et al.*, 1983), to characterise and to purify chromosomes (Gray and Langlois, 1986) and to select heterokaryons after somatic hybridisation (Hammatt *et al.*, 1990). Flow cytometry can be employed as a rapid large-scale screening method for estimating chromosome numbers. Chromosome numbers of somatic hybrids within the *Brassicaceae* could be determined with an accuracy of ± 10 % (Fahleson *et al.*, 1988).

Numerous studies have shown that the DNA content per genome is usually constant and therefore characteristic for each species. However, considerable interspecific variation in DNA content per genome has been noted (Bennett and Smith, 1976).

In the present study, flow cytometric analysis has shown that leaf material of spinach Breeding Line E contained an even proportion of G_0/G_1 cells and of G_2/M cells. A similar fluorescence histogram of the DNA content was obtained for seed propagated plants germinated from original seeds of Breeding Line E and for plants germinated from seeds obtained from regenerated plants, producing the R_1-generation together with their progeny, giving the R_2-generation. The high proportion of G_2/M cells found in the leaf tissue may be characteristic for spinach cultivars as they were observed in the original diploid breeding line as well as in the seed progeny derived from regenerated plants and may be a result of high mitotic activity. However, a high proportion of G_2/M cells may also be indicative of tetraploidy. Jay de Rocher *et al.* (1990) found in their study on *Mesembryanthemum crystallium*, for example, that all analysed tissues were multiploid.

The loss of DNA observed in regenerated plants from root explants (R_0-generation) was not detected in their progenies. The occurrence of this phenomenon may be caused by the physiology of the leaves used, considering that leaf material of regenerants was older than that of germinated seedlings.

CHAPTER 6

Transformation studies on spinach (*Spinacia oleracea* L.)

6.1 Introduction

Genetic transformation offers an alternative strategy in plant breeding to introduce single gene traits into crop plants with the goal of plant improvement. The use of this novel approach offers the possibility of transferring genes from a variety of organisms, for example from plants, microorganisms or animals, to a target species (Perani *et al.*, 1986).

The *Agrobacterium tumefaciens*-mediated transformation system is the most widely used method for introducing foreign genes into dicotyledonous plants, the latter being natural hosts of the soil-borne bacterium. *Agrobacterium* carries a large virulence plasmid, the Ti (tumor-inducing) plasmid, that possesses the ability to insert a part of its DNA into the chromosome of the host plant cell. The transferred DNA, the T-DNA, on the Ti plasmid can be replaced with genes of interest for crop improvement (Chilton *et al.*, 1980).

To date, no reports are available on the genetic manipulation of spinach. Within the *Chenopodiaceae*, only sugar beet has been transformed using the *Agrobacterium tumefaciens* strain LBA 4404 by inoculation of shoot base tissue (Lindsey and Gallois, 1990).

The production of transgenic plants requires stable integration of the T-DNA into a recipient nuclear genome and, subsequently, the regeneration of shoots. In the present study, the effects of pTOK47 have been investigated in conjunction with the binary vector pMOG23 (Sijmons *et al.*, 1990) on the transformation of spinach root segments or excised regenerating spinach tissue. The hypervirulent helper pTOK47 contains the virulence genes (*vir* B, *vir* C and *vir* G), which increase the ability of *Agrobacterium tumefaciens* to transform plants (Jin *et al.*, 1987). The transformation method applied was adopted from Curtis *et al.* (1994), who successfully transformed various lettuce cultivars using *Agrobacterium tumefaciens*.

The aim of the present study was to evaluate the susceptibility of spinach to *Agrobacterium* mediated transformation, and to find a method for gene transfer into organogenic tissues of spinach. The establishment of a transformation system for spinach will be essential for the subsequent insertion of agronomically important genes into this leafy vegetable.

6.2 Materials and Methods

6.2.1 Plant materials and culture conditions

Four types of plant material of Breeding Line E and of the variety 'Longstanding' were used for inoculation with *Agrobacterium*:

- Root tip and middle segments, each 5 mm in length, from germinated seedlings.
- Root tip and middle segments, each 5 mm in length, from established axenic shoot cultures. Root segments were incubated on Nitsch and Nitsch (1969) shoot regeneration medium for 2 or 8 weeks, before the root material was used for experimentation.
- Cubes (3 mm x 3 mm), comprising shoot buds, callus and roots of regenerating plant material originally initiated from root explants, or newly produced roots excised from regenerating plant material. The regenerating plant tissue used was 12, 18, 20 and 24 months old.

All plant materials were transferred to 20 ml aliquots of Nitsch and Nitsch (1969) culture medium containing 3 % (w/v) sucrose, 0.75 % (w/v) Oxoid agar (pH 6.2) and supplemented with 20 µM NAA and 5.0 µM GA_3 2 d before inoculation with *Agrobacterium tumefaciens*. Four explants were cultured in 60 ml capacity screw-capped Sterilin polystyrene containers at 25°C under a 16 h photoperiod using 36 W White Pluslux 3500 fluorescent tubes, giving 42 ± 14 µE $m^{-2}s^{-1}$ of irradiance.

6.2.2 Tests for antibiotic tolerance

i. Carbenicillin

Tip and middle root sections from 8 week-old shoot cultures of Breeding Line E were incubated on Nitsch and Nitsch (1969) medium containing 20 µM NAA and 5.0 µM GA_3. Carbenicillin (Sigma) was added to the culture medium in concentrations of 0, 100, 200, 300, 400, 500 µg/ml. Shoot regeneration from the root explants, fresh weight, and further root development was scored after 12 weeks incubation. In 2 sets of replicates, 10 root explants were exposed to each carbenicillin treatment.

ii. Kanamycin sulphate

Cubes of regenerating plant material of Breeding Line E comprising roots, callus and shoot buds were incubated on Nitsch and Nitsch (1969) medium containing 20 µM NAA and 5.0 µM GA_3. Kanamycin sulphate (Sigma) was included in the culture medium at concentrations of 0, 50, 75, 100, 125 and 150 µg/ml. Shoot development and further root growth were assessed after 4 weeks incubation. Ten explants in 2 sets of replicates were tested against each kanamycin treatment. Data obtained from both antibiotic tests were statistically processed as described in section 3.2.9.

6.2.3 Bacterial strains and plasmids

The bacterial strains used for spinach transformation were provided by the University of Nottingham. The binary vector pMOG 23 (Sijmons *et al.* 1990) contained the chimaeric *nos.npt*Ⅱ.*nos* gene located between the left and right border sequences, which confers resistance to kanamycin in plants. A CaMV35S *gus*-intron reporter gene (Vancanneyt *et al.*, 1990) was inserted in a multiple cloning site between the border sequences. This reporter gene enabled transformed plant cells to degrade the substrate X-Gluc (5-bromo-4-chloro-3-indoyl *β*-D-glucuronic acid) to be identified by their blue colouration through the production of an indigo dye at the site of the reaction. Through triparental mating, pMOG 23 was introduced into *Agrobacterium tumefaciens*. The resulting strain was designated '0065'. The plasmid TOK 47 (Jin *et al.*, 1987) was introduced into '0065' generating the strain '1065'.

Both *A. tumefaciens* strains were tested for spinach transformation, evaluating the effect of the hypervirulent plasmid on transformation by using strain '1065'.

6.2.4 Initiation of bacterial cultures

Bacterial cultures were initiated from -70°C glycerol stocks by streaking onto Luria broth (Sambrook *et al.*, 1989), which was semi-solidified with 0.8 % (w/v) agar (Sigma). The bacterial culture medium was supplemented with 100 µg/ml rifampicin, 50 µg/ml kanamycin sulphate and 5 µg/ml tetracycline-HCl. The cultures were incubated for 1-2 days at 25°C in the dark to stimulate multiplication of the bacteria.

Liquid cultures were established by transferring bacteria from agar plates to 10 ml or 20 ml of Luria broth in 30 ml capacity screw-capped tubes or 100 ml capacity conical flasks, respectively. The liquid medium contained 40 µg/ml rifampicin, 50 µg/ml kanamycin sulphate and 2 µg/ml tetracycline-HCl. The liquid cultures were incubated in the dark at 28°C for 16 h on a horizontal rotary shaker (150 rpm). The bacterial cultures were grown to an optical density ($O.D._{600}$) of 1.10-1.75, before being used to inoculate spinach explants.

6.2.5 Co-cultivation of organ segments with *Agrobacterium tumefaciens*

Root segments from spinach cultures or cubes of regenerating plant material were floated in a 1:10 (v/v) dilution of an overnight culture of *Agrobacterium* for 10 min or were dipped in a 1:1 (v/v) dilution of the bacterial suspension for 2-3 sec. Excess bacterial solution was removed by blotting on sterile filter paper. Liquid Nitsch and Nitsch medium (1969) was used for the dilution of the bacterial overnight cultures.

Spinach explants and *Agrobacterium* were co-cultivated for 2 d, before transfer to antibiotic-containing culture medium. The Nitsch and Nitsch (1969) medium was supplemented with 100 µg/ml carbenicillin, 100 µg/ml cefotaxime (Sigma) and 50 µg/ml kanamycin sulphate. Plant growth regulators were added at concentrations of 20 µM NAA and 5.0 µM GA_3.

After 7 d, carbenicillin was omitted from the culture medium. Explants were transferred to carbenicillin-containing medium for further 7 d, if bacterial growth recurred.

After 4 weeks of culture, the explants were transferred to Nitsch and Nitsch (1969) regeneration medium containing 50 µg/ml kanamycin sulphate in order to maintain the selection pressure on transgenic shoots.

In each conducted experiment, 12 control explants were cultured on regeneration medium devoid of antibiotics and another 12 non-inoculated explants were cultured on medium containing the above combination of antibiotics.

6.2.6 Histochemical assay of GUS-expression in inoculated root segments and regenerating plant material

Seven d after co-cultivation with *Agrobacterium tumefaciens*, 3 randomly chosen explants from each treatment were tested for GUS expression and after 8 weeks of culture, all explants were subjected to the histochemical assay developed by Jefferson *et al.* (1987):

- 2 ml Eppendorf tubes were filled with 500 µl sodium phosphate buffer (50 mM NaH_2PO_4, pH 7.0),
- 1 explant of regenerating plant material or 2 root segments were placed in each Eppendorf tube,
- 25 µl of X-Gluc solution (10 mg of X-Gluc solved in 1 ml of ether-glycol-mono-ether) was added to the buffer.

The chemicals, together with the explants, were vortexed and incubated in the dark on a horizontal rotary shaker (200 rpm) at 37°C for 24 h. Blue coloured areas resulting from the histochemical staining, were scored on different sites of the explants.

6.2.7 Light microscopy studies on GUS-expressing plant tissue

In order to determine the location of GUS expression within the *Agrobacterium* transformed plant tissues, specimens were prepared as described by Constable (1982) and embedded in Agar Resin 100 (Agar Aids Ltd., Stansted, UK). The protocol for the preparation of the specimens was as described in section 5.2.4.

Sections, 4.0 µm in thickness, were cut on glass knives and collected on glass slides for light microscopy.

6.3 Results

6.3.1 Sensitivity of spinach root explants to the antibiotic carbenicillin

Callus growth at the cut ends of the root explants of Breeding Line E and lateral root development was observed in all carbenicillin treatments. The fresh weight of the explants, however, was very significantly decreased after 12 weeks incubation on carbenicillin-containing culture medium compared to the control (Figure 6.1). Shoot regeneration was reduced with increasing amounts of the antibiotic and the inclusion of 400 µg/ml carbenicillin and above inhibited the production of adventitious shoots (Table 6.1).

Table 6.1: Influence of carbenicillin on the shoot regeneration from root explants of Breeding Line E.

Carbenicillin (µg/ml)	Shoot regeneration (%)	Mean no. of shoots/reg. root explant (mean values ± SD)
0	50.0	2.80 ± 1.30
100	30.0	4.00 ± 2.65
200	20.0	3.33 ± 2.08
300	20.0	2.83 ± 1.72
400	0	0
500	0	0

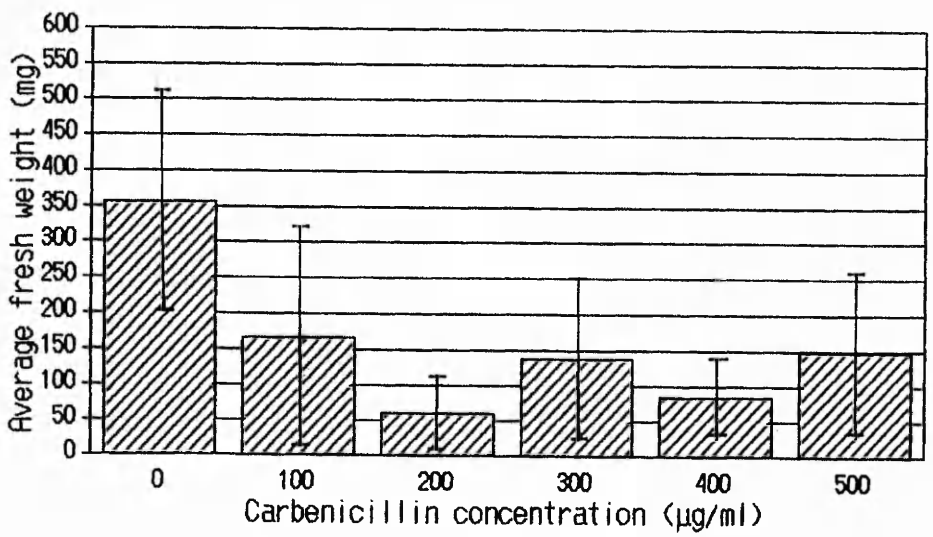


Figure 6.1: The influence of carbenicillin on the fresh weight of root explants of Breeding Line E after 12 weeks of culture (mean values ± SD).

6.3.2 Sensitivity of regenerating spinach tissue to kanamycin sulphate

Adventitious shoot production was maintained by the control explants in the absence of kanamycin and shoot buds developed into shoots.

Root and callus growth continued from explants in all kanamycin treatments. However, shoot development was inhibited when the culture medium contained kanamycin (Table 6.2). Previously initiated shoot buds on explants incubated on kanamycin-containing medium, exhibited a stressed appearance, with dark red colouration caused by increased anthocyanin production in the plant tissue (Plate 6.1).

Table 6.2: Influence of kanamycin sulphate on the maintenance of shoot regeneration of explants of Breeding Line E.

Kanamycin (µg/ml)	Shoot regeneration (%)	Mean no. of reg.shoots/explant (mean value ± SD)
0	25.0	1.6 ± 0.89
50	0	0
75	0	0
100	0	0
125	0	0
150	0	0

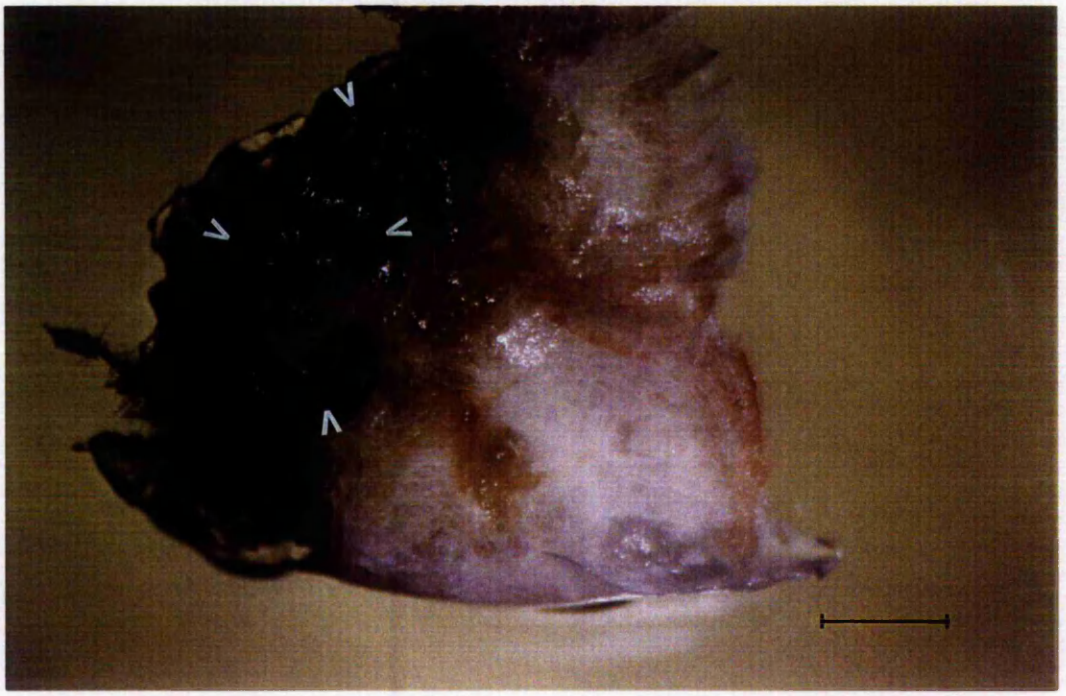


Plate 6.1: Cultured plant tissue of Breeding Line E demonstrates the formation of shoot buds containing high levels of anthocyanin (arrows) following 4 weeks incubation on culture medium containing 50 µg/ml kanamycin (bar = 0.15 cm).

6.3.3 GUS expression in spinach root segments after co-cultivation with *Agrobacterium tumefaciens*

In order to investigate whether the GUS reporter gene had been successfully transferred into cells of spinach root explants, all explants were assayed histochemically for GUS activity 8 weeks after inoculation with *Agrobacterium* (Table 6.3).

Seedling root explants of Breeding Line E co-cultivated with *Agrobacterium tumefaciens* strains '0065' or '1065' failed to exhibit GUS activity, which is normally shown by a blue colouration of the explants. Root explants from established shoot cultures of Breeding Line E and 'Longstanding', which were incubated for 14 d on Nitsch and Nitsch (1969) regeneration medium prior to their co-cultivation with *Agrobacterium*, were also found to be unresponsive to the *Agrobacterium* treatments. However, root explants from established shoot cultures of 'Longstanding', which were incubated for 56 d before co-cultivation exhibited GUS activity after treatment with *Agrobacterium* strain '1065' when the latter was used in a 1:10 dilution (Plate 6.2. A).

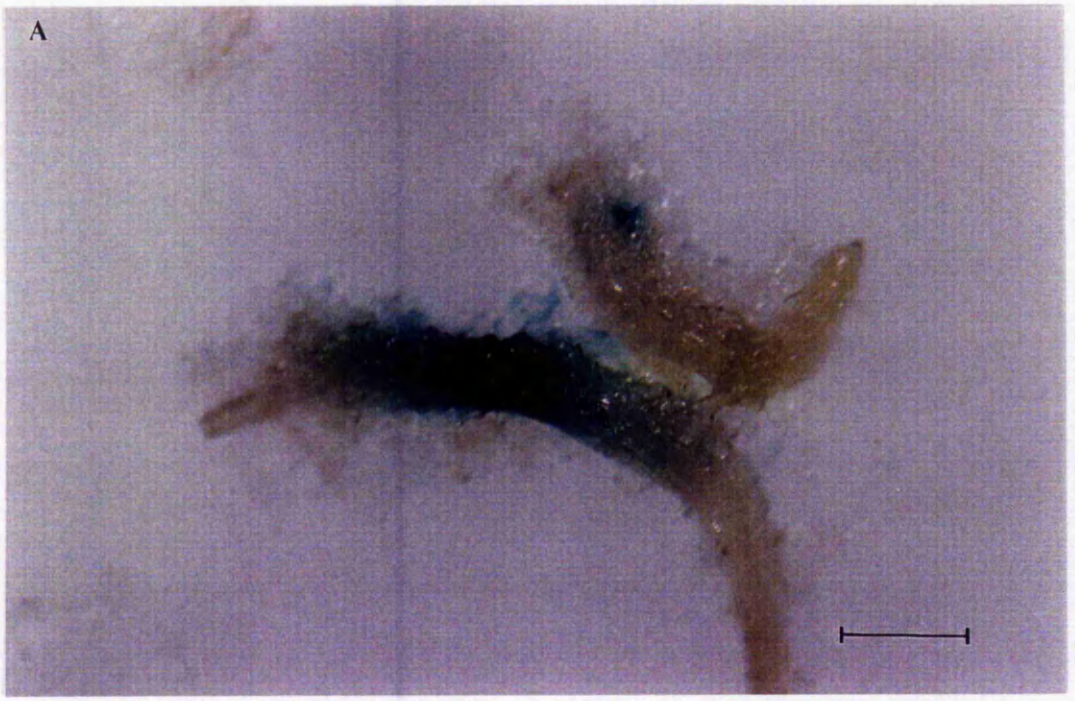
Root explants derived from regenerating plant material, which had been maintained for 20 months, responded with histochemical blue staining when co-cultivated with both dilutions of the *Agrobacterium* strain '1065' (Plate 6.2 B).

Control explants cultured on medium without antibiotics continued to regenerate adventitious shoots. Explants grown on medium containing the antibiotic combination of carbenicillin, kanamycin and cefotaxime were strongly inhibited and failed to develop adventitious shoots. *Agrobacterium*-inoculated root explants cultured on antibiotic-containing culture medium started to deteriorate after 4 weeks of culture and shoot regeneration could not be observed.

Table 6.3: Analysis of root explants for GUS expression eight weeks after inoculation with *Agrobacterium tumefaciens*.

Cultivar	Bacterial strain	Dilution	Number of explants	GUS-positives
[1]E	0065	1:1	28	0
	0065	1:10	28	0
	1065	1:1	28	0
	1065	1:10	28	0
[2]E, LS	0065	1:1	36	0
	0065	1:10	36	0
	1065	1:1	36	0
	1065	1:10	36	0
[3]LS	1065	1:1	24	0
	1065	1:10	24	3
[4]LS	1065	1:1	12	1
	1065	1:10	15	3

[1]seedling root sections. [2]root explants from established shoot cultures incubated for 14 d, before treatment. [3]root explants from established shoot cultures incubated for 56 d, before experimentation. [4]root explants from 20 month-old regenerating plant material. E=Breeding Line E; LS='Longstanding'.



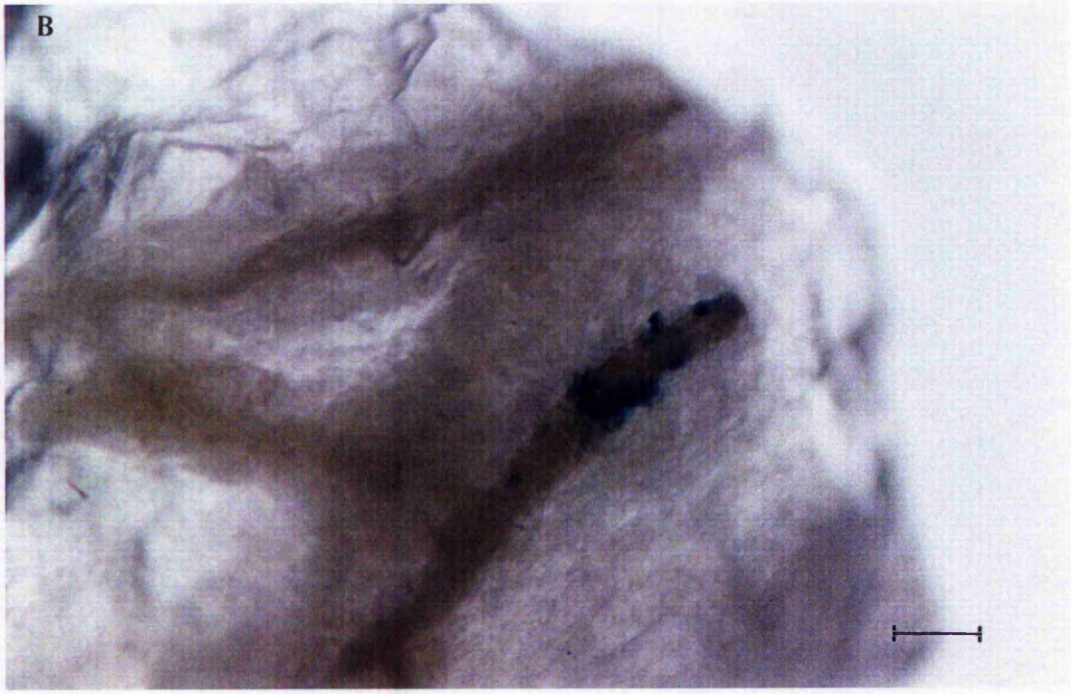


Plate 6.2: (A) Fifty six d old root explants from shoot cultures of 'Longstanding' and (B) root explants derived from 20 month old regenerating plant tissue of 'Longstanding' showing GUS activity after co-cultivation with *A. tumefaciens* '1065' in a 1:10 dilution (bars = 0.3 cm).

6.3.4 GUS expression in regenerating plant tissue of the spinach cultivar 'Longstanding' after co-cultivation with *Agrobacterium tumefaciens*

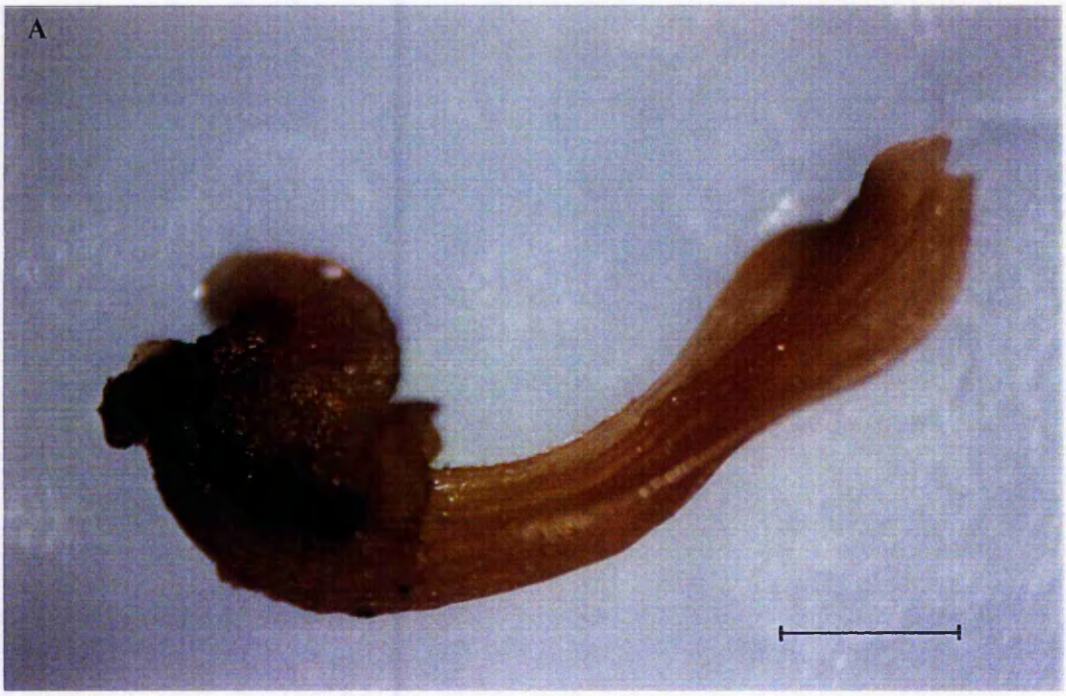
Mature, regenerating plant tissue of 'Longstanding' was found to be responsive to both *Agrobacterium* strains, although transformation was higher with strain '1065'. Of the 2 inoculation methods used, a dilution of 1:10 applied for 10 min was more effective, although the 'quick dip' method in a 1:1 bacterial dilution also resulted in gene transfer as confirmed by a positive GUS assay (Table 6.4). Blue GUS staining within the regenerating tissues could be detected in adventitious roots and shoots. In particular, the shoot bases of adventitious shoots showed areas of blue colouration (Plate 6.3 A). Longitudinal sections of tissue exhibiting blue GUS staining confirmed the location of transformed cells (Plate 6.3 B). Mitotic cells are fully stained, whereas older, vacuolated cells showed blue colouration beneath the cell wall (Plate 6.4).

Control tissue cultured on antibiotic-free medium maintained its regenerative capacity, whereas tissue on antibiotic containing medium became necrotic and ceased adventitious shoot production. *Agrobacterium*-inoculated tissues reacted in the same way, and transformed shoots could not be maintained *in vitro*.

Table 6.4: Analysis of explants of regenerating tissue for GUS expression eight weeks after inoculation with *Agrobacterium tumefaciens*.

Cultivar	Bacterial strain	Dilution	Number of explants	GUS-positives
[1]LS	0065	1:1	12	0
	0065	1:10	12	0
	1065	1:1	16	0
	1065	1:10	16	3
[2]LS	1065	1:1	16	3
[3]LS	0065	1:1	36	1
	0065	1:10	36	0
	1065	1:1	36	2
	1065	1:10	36	3
[4]LS	1065	1:1	6	1
	1065	1:10	12	3

Explant age before inoculation: [1]12 months [2]18 months [3]20 months [4]24 months.



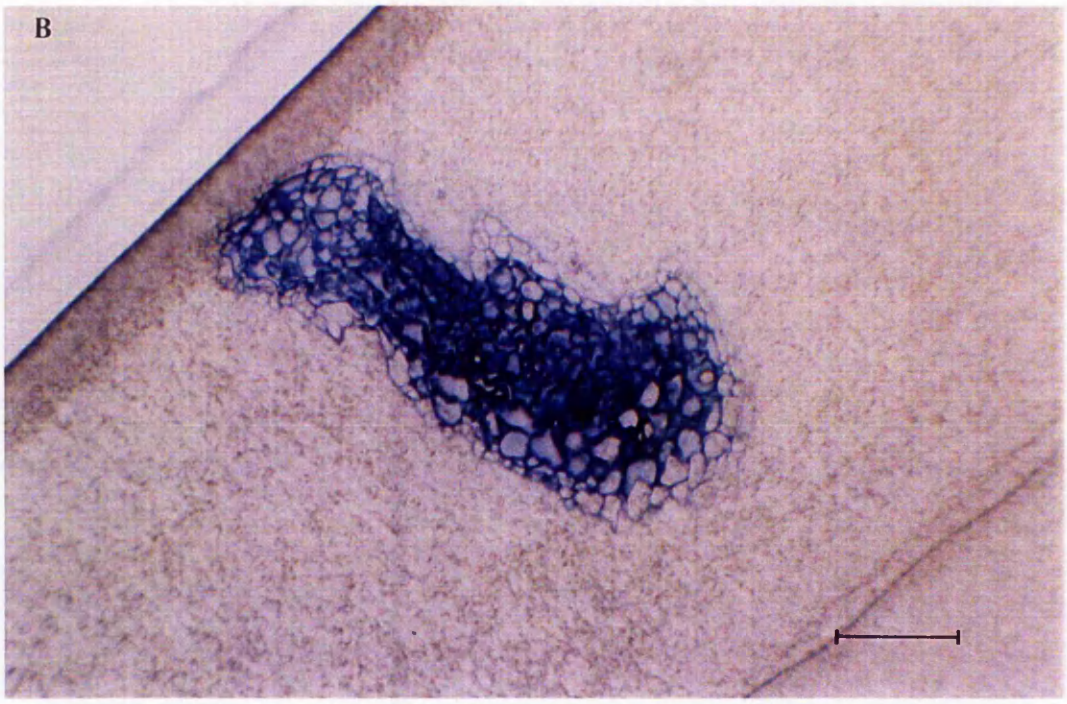


Plate 6.3: (A) Adventitious shoot derived from 18 month-old regenerating plant tissue of 'Longstanding' after co-cultivation with *A. tumefaciens* strain '1065' in a 1:10 dilution, exhibiting GUS staining at the stem base (bar = 0.1 cm).

(B) A longitudinal section of this stem base confirms the location of transformed cells (bar = 250 µm).

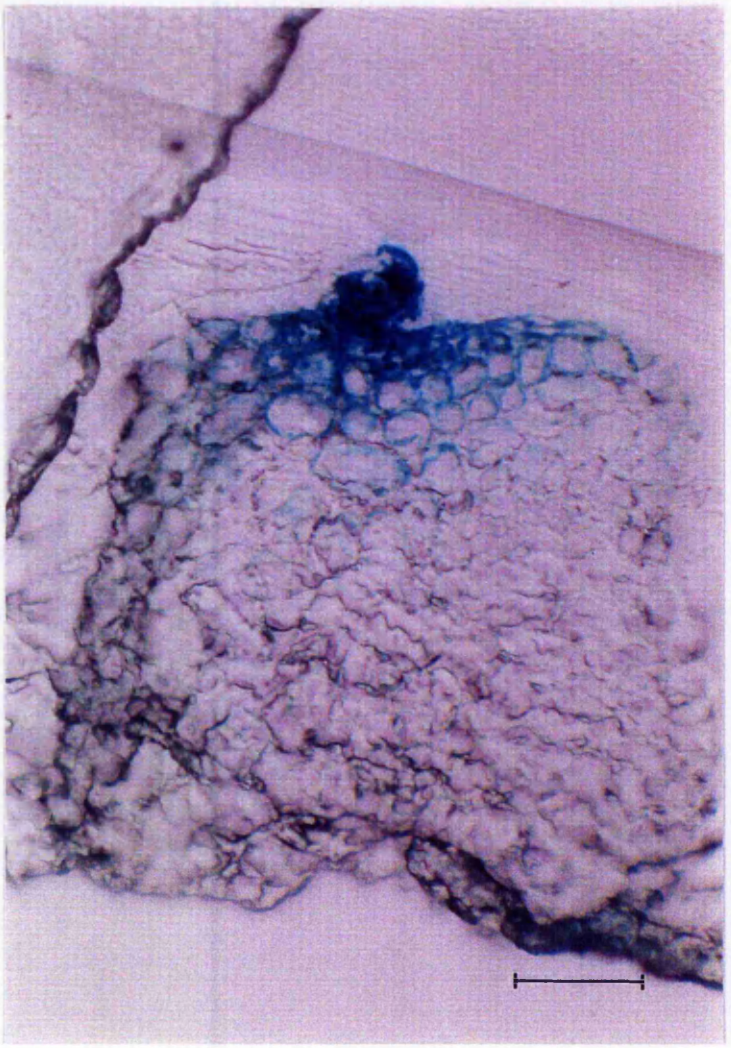


Plate 6.4: Section through 18 month-old regenerating plant tissue of 'Longstanding' after co-cultivation with *A. tumefaciens* strain '0065' in a 1:1 dilution, showing GUS stained shoot primordia (bar = 250 µm).

6.4 Discussion

In this study, various factors have been determined for the development of a successful *Agrobacterium*-mediated transformation of spinach. The age of the cultured explants used for transformation studies on spinach was found to be a crucial factor for gene transfer. Seedling roots and root explants from established cultures of Breeding Line E and 'Longstanding' incubated on Nitsch and Nitsch (1969) shoot regeneration medium for 14 days were non-responsive to treatments with both *Agrobacterium* strains. However, root explants excised from established shoot cultures of 'Longstanding' incubated for 56 days prior to co-cultivation with *Agrobacterium* proved to be susceptible to transformation, as shown by GUS histochemical analysis. Regenerating spinach tissue, which had been maintained for up to two years in culture, achieved the highest transformation rate with 25 % of explants being GUS-positive. These results suggested that explants have to reach a certain physiological stage, before they are able to undergo genetic manipulation. Braun (1975) proposed that actively dividing cells are more susceptible to *Agrobacterium* infection, resulting in an enhanced transformation rate. This suggests, that in this study the prerequisite of actively dividing cells for successful transformation had been met by the use of root explants, in which adventitious shoot production had been initiated. A parallel can be drawn to sugar beet, which is regarded to be recalcitrant to *in vitro* manipulation. Lindsey and Gallois (1990) obtained transformed sugar beet plants from cultured basal stem tissue, which possesses high morphogenetic capacity.

In the present study, limited transformation was achieved by using the strain '0065', but the hypervirulent strain '1065', containing pTOK47, was more effective as demonstrated by the GUS assays.

Another factor to be considered was the bacterial dilution employed. A ten minute exposure of the spinach explants to a 1:10 bacterial dilution of strain '1065' led to a higher transformation rate than a 'quick dip' in a 1:1 bacterial dilution. A similar observation was made by Curtis *et al.* (1994), who generated more kanamycin-resistant shoots when lettuce cotyledons were infected with a 1:10 bacterial dilution of the strain '0065'.

Although this preliminary success in transformation of spinach with the binary vector containing both T-DNA border sequences could be obtained, a completed transformation system for spinach, including the culture and *ex vitro* establishment of transgenic shoots, remains to be established. After a culture period of approximately eight weeks, the *Agrobacterium*-treated explants deteriorated and died. The reason for these events may be because of the composition of the culture medium containing a combination of antibiotics. In preliminary experiments, it was shown that carbenicillin had an inhibitory effect on the regeneration of spinach root explants and the development of regenerating tissue. This response may have been exaggerated by the addition of the antibiotics cefotaxime and kanamycin to the culture medium. Valvekens *et al.* (1988) reported sensitivity of *Arabidopsis thaliana* root explants to cefotaxime and carbenicillin and suggested to replace cefotaxime by vancomycin in transformation experiments to prevent *Agrobacterium* growth, but not shoot development, after co-cultivation.

Although in earlier experiments no evidence was found of sensitivity of spinach tissue to cefotaxime, a reappraisal of the antibiotic concentrations applied may be necessary. In order to overcome this problem, de Laat (1994) recommended washing *Agrobacterium*-inoculated explants in liquid culture medium containing antibiotics for 24 h prior to transfer of the explants to semi-solidified culture medium supplemented with kanamycin sulphate only.

Direct gene transfer methods might be exploited in future through chemical means as described by Davey *et al.* (1980) for *Petunia* protoplasts, electroporation (Dekeyser *et al.*, 1990) or by mechanical means such as microinjection (Crossway *et al.*, 1986) and high velocity bombardment of cells with microprojectiles (Koziel *et al.*, 1993). Potential target areas could be spinach root explants having entered the regeneration process or already initiated adventitious shoot buds, which, in the present study, were shown to be structures amenable to *Agrobacterium*-mediated transformation.

CHAPTER 7

GENERAL DISCUSSION

7.1 Micropropagation of spinach (*Spinacia oleracea* L.)

The main objective of the present study was to establish a comprehensive *in vitro* regeneration system for spinach. The first task was the initiation of tissue cultures from commercially important varieties, including breeding lines and commercial cultivars. After the establishment of asepetic cultures, the emphasis was placed on the clonal propagation of different spinach cultivars.

The initiation of tissue cultures proved to be problematic due to high levels of fungal and bacterial contamination of the spinach seeds, especially seeds of the commercial cultivars. Consequently, a sterilization method was devised, using 25 % (v/v) 'Domestos' solution for 20 min, which limited contamination. The heavy bacterial contamination of commercial seeds was lowered by addition of the antibiotic 'Baypen' at a concentration of 100 $mg l^{-1}$ in the germination medium. This concentration of 'Baypen' used was high, but as the seeds were germinated at a temperature of 5°C, this tended to reduce the activity of the anti-bacterial agent. The sterilzation and germination method developed was considered satisfactory in achieving high germination rates combined with considerably reduced infection of the seedlings.

An alternative method for seed sterilzation has been suggested by Watts *et al.* (1993), who found that bacterial and fungal contamination of wheat seeds could be removed by pre-soaking seeds in water for at least 8 hours, followed by a hot water treatment at 45 - 60°C for 5 - 10 minutes. No further surface sterilization was needed. This method may be advantageous for spinach seeds, the use of phytotoxic chemicals would be avoided and cultures could be grown without antibiotics. Consequently, carry-over effects would be eliminated which might interfere with subsequent plant transformation studies using *Agrobacterium* ssp..

Micropropagation is usually based on inducing axillary branching *in vitro*, because multiplication via adventitious shoot buds may result in somaclonal variation (De Klerk, 1990). The growth of axillary buds is often inhibited by apical dominance which can be broken by the removal of the apical meristem or by changing the endogenous hormone level (Hillman, 1984). It is generally considered that a high auxin : cytokinin ratio results in the maintenance of apical dominance and a low ratio in the activation of axillary buds (Wickson and Thimann, 1958). For example, multiple shoot formation in *Pisum sativum* has been achieved by a combination of 20 µM BAP with 0.1 µM NAA (Griga and Stejskal, 1994).

In the present study, plant growth regulator combinations of auxins IAA, IBA and NAA and cytokinins kinetin, BAP and 2iP were tested in order to stimulate axillary shoot development. The conventional approach of increasing the cytokinin levels in the culture medium in order to obtain multiple shoot growth resulted in the production of large amounts of stem based callus and all tested spinach cultivars demonstrated hyperhydricity at higher levels of cytokinins. The cytokinins kinetin, BAP and 2iP, with increasing levels of concentration, changed the plant shape from an elongate to a rosette type and axillary shoot formation was not encouraged, if not prevented. Although a combination of low concentrations (1.0 µM) of kinetin or BAP in combination with any of the auxins was possible for the culture of spinach, the highest percentage of plants producing axillary shoots occurred on medium containing auxins only. IAA and NAA had similar effects on plant development, but most axillary shoots were obtained with 1.0 µM NAA. An average of 1 - 3 axillary shoots was produced per plantlet. All tested commercial cultivars and breeding lines responded in a similar way to the applied treatments and genotypic differences were not obvious.

The micropropagation protocol developed for spinach contrasts to that for sugar beet (*Chenopodiaceae*). Ritchie *et al.* (1989) induced multiple shoot growth in sugar beet by addition of 1.0 µM BAP to the MS-based culture medium, whereas cytokinins were not found to be essential for shoot cultures of spinach.

De Klerk (1992) has suggested another possibility of breaking apical dominance in plants and found that axillary buds of apple could be released by treatments with fluridone, an inhibitor of abscisic acid synthesis. Furthermore, silver thiosulphate was found to encourage the growth of axillary buds in rose and apple (Van Telgen *et al.*, 1993). These two approaches may be worthwhile testing on cultured shoots of spinach.

The present study showed that axillary shoots of spinach could be excised and cultured *in vitro*. The addition of 1.0 µM NAA to the culture medium was found to be required for routine subculture, but further investigations are needed to confirm the optimum concentration of this hormone. Vegetative growth, however, was difficult to maintain, because shoots readily flowered after 6 - 8 weeks of culture, followed by seed set. Being an annual vegetable, similar flowering behaviour can be observed in the conventional growth of spinach, completing the life cycle within one growing season.

Lowering the culture temperature from 25°C to 15°C significantly reduced flower production in culture. Although the number of axillary shoots was not increased by the change in temperature, the number of usable axillary shoots was increased, with less plants carrying induced inflorescences. A culture temperature of 15°C was best for plant quality. Plant growth, however, was strongly inhibited when the culture temperature was further reduced to 5°C, but offered a possibility for plant storage without adversely effecting the subsequent use of shoots.

The effect of light duration was studied on spinach cultures. Spinach is a typical long day plant with a critical day length of 14 h. *In vitro*, a photoperiod of 16 h appeared optimal. Although flower induction is promoted at this photoperiod, plant quality was best under such conditions. However, the flower promoting effect of light could be counteracted by reduction of the culture temperature to 15°C.

Three main parameters need to be determined for the establishment of successful spinach tissue cultures, the encouragement of axillary shoot growth and the production of a high quality plantlets. Best results were achieved when the MS-based culture medium was supplemented with 1.0 µM NAA and the cultures ideally kept at 15°C under a 16 h photoperiod.

The possibility of enhancing axillary shoot production of spinach was investigated by the use of a double phase culture system employing semi-solid and liquid culture media. This culture method had been previously applied to *Beta vulgaris* and resulted in multiple axillary shoot production (Short and Davey, 1990). The addition of liquid medium to established shoot cultures of spinach was tested and either 5 ml of liquid medium used for a duration of 7 or 14 days or 10 ml for a duration of 7 days was possible. Unfortunately, cultures did not benefit from this culture regime and multiple shoot formation did not occur.

It would be interesting to study the effect on tissue physiology regarding the exchange of components and nutrients between plant tissues and culture medium. From such investigations, conclusions may be drawn which would allow tissue culturists to refine the composition of the liquid phase, so that compounds readily taken up by cultures could be supplied in the liquid medium.

A factor detrimental to the plant development in double phase culture was the frequent occurrence of hyperhydricity. Rossetto (1993) found that hyperhydricity could be reduced in cultures by facilitating gaseous exchange by making a hole in the propylene lids of the culture vessels, which was covered with double layer of filter paper. In order to overcome hyperhydricity in cultures of *Dianthus caryophyllus*, a nutrient mist bioreactor for the formation of multiple shoots has been constructed by Woo and Park (1993). The mist regime produced morphologically normal shoots without signs of hyperhydricity. This micropropagation system might be of use for future large-scale micropropagation of spinach as this plant is highly sensitive to hyperhydricity.

The *in vitro* culture and micropropagation of spinach may be further improved by modification of the culture medium. There are many reports that additions of activated charcoal to both liquid and semi-solid media is beneficial in many culture systems. Developmental inhibitors which result from autoclaving of sucrose-containing media or toxic metabolites can be adsorbed by activated charcoal, thereby allowing growth (Weatherhead *et al.*, 1978). Growth inhibition in date palm could be overcome by the additon of activated charcoal (Reuveni and Lilien-Kipnis, 1974). However, Ebert and Taylor (1990) reported that activated charcoal tends to reduce the availability of auxins and cytokinins in culture media. As a result of their assessment of changing 2,4-D concentrations in liquid and semi-solid media caused by additions of acitvated charcoal, a maximum concentration of 2.5 $g\,l^{-1}$ of activated charcoal was suggested to use in order to avoid an excessive removal of media components.

A review of the carbohydrate source used in the culture of spinach could be advantageous. In all experiments in the present study, a supplement of 3 % (w/v) sucrose to the culture media was chosen. Sucrose is the most generally used carbohydrate for the growth of plant cell cultures, although, in a number of instances, it has been shown that other sugars are equally effective (Maretzki *et al.*, 1974).

Apart from a possible use of fructose or maltose, a substitiution of glycerol for sucrose may be considered. Glycerol was found to increase embryogenesis and chlorophyll synthesis in sweet orange (Vu, 1993).

Al-Khayri *et al.* (1992d) reported an improvement in the growth of spinach tissue cultures with coconut water. The addtition of 15 % (v/v) coconut water resulted in increased shoot regeneration from callus and also in an improved growth of regenerated shoots. In conclusion, different additions to the culture medium for spinach cultures may be of benefit for both axillary shoot cultures and the culture of adventitious shoots.

7.2 The development of a regeneration system for spinach

After the establishment of axenic shoot cultures of spinach, the second objective of this study was the production of adventitious shoots. Various explant sources, including hypocotyls, cotelydons, leaves, stem segments, infloresecences and roots were tested for their organogenic capacity. Roots from axenic cultures or roots induced on hypocotyls proved to be the only explant source giving rise to adventitious shoots.

Earlier publications reported adventitious shoot production from different explant sources of spinach. Hypocotyl segments were found to be organogenic, and callus formation and root production were observed prior to bud formation (Sasaki, 1989; Mii *et al.*, 1992). Callus derived from spinach leaves or germinating seedlings gave rise to adventitious shoots and root proliferation was also observed before bud formation (Al-Khayri *et al.*, 1991a,b, 1992a). In the present study, histological analysis has unequivocally shown that regenerated shoots were derived directly from the root explants, without passing through a callus phase. It is possible that in previous reports false conclusions have been drawn concerning the origin of shoots from cultured hypocotyls or callus tissue.

Shoot regeneration from root explants has been reported in many plant species. The plant growth regulator concentrations used to induce shoot formation were usually high in cytokinin and low in auxin. Xiang-Can *et al.* (1989) obtained shoot regeneration of flax from root explants induced on cotyledons. The MS-based culture medium was supplemented with 0.02 mg l^{-1} NAA, 1.0 mg l^{-1} BAP and 20 mg l^{-1} adenine. Lazzeri and Dunwell (1984) obtained shoot regeneration on basal seedling root segments of *Brassica oleracea* and *Brassica napus*. The use of BAP or zeatin has been shown to reduce shoot formation, whereas kinetin supplements at 0.15 mg l^{-1} promoted shoot regeneration. *Limnophila indica* was regenerated from roots derived from shoot cultures in liquid Nitsch and Nitsch (1969) medium devoid of plant growth regulators (Rao and Ram, 1981).

In contrast to these studies, the trend in previous work on spinach has been to use high auxin concentrations with little or no cytokinin to stimulate shoot regeneration. The ratio of plant growth regulators used in the present study is not typical of that required for shoot regeneration in most other *in vitro* cultured tissues. A combination of 20 µM NAA and 5.0 µM GA_3 was necessary to induce adventitious shoots in root explants of spinach, with tip and middle root sections of roots displaying the highest shoot regeneration capacity.

Kinetin supplements reduced lateral root production on hypocotyl explants. In addition, kinetin inhibited shoot regeneration on root explants derived from hypocotyls and from axenic cultures of the tested breeding line and was not found to be essential for shoot regeneration in the commercial cultivar.

The role of GA_3 in tissue culture systems is still not very clear. A beneficial effect is not always achieved, as GA_3 decreased the formation of shoots from *Albizzia julibrissin* hypocotyl explants (Sankhla *et al.*, 1993). In contrast, the addition of GA_3 to the culture medium proved to be very beneficial for shoot regeneration of spinach, which was also demonstrated in studies by Al-Khayri *et al.* (1992c).

Al-Khayri *et al.* (1991b) found, in a different study on shoot regeneration from leaf-derived callus, a varying response in four spinach genotypes of between 2.4 % to 32.5 % regeneration when cultured on MS-based medium containing 2.0 $mg\,l^{-1}$ kinetin and 0.5 $mg\,l^{-1}$ 2,4-D. In the present study, all breeding lines and commercial cultivars tested responded in a similar way to treatments of NAA (20 µM) and GA_3 (5.0 µM) and shoot regeneration was found to typically originate from root explants cultured on cytokinin-free medium. Breeding Line E achieved a regeneration rate of 33 % and 'Longstanding' of 63 %. This indicates that genotypic differences do exist, but the high level of regeneration suggests that this system has the potential of being generally used for the successful regeneration of shoots in spinach cultivars.

In addition to the hormones used in this study, the composition of nutrient salts in the culture medium may have contributed to the shoot regeneration response of the

root explants. The Nitsch and Nitsch (1969) nutrient salts, which were used for organogenesis studies, contained 720 $mg\,l^{-1}$ ammonium nitrate and 950 $mg\,l^{-1}$ potassium nitrate. In marked contrast, the commonly used Murashige and Skoog (1962) nutrient salts contained significantly larger amounts of nitrate, such as 1650 $mg\,l^{-1}$ ammonium nitrate and 1900 $mg\,l^{-1}$ potassium nitrate. While high amounts of nitrate are thought to be beneficial for general plant growth, reduced nitrogen has been shown to be essential for organogenesis in several tissue culture systems (Dougall, 1981). Therefore, the Nitsch and Nitsch (1969) culture medium may better meet the needs for the regeneration of spinach by offering a relative higher amount of reduced nitrogen in comparison to the MS-based medium.

Although the initial shoot regeneration response of spinach root explants was acceptable, different options remain to be investigated in order to further improve on rates of shoot regeneration.

Temperature has been shown to influence shoot organogenesis in many plant cultures. Ritchie *et al.* (1989) found that raising the culture temperature from 25°C to 30°C for axenic shoots of sugar beet (*Chenopodiaceae*) increased leaf production, and excised petioles cultured at the same temperature regenerated significantly more adventitious shoots. A cold pre-treatment of carrot stock plants at 4°C for 1-2 months resulted in calli, derived from stock plant roots, producing somatic embryos earlier and generating more embryos per callus than cultured material at higher temperatures (Krul, 1993). Derived from these results, a temperature change applied to spinach cultures before or after the excision of root explants could result in a higher regeneration rate.

A different method, in order to improve on the regeneration rate of spinach, may be adapted from Malik *et al.* (1993). In their study, seeds of the legume *Lathyrus* were germinated on medium containing BAP. Multiple shoots were regenerated from epicotyl explants following this pretreatment, whereas without the preconditioning step the regeneration process failed. This notion could be tested for spinach seed germination, adding NAA and GA_3 to the germination medium.

Explant sources, other than axenic cultures or hypocotyls, are another parameter which may be tried for the induction of lateral roots for subsequent adventitious shoot production. Cotyledons, for example, have often been employed for studies of organogenesis. Jain *et al.* (1988) obtained shoot formation from calli derived from cotyledons of *Brassica* species, while Gambley and Dodd (1990) produced adventitious shoots directly in cucumber cotyledons.

In preliminary experiments, all explant sources used failed to produce shoots, but occasional root formation was observed on leaf lamina or stem based callus. This low response may be be overcome by culturing different tissue explants on Nitsch and Nitsch (1969) culture medium supplemented with 20 µM NAA and 5.0 µM GA_3. Thereby, lateral roots could be produced which may be used for studies of organogenesis in spinach.

An alternative culture method, the use of feeder cultures, could be tested which is frequently applied to protoplast culture or microspore culture. The use of microspore feeder cultures led to a tenfold increase of embryo yield in low density microspore cultures of *Brassica napus* (Huang *et al.*, 1990). Curtis *et al.* (1994) obtained higher adventitious bud formation from cotyledons of different lettuce cultivars when cultured with a petunia nurse culture. Advantages for the initiation of adventitious shoots from root explants of spinach may be derived by the use of feeder cultures and a higher initial regeneration response might be achieved.

In the present study, however, a regeneration system for spinach has been established, which provides a continuous supply of shoots that may be harvested and taken into culture. Regenerating spinach cultures were maintained for a period of over two years without noticeable decline in their shoot regeneration capacity.

The final phase of *in vitro* culture involves the rooting of shoots, prior to the transfer of *in vitro*-derived plantlets to the glasshouse. The importance of well developed roots before transfer to compost has been highlighted by Conner *et al.* (1992). Micropropagated asparagus plants with 3-4 cm long storage roots had a significantly higher survival rate under glasshouse condition and required less time for the initiation of new shoot growth in soil culture than those with less developed roots.

The production of spinach plantlets *in vitro* from individual, adventitious shoots was attempted with additions of NAA or IBA to the culture medium, or with medium devoid of plant growth regulators, in order to stimulate root growth. Al-Khayri *et al.* (1991 a) rooted regenerated shoots of spinach on medium containing 1.0 $mg\,l^{-1}$ IBA under a 10 h photoperiod. In the present study, shoots were rooted under a 16 h photoperiod and treatments with IBA resulted in a very low percentage of rooted shoots. A possible explanation is offered by Krieken *et al.* (1990). In their study on rooting of micropropagated *Malus*, rapid photo-inactivation of IBA under the influence of light was observed, whereas no photo-inactivation occurred in the dark.

Thus, it is likley that, in the present study, IBA was probably photo-inactivated and hence the induction of roots prevented. Most shoots produced roots when grown on MS-based culture medium supplemented with 1.0 µM NAA. Unrooted shoots that had developed at least 8 leaves showed the ability to develop roots in compost, but did not survive the acclimatisation process. In contrast, Donnan *et al.* (1978) established unrooted tissue cultured plants of *Saintpaulia* and *Begonia* in the glasshouse. The conditions used in this study were low light levels, high humidity and moderate temperatures.

Fifty percent of regenerated spinach plants survived the transfer to the glasshouse, but only 30 % of the plants continued to grow to maturity. These results reveal the necessity for continued investigations on the acclimatisation of spinach plantlets. *In vitro* rooting in some plants has been shown to be initially improved by additions of phloroglucinol (1,3,5-trihydroxybenzene). Hammatt (1993) reported that inclusion of phloroglucinol in the culture medium, in addition to auxin or on its own, promoted rooting of woody plant species.

Furthermore, in conventional culture, spinach is known as a cool season crop and has shown an affinity to a culture temperature of 15°C *in vitro*. The temperature in the glasshouse during the acclimatisation process used was markedly higher, which is likely to have had a detrimental effect on the cultures, especially considering that the material was subjected to a temperature increase of approximately 10°C upon transfer from *in vitro* to *ex vitro* conditions. This temperature rise is likely to have caused a substantial water loss and probably contributed to the low transfer rate. In addition, the maintenance of high humidity during acclimatisation proved to be difficult to control by the use of plant trays and covers. Acclimatisation of *in vitro* grown spinach plants may be more successful under a controlled environment, with the option to keep temperature low and humidity levels regulated.

Additional factors leading to the production of hardier plants in the final phase of culture are discussed by Roberts *et al.* (1994), which should be reviewed and considered for further investigations on the culture of spinach. General requirements for a successful transfer to the glasshouse are improved water conservation by shoots, achieved by stomata that close when water-stressed, and increased wax formation on the cuticle, a root system that replaces water lost by the shoots, and improved carbon dioxide-fixation to support autotrophic growth after transplantation. Rooting in cellulose plugs (Sorbarods, Baumgartner Papiers S.A., Switzerland), was suggested

by Short *et al.* (1987). Cellulose plugs have been shown to improve the ability of the root system to replace water lost by shoots, while plants can be transferred to compost without being removed from the cellulose plug, thereby protecting the root system during transplantation.

A range of methods have been used to reduce the relative humidity in culture vessels, including the increase of agar concentration in the culture medium, the use of culture vessels with porous closures (Short *et al.*, 1987) and cooling the bottom of the culture vessel (Maene and Debergh, 1986). These treatments were found to promote surface wax development and to limit excessive transpiration.

Plantlets are traditionally cultured *in vitro* under light of low irradiance, on media containing sucrose and in closed culture vessels, in which CO_2 concentrations fall to low levels. If plantlets are exposed to high irradiance and CO_2 enrichment, mixotrophic growth occurs on sucrose-supplemented medium and autotrophic growth occurs on sucrose-free medium (Kozai, 1991). In order to utilize such conditions, models for *in vitro* culture under glasshouse conditions have been presented by Maene and Debergh (1986). However, under the conditions used in this study, acclimatized spinach plants could be grown to maturity and proved to be fertile. Seeds collected from these plants were fully viable and produced mature plants, producing viable seeds for the next progeny.

No comprehensive investigation was undertaken to determine any variation in the regenerated plants and their progenies and would be worthy of a future study. Plants derived from tissue culture and their progenies can be screened for somaclonal variation by means of morphological, cytogenetical, biochemical and molecular tests. The production of plants that are not true-to-type potentially reduces the commercial value of tissue culture-derived products, although somaclonal variation is regarded as a source of variation which can be exploited for crop improvement, but is unpredictable as a breeding tool (Karp, 1993). Skirvin (1978), however, stated that regenerants directly induced on the explant without an intervening callus phase are less likely to be genetically changed than those derived from callus.

In the present study, regenerated plants displayed a variable growth response after acclimatisation, but plants that grew to maturity were very similar in character.

In order to ascertain any existing genetic variability more precisely, analysis of a larger population of acclimatized regenerants would be neccessary. The first seed propagated progeny, however, was homogenous, true-to-type and morphological differences were not obvious. This applied also to the second seed propagated progeny, with the only difference that those plants appeared less vigorous.

Flow cytometric analysis was used to determine ploidy levels of the original plant source, *in vitro* regenerated plants and seed-propagated progenies derived from regenerated plants. Leaf tissue from all plant generations displayed an even proportion of G_0/G_1 cells and G_2/M cells. All seed propagated spinach plants appeared to have the same DNA content, whereas a loss of DNA was detected in the *in vitro* regenerated plants. This observation will need to be verified by a more comprehensive study.

In order to substantiate the genetic relation between regenerated plants and their progenies, a further, more detailed investigation is required. A future study should use an increased sample size for flow cytometric analysis, and, in addition, it would be interesting to follow ploidy levels throughout the regeneration process starting with the anaylsis of root explants to regenerated plants and their progenies. This approach would provide a more precise knowledge concerning ploidy levels and potential genetic aberrations.

An investigation using such an approach could be complemented by the use of chromosome counts as a useful tool to determine whether plant tissues are high in mitotic activity or naturally contain an even proportion of diploid and tetraploid cells. Karyological analysis of regenerants would reveal significant changes, including alterations in ploidy levels, as well as structural rearrangements.

Results from such an investigation may relate to a study on plant regeneration from sugar beet hypocotyls conducted by Jacq *et al.* (in press). Flow cytometric analysis showed that hypocotyl explants of diploid and tetraploid sugar beet plants contained cells with different ploidy levels and that initiated callus reflected the nuclear condition of the cells of the hypocotyl. Most regenerated plants eligible for transfer to the glasshouse were diploids, indicating that diploid callus cells preferentially produced plants.

7.3 Transformation studies on spinach

Primrose (1991) distinguishes between non-recombinant and recombinant approaches to biotechnology and plant breeding. Non-recombinant approaches include the exploitation of spontaneous or induced variation in cultured plant cells or tissues and the use of intra- and interspecific protoplast fusion. Recombinant approaches include genetic engineering, which involves the introduction of genes into a plant and gene manipulation in the guise of protein engineering, which can be used to make substantial alterations to key cellular proteins. Such changes are unlikely to occur spontaneously. Amongst others, cloning vectors are required for recombinant approaches, those that are based on the Ti-plasmid of the plant pathogen *Agrobacterium tumefaciens* being the most commonly employed.

In the present study, the establishment of a reproducible regeneration system for spinach was accomplished and therefore, the prerequisite for the application of plant transformation methods provided. The aim was to evaluate the susceptibility of spinach to *Agrobacterium*-mediated transformation. The transformation method applied was adopted from Curtis *et al.* (1994), who used two *Agrobacterium tumefaciens* strains (designated '0065' and '1065') for the transformation of lettuce. This method was successfully applied to spinach and transformed, regenerating plant tissue was obtained. Histochemical and histological analyses proved unambiguously that spinach tissues were susceptible to *Agrobacterium tumefaciens*-mediated gene transfer. Transgenic shoot cultures, however, failed to be raised, because of the loss of the transformed, regenerating tissue.

A number of opportunities for further investigations on the transformation of spinach are open to exploration. Firstly, a modification of the applied transformation method may result in a higher transformation rate and possibly also in the development of transgenic shoots.

Prior to any infection with *Agrobacterium*, a determination of suitable antibiotics and their concentrations is necessary in order to eliminate the bacterium after co-cultivation, without harming the plant tissue. The antibiotics cefotaxime and carbenicillin are commonly used for transformation studies. The consideration of the toxicity of these anitbiotics is critical when designing transformation schemes for selection and recovery of transgenic plants.

Only cefotaxime is known to have occasionally a positive impact on stimulating shoot growth and organogenesis, for example in *Malus* cultivars (Yepes and Aldwinckle, 1994) or in finger millet (Eapen and George, 1990). Although no effect of cefotaxime on tissue explants of spinach has been found in the present study, it may be useful to re-examine a potential positive or negative influence on spinach tissue explants, especially with view to the regeneration of transgenic shoots. Carbenicillin and kanamycin had a phytotoxic effect on spinach tissue. The use of different antibiotics, such as vancomycin, has been suggested by Valvekens *et al.* (1988) in order to overcome sensitivity of *Arabidopsis* root explants to carbenicillin and cefotaxime. More extensive tests, therefore, regarding the use of individual antibiotics, the optimum concentration to be applied and their possible use in combination with other antibiotics are needed, in order to provide efficient means to control the growth of *Agrobacterium*, to provide reliable selection systems and to allow transformed shoots to develop.

Further improvements within the applied transformation method may be sought in the bacterial dilution and the incubation time. The highest transformation rate was achieved with a 1:10 (*vir*) bacterial solution for a period of 10 minutes, but improvement may be obtained by application of a weaker bacterial solution for a longer incubation period or *vice versa*. In addition, instead of treating explants in a stationary solution, gentle agitation may facilitate a more effective contact with the bacteria.

A different method for *Agrobacterium tumefaciens* transformation could be the injection of the bacterium into the shoot apex of spinach seedlings as demonstrated by Ulian *et al.* (1988), who transformed petunia seedlings by injecting a bacterial solution into shoot apical tissues.

Apart from trying different *Agrobacterium tumefaciens* strains, the use of *Agrobacterium rhizogenes* offers an alternative for transformation studies with spinach. Sugar beet plants were induced to produce roots on their petioles after infection with *Agrobacterium rhizogenes*, which in turn gave rise to transformed shoots (Ritchie *et al.*, 1991).

In the present study, spinach shoots regenerated from root explants. *Agrobacterium rhizogenes*-mediated transformation may result in abundant root growth, from which transgenic shoots might be regenerated.

Direct gene transfer methods should be considered for future studies on spinach and should include chemically-mediated DNA uptake, electroporation, microinjection and biolistic methods. Microinjection and the use of biolistics have the advantage that they can be applied to multicellular structures. Neuhaus *et al.* (1987) produced transgenic rapeseed plants by microinjection of DNA into microspore-derived embryoids. Klein *et al.* (1987) circumvented limitations of biological vector systems by delivering nucleic acids into plant cells using high-velocity microprojectiles. The latter were used to carry RNA and DNA into the epidermal tissue of onion and these molecules were subsequently expressed genetically. This gene delivery device has been modified since and was used to generate transgenic corn, resistant to insects (Koziel *et al.*, 1993) and viral infection (Murry *et al.*, 1993).

Gene transfer by means of electroporation has been applied by Abdul-Baki *et al.* (1990). In their study, DNA was transferred into germinating pollen grains of *Nicotiana gossei*. Transformed pollen can be applied to stigmas of compatible flowers with the potential of producing viable transgenic seeds. Ion beam-mediated DNA delivery, as a novel technique, has been used by Zenglian and Jianbo (1994). They produced transgenic rice plants after transfer of a hygromycin-resistance gene into mature rice embryos by application of this novel technique.

Transformation rates obtained by direct gene transfer methods may be enhanced by irradiating the target tissue with X-rays either before or after transformation. After irradiation of *Petunia hybrida* and *Brassica nigra* protoplasts, a six- to sevenfold increase in transformation rates was achieved (Köhler *et al.*, 1990).

These examples provide good reasons for reviewing the scope and range of methods to be used for genetic modification by direct gene transfer in a future study on spinach.

7.4 Conclusions

The prime objectives of the present study have been met by establishing a comprehensive and reproducible regeneration system for spinach. It proved difficult to maintain spinach as a shoot culture, whereas a continuous supply of new shoots could be obtained by adventitious shoot production. Axillary shoot production, adventitious shoot production and the formation of spinach plantlets were regulated by addition of the auxin NAA. This suggests that spinach may have a large endogenous pool of cytokinin, thereby obviating its need for an exogenous supply of this hormone. This statement may be verified by determination of the actual endogenous cytokinin level, for example, by HPLC analysis.

Figure 7.1 summarizes the developed micropropagation system for spinach. Closed lines mark accomplished culture steps, which bear the potential for further improvements as discussed earlier. Dotted lines, however, indicate areas to which future research may be directed. Spinach has been shown to respond positively to *Agrobacterium*-mediated transformation and with the subsequent completion of a transformation system, resulting in the production of transgenic shoots, the introduction of agronomically important genes may be attempted.

Spinach readily flowers *in vitro* and sets seeds which are viable. This feature is potentially of great value for future work. Spinach is normally wind pollinated and therefore subjected to cross-pollination. Cross-pollination can be avoided in an enclosed culture system, thereby supporting the maintenance of homozygous breeding lines. Plants can be grown to maturity producing fertile plants and potentially fertile transformants. The insertion of foreign genes can be followed by analysis of inheritance either *in vitro*, or seeds could be used for glasshouse screening of seed-derived plants.

Molecular techniques have considerable potential for crop improvement and may be exploited in the breeding process, with the specific objective of producing controlled increase in variability. This strategy could be used in spinach to modify characteristics including qualitative and quantitative improvements of yield, enhanced disease resistance and improvement of processing qualities.

The basis for such an approach has been provided by this study through the establishment of a regeneration system for spinach and the demonstration that spinach is amenable to *Agrobacterium*-mediated transformation. The reproducible plant regeneration procedure developed in the present study may be of commercial value, with application to spinach breeding in pursuit of the production of an improved crop plant.

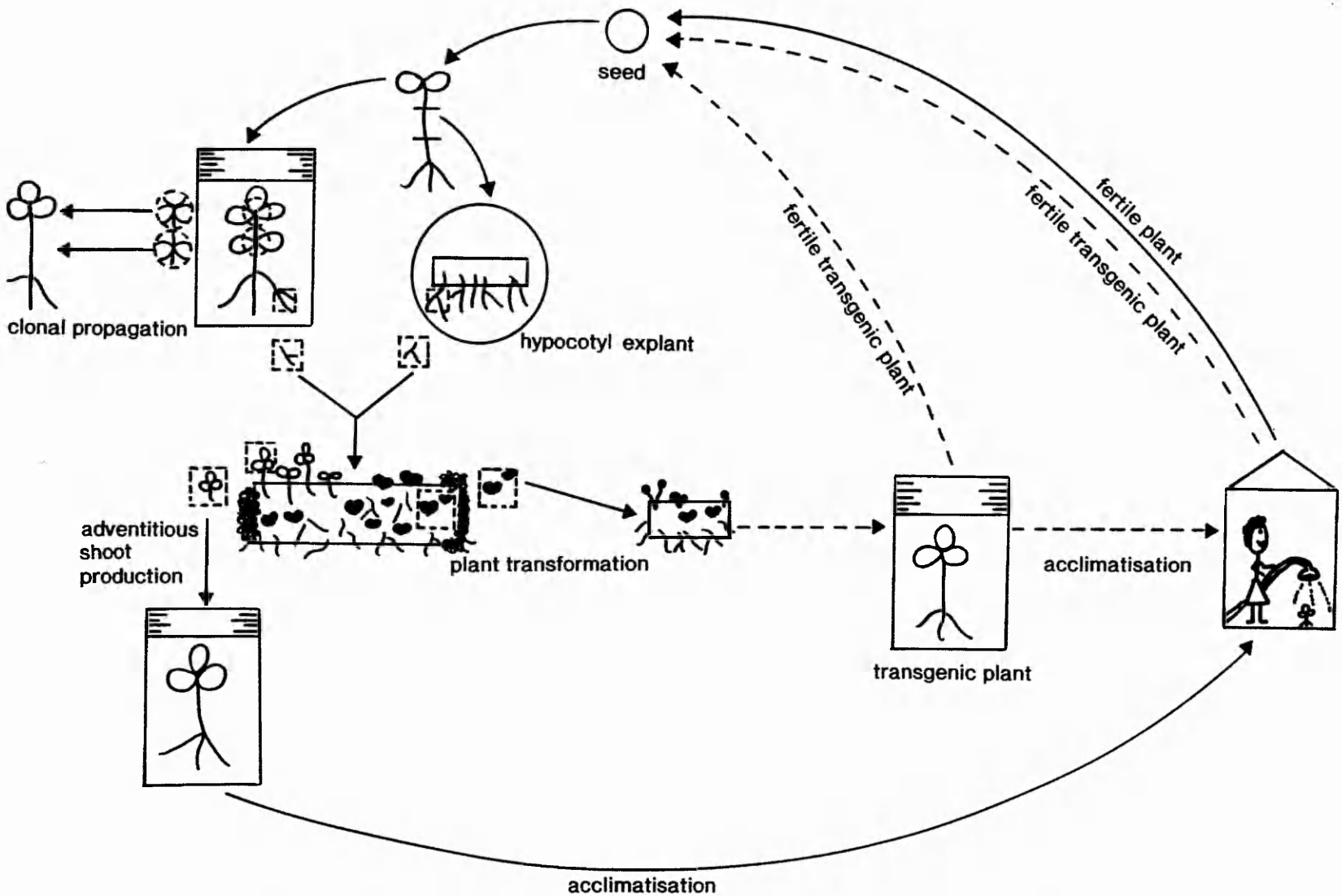


Figure 7.1: Illustration of the developed micropropagation system for spinach. Closed lines symbolize accomplished culture steps and dottet lines indicate areas for future research.

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