Variation arising through tissue culture in soft fruits

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ABSTRACT

The study mainly concerns the potential of the tissue culture for mass propagation and to evaluate the trueness to type of regenerated plants from tissue culture using appropriate molecular markers. Three soft fruits species *Ribes, Fragaria* and *Rubus* were used in this study. The study has three main sections dealing with an evaluation of appropriate molecular marker systems to study the plants regenerated via micropropagation, callus culture and regeneration and tissue culture and regeneration.

The first section evaluates the potential of SDS-PAGE protein electrophoresis and RAPD-PCR as markers to distinguish among clonally propagated cultivars of *R. nigrum*. SDS-PAGE was able to distinguish only four out of ten cultivars tested. RAPD-PCR was able to distinguish all the cultivars studied using only two primers. The data generated by RAPD-PCR and from pedigree information was used to examine the relatedness among the cultivars studied. RAPD-PCR was further used to examine the purity of the cultivar Baldwin collected at various locations in the UK. Polymorphism was detected and differences were found between the sub-samples of a single cultivar.

The second section deals with the multiplication of *Rubus, Ribes* and *Fragaria* by micropropagation. The effect of culture cycle on the plants regenerated was evaluated using RAPD-PCR. *Ribes* did not show any variation until the 14th generation cycle but in the 15th and 16th cycles variation was detected from 6.2% and 13.4% respectively. Considerable variation was detected in *Rubus* starting with the 4th sub-culture and was at a maximum in the 7th sub-culture. In *Fragaria*. all plants at sub-culture 3 were evaluated and variation was detected between them. The relevance of such variation on the release of material of all three species is discussed in relation to certification scheme requirements.

The third experimental section evaluates the potential of callus as explant source for the multiplication and regeneration of plants in *Ribes* and *Fragaria* species. The study also describes investigations in the optimum growth regulators concentrations for callus culture and subsequent plant regeneration. Both the explant type leaf disc and leaf petiole showed successful callus induction in *Ribes* cultivars. However, plant regeneration was not successful in the cultivars and explant sources studied. In *Fragaria* regeneration was easily achieved from leaf disc callus and the plants were evaluated for trueness to type using RAPD-PCR This indicated variation ranges from 0.68 to 22.80%.

The final discussion reviews micropropagation, callus culture and regeneration and their application to mass propagation. The use of molecular marker systems to evaluate multiplication methodology is discussed both in terms of the needs of the soft fruits industry and as the general approach to the evaluation of progeny of clonally propagated species.

Dedication

This work is dedicated to

my late father Fayyaz Ali Khan.

DECLARATION

This is to declare that this thesis has been composed entirely by myself and has not been accepted in any previous application for a degree. All the work carried out herein was also carried out by me. All sources of information have specifically been acknowledged by means of reference.

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitraily primed PCR
AT	Adenine, thymine
BAP	6-Benzyl Amino Purine
bp	Base pair
CRD	Completely Randomised Design
CTAB	Cetyltrimethylammonium bromide
cv	Cultivar
2,4-D	2,4-Dichlorophenoxyacetic Acid
dATP	2 'Deoxyadenosine 5' -triphosphate
dCTP	2 'Deoxycytosine 5' -triphosphate
dGTP	2 'Deoxyguanosine 5' -triphosphate
DNA	Deoxyribonucleic Acid
dNTP	2'Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	2'Deoxythymidine 5' - triphosphate
$EDTA(Na)_2$	Ethylenediaminetetra-acetic acid, disodium salt
EtBr	Ethidium bromide
g	Relative Centrifuge force (RCF)
GC	Guanine, cytosine
HCl	Hydrochloric acid
IAA	Indol-3-Acetic Acid
IBA	Indol-3-Butryic Acid
kb	Kilobase
KCl	Potassium chloride
$MgCl_2$	Magnesium choloride
$MgSO_4$	Magnesium sulphate
MS-media	Murashige and Skoog (1962) basal media
NAA	Naphthaleneacetic Acid

NaCl	Sodium chloride
ng	nanogram
NaOH	Sodium hydroxide
O.D.	Optical density
P	Probability
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RCF	$(1.12x10^{-5})(rpm)^{2r}$ r = radius in cm measured from centre of spindle to bottom of rotor bucket
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions Per Minute
SAC	Scottish Agricultural College
SCRI	Scottish Crop Research Institute
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus*
TBE	0.089M Tris, 0.089M boric acid, 0.002M EDTA
TE	10mM Tris HCl, 1mM EDTA
Tm	DNA melting temperature
Tris	Tris (hydroxymethyl) aminomethane
μl	microlitre
UV	Ultraviolet

CHAPTER 1.

INTRODUCTION

1.1. GENERAL INTRODUCTION

Pakistan has an agriculture-based economy, in the last few years much progress has been made in agricultural research. Although a number of high yielding, fertiliser responsive traditional crop varieties produced through the conventional breeding programmes have been released, the country is still facing many problems in sustaining crop production with conventional crops. As the population is increasing at an alarming rate (annual population growth rate 2.7%), there is a need to explore non-conventional crops of high nutritive value in order to provide such nutrition and to satisfy the needs of the population for variety in food sources.

Soft fruits are a relatively recent introduction to Pakistan. Strawberry production started in Faisalabad in the Punjab province in 1964-65 and expanded for several years (AHMAD. D. Personal communication). The plants could not withstand the high summer temperature, preventing the production of planting materials for the next year. In the Northern region of Pakistan where summer temperatures are relatively cool, these crops can be easily grown as perennials. Recently certain growers in the Swat district of North West Frontier Province (NWFP) have started cultivating this fruit crop which is then distributed to the hotels of Islamabad and other big cities. In the most fertile land of the Punjab province this crop can also be grown if the supply of planting material for the next season's crop can be ensured. One strategy is to establish nurseries in the Northern areas to multiply the material vegetatively and supply planting material to the growers across the country. Another alternative is to exploit the potential of tissue culture, so that

plant material can be multiplied very quickly in a limited space all year around and supply it to the growers locally.

The general aims of this study are to evaluate the potential of *in vitro* culture as a rapid multiplication system and to evaluate the ability of the system to give true-to-type plants using appropriate marker systems. Strawberry, raspberry, and blackcurrant were selected for the study as these are the most important and economically valuable crops among the soft fruit group.

1.2. TRADITIONAL METHODS OF PROPAGATION

Cuttings are the most important means of propagation in soft fruits. The methods are simple and do not require the special techniques necessary with propagation of other perennial crops such as grafting, budding, or micropropagation. Different types of cutting are used for different soft fruit species.

1.2.1. HARDWOOD CUTTINGS

These are cuttings made of matured dormant hard wood after the leaves have been abscised and before new shoots emerge in the Spring. These are more common in soft fruits such as blackcurrant (MAFF, 1981) and red currant. Normally this takes the form of planting un-rooted hardwood cuttings (20-30 cm long) in the field (Wainwright, and Hawkes, 1984). These have been stored, in moist sand/sawdust at about 1.6 °C (35°F) then transplanted in early Spring. The plant can be transplanted to its permanent location in one or two years.

1.2.2. SOFT WOOD CUTTINGS

Cuttings prepared from the soft, new Spring growth about 3-8 cm long with two or more nodes are placed in a cold frame in prepared soil at distances of 15 cm square, during May. The soil is well watered and a light source placed over the frame which is closed and shaded until after rooting. Soft wood cuttings generally

root easier and quicker than the other types but require more attention and equipment and are used in raspberry.

1.2.3. ROOT CUTTING

Soft fruits such as raspberries can also be propagated by root cuttings (Torre, 1979; Hudson, 1955). The length of root cuttings varies from 5 to 15 cm for thin and thick roots subsequently. With care, good strong healthy plants can be produced in one year. Best results with root cuttings are likely to be attained if the root pieces are taken from young stock plants in late Winter or early Spring when the roots are well supplied with storage food but before new growth starts.

1.2.4. TIP LAYERING

Tip layering is a natural method of propagation and is characteristic of raspberries and blackberries. Healthy young plants are set 3.6 m apart to give room for subsequent layering. Rooting takes place near the tip of the current season's shoot. The shoot tip recovers upward to produce a sharp bend in the stem from which roots develop.

1.2.5. MOUND LAYERING OR STOOLING

Stooling is a form of layering used commercially to produce blackcurrant plants before new growth starts in the following Spring. All plants are cut back to 2.5 cm above ground level. Two to five new shoots usually develop from the crown in the second year; more in later years. Loose soil is drawn up around each shoot to one half its height. Stool shoots should have rooted sufficiently by the end of the growing season to be separated from the parent stool. The rooted shoots are cut and transplanted directly to the nursery row.

1.2.6. RUNNERS

A runner is a specialised stem that develops from the axils of a leaf at the crown of a plant. It grows horizontally along the ground, and forms a new plant at one of the nodes. The strawberry is a typical plant species propagated in this way. In most

strawberry cultivars, runner formation is related to the length of the day and temperature (Hartmann et al., 1990). In propagating by runners, daughter plants are dug when they become well rooted and then transplanted to the desired locations

1.2.7. CROWN DIVISION

Everbearing strawberry cultivars that produce few runners may also be propagated by crown division. Certain cultivars may produce 10-15 strong crowns per plant by the end of the growing season. In the Spring such plants are dug and carefully cut apart, each crown may then be used as a new plant.

1.3. GENETIC VARIATIONS IN VEGETATIVE PROPAGATION SYSTEMS

All vegetative propagation systems aim to produce progeny which are identical to the mother clone. However, even with the traditional systems of vegetative propagation, variation can arise as discussed below.

1.3.1. MUTATION

Changes in the genetic composition can occur within a somatic (vegetative) cell and, if followed by mitotic division may lead to permanent changes in the clone if subsequently daughter cells occupy a substantial portion of a growing point. There are many types of mutational changes that can occur such as point mutation, gross structural changes of chromosomes deletions, duplications, inversions, additions or subtraction of one or several chromosomes of a set (aneuploidy) or multiplication of an entire set of chromosomes (polyploidy). Cytoplasm also contains DNA, which is independently involved in determining plant characteristics (Jinks, 1964). Permanent changes can occur in them and lead to changes within a clone. Any single mutation or chromosomal change in itself, is a relatively rare event. However, since vegetative growth of a clone involves billions of cell divisions, the chances that some type of spontaneous change will occur within a clone are reasonably good.

1.3.2. CHIMERAS

Many bud mutations are mixtures of mutated and non-mutated tissues which occupy distinct layers or sectors of the plant. These combinations are called chimera. A chimera is a plant or part of a plant, composed of two or more genetically distinct tissues growing adjacent to each other as part of a composite plant. A chimera may originate by spontaneous mutation in the cell of the plant within one of the layers of the growing point.

This change does not necessarily affect the entire growing point but only those parts of the stem resulting from further division of the mutated cells. There are several kind of chimeras.

1.3.2.1. SECTORAL CHIMERAS

In this type the growing point of the shoot is composed of two genetically different tissue situated side by side occupying distinct sectors of the stem; leaves and lateral buds arising from such a shoot may be composed of two tissues combined in various ways depending upon their location.

1.3.2.2. PERICLINAL CHIMERAS

In this type, tissues of one genetic composition occur as a relatively thin "skin" one or several cell layers in thickness over a genetically different "core". Figure 1.1. This is the most common and relatively stable chimeral type. The genus *Rubus* for example has numerous thornless blackberry forms in which the epidermal layer lacks the gene for thorns (Darrow, 1928). Such plants usually retain this characteristic if propagated by stem cuttings or by tip layering. It is significant that the adventitious roots arising endogeneously beneath the mutated tissue give rise to a root system of non-mutated tissues

1.3.2.3. MERICLINAL CHIMERAS

This type is similar to the periclinal chimeras except that the outer layer of a different tissue does not extend completely around the shoot, occupying only a

segment of the circumference (Figure 1.1). This type is the most common to occur naturally since a mutation in a single cell of a growing point would give rise to such a pattern of development.

1.3.2.4. BUD SPORTS

A branch which shows changes in one or more inheritable characters that can be propagated by asexual means is termed a bud sport or bud mutation. It can originate by any of the somatic mutations or chromosomal changes mentioned earlier. Somatic variants are starting points of new clones and if introduced cultivars are given names.

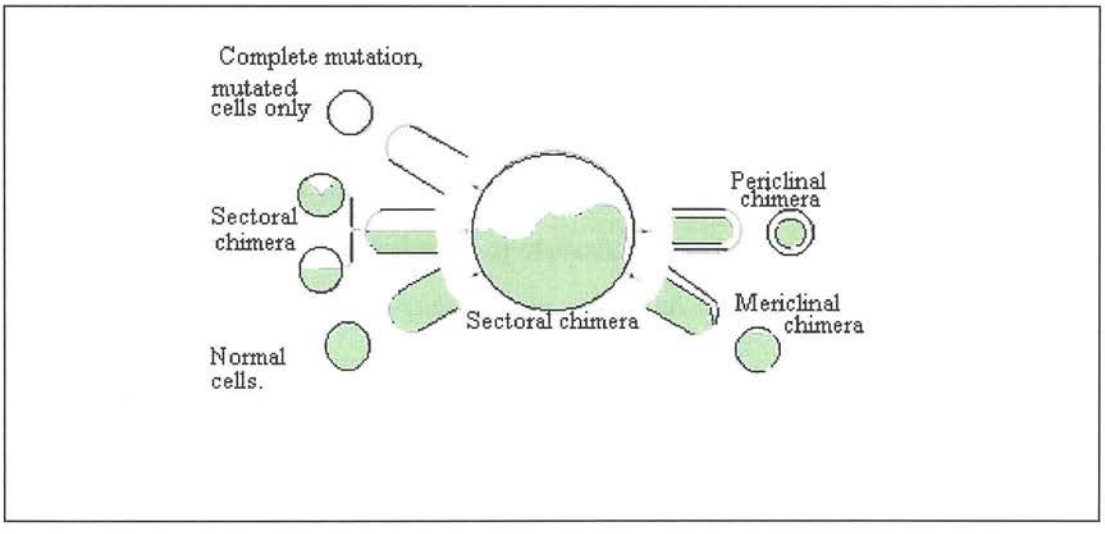


Figure 1.1. Types of chimeras. Bud arising at different positions on a sectorial chimera may produce shoots consisting entirely of mutated cells or entirely of non-mutated cells; or the shoots may be a sectorial, mericlinal, or periclinal chimera, depending upon their location.

1.4. ALTERNATIVE METHODS OF PROPAGATION

With the explosive development of the amenity plant trade, the pressure on the horticulture trade to produce vast numbers of plants for specific markets has increased. This has put pressure on research to produce plants more quickly. In particular, to understand better the major biological factors and processes that

affect plant growth, such that it becomes possible to manipulate plant development in desired directions (Dixon, 1987).

Conventional methods of vegetative propagation have allowed unique heterozygous plants of many species to be multiplied and perpetuated for breeding material or for direct use as desirable cultivars. *In vitro* propagation enables a far wider range of species. Potentially all species of higher plants can be cloned rapidly under highly controlled conditions. The ability to grow plant tissue, such as callus, cell suspension, and various plant organs, such as stem, flower, roots, and embryo more or less indefinitely, has been utilised in scientific laboratories for many decades as a research tool for geneticists, botanists and plant pathologists (Dixon, 1987).

1.4.1. TISSUE CULTURE

Tissue culture is based on the principle of totipotency, i.e., the concept that every living cell has the genetic potential to reproduce the entire organism (Haberlandt, 1902). Since then the field of plant tissue, cell, and organ culture has grown to be of immense size, commercial importance and scope.

The term micropropagation is used specifically to refer to the applications of tissue culture techniques "to the propagation of plants starting with very small plant parts grown aseptically in a container where the environment and nutrition can be rigidly controlled". (Hartmann and Kester, 1990; Dixon, 1987; Smith and Drew, 1990). In practice, many propagators use the term micropropagation and tissue culture interchangeably to mean any plant propagation procedure utilising aseptic culture. A synonymous term is *in vitro* culture. Micropropagation and tissue culture begin with the excision of a small piece of plant followed by sterilising and placing it into aseptic culture. The term used for this propagule to start the process is "explant". To ensure successful production the tissue material requires the normal growth elements in the form of mineral salts; carbon is added in the organic form as sugar. Other material required are amino acids, B vitamins and growth hormones. To this

solution agar is added to provide support for the plant material. If the culture is submerged, then aeration can be provided by shaking the flask in a reciprocating or rotating shaker. The important factor with this method is absolute sterility, as the plant material used has no epidermal layer to protect against bacterial and fungal contaminants.

Micropropagation has many advantages over traditional propagation techniques some of which are listed below:.

- Rapid clonal multiplication.
- New varieties or introductions can be bulked up to enable larger quantities to be released.
- Plant materials can be more easily exchanged between countries when grown in aseptic culture.
- The production and dissemination of disease free stock can be enhanced.
- Plant propagation can be accurately programmed.
- Plants that were difficult or could not be propagated traditionally can often be propagated *in vitro*.
- Stock plants can be stored for extended periods *in vitro*.

1.4.2. BRIEF HISTORY

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. (George, 1993). Although strictly it refers to the culture of an undifferentiated mass of plant cells only (Duncan and Widholm, 1986; Street, 1977; George and Sherington, 1984), plant tissue culture techniques have become a powerful tool for studying many basic and applied problems in plant biology.

Living organisms vary greatly in their complexity, life-cycles, and mode of propagation and reproduction. Regardless of these differences, such organisms possess or have possessed during their long evolutionary history, the common feature of being represented at some stage by a single cell. All complexities and

events occurring during their life-cycle must unfold from this single unit. The cell must therefore contain all the information necessary for the organism to grow and reproduce in its environment. In this sense the single cell can be called totipotent.
The first theoretical attempts to explain the complexity of multicellular organisms were put forward in 1838 by two German biologists, M.J.Schleiden and T. Schwann (Thomas and Davey, 1975). Although they realised that complex interactions must occur between different cells, tissue and organs, they suggested that each cell is an independent unit capable of forming a new organisms. They clearly implied that differentiated cells of a multicellular organism still retain the information which was present in the first single cell, the fertilised egg.

The first botanist to realise the significance of the totipotancy of plant cells was G. Haberlandt, who, in 1902, enunciated ways in which this property, if it existed could be exploited. Haberlandt begins, "To my knowledge no systematically organised attempts to culture isolated vegetative cells from higher plants in simple nutrients solutions have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary unit possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular organisms are exposed". He concluded by writing "Without permitting myself to pose further questions, I believe, in conclusion, that I am not making too bold a prediction if I point to the possibility that, in this way, one could successfully cultivate artificial embryos from vegetative cells". (Thomas and Davey, 1975).

The first major steps into experimental plant tissue culture were made in 1922 by W.J. Robbins in America and by W. Kotte, a student of Haberlandt. (Thomas and Davey, 1975). They used intact meristems excised from roots of grass seedlings. When transferred to a liquid culture medium composed of inorganic salts and glucose the excised roots tips grew vigorously, and gave rise to small root systems sometimes bearing laterals. Unfortunately, the growth of these roots rapidly

declined in culture, and virtually ceased, even if meristems were excised and transferred to fresh medium. The cultural conditions clearly did not permit continuous growth. However, these studies laid the foundation of plant tissue culture as it exists today.

The first successful cultures were those of P.R. White (1934), who isolated and grew for prolonged periods excised root tips of tomato (*Lycopersicon esculentum*) in liquid medium containing inorganic salts, yeast extract and sucrose. Since then many research workers have examined and refined the nutritional and environmental requirements of plant cells in culture, with the work of Murashige and Skoog (1962) providing the basis of most tissue culture media in current use.

Murashige (1974) showed that nutritional and physical parameters can affect the process of plant regeneration and the discovery of cytokinins and the hormonal control of shoots and root regeneration from tobacco callus by Skoog and his co-workers in 1948, established the basis of manipulating organ initiation and provided the principle on which all micropropagation depends. An important advance in the culture of single cells was made in 1960 when Bergmann showed that it was possible to obtain cell suspension with a high proportion of single cells using a simple filtration technique to remove the larger cell aggregates. The single cells could be mixed with agar, cultured in petri dishes, and induced to grow. Later it was shown that the calli obtained from fully differentiated leaf cells could be induced to produced embryoides. The ability to culture single cells provides opportunities to isolate normal and mutant single cells.

1.4.3. MICROPROPAGATION

Soft fruits are traditionally propagated by vegetative means. The most readily identifiable weakness of traditional propagation is the slow rate of multiplication. In addition, it needs specialised conditions in order to maintain disease-and virus-free propagation material. With the introduction of new cultivars and the limited supply

of virus-free clones of existing cultivars, there is a need for a more economical, efficient and practical method of propagation using available plant material. One method that has been used is micropropagation (Boxus, 1974; Boxus et al., 1977; Damiano, 1980). It involves the production of plants from very small plant parts (meristem) grown aseptically *in vitro*, where environment and nutrition can be rigidly controlled. The application of tissue culture techniques to the regeneration and propagation of whole plants is a more recent development. George Morel (1960) pioneered the application of shoot tip culture as a clonal multiplication tool.

1.4.4. CALLUS CULTURE

Micropropagation although attractive in comparison to traditional methods, can be unresponsive to needs. An alternative would be the exploitation of callus culture and regeneration. Callus is a coherent and amorphous tissue, usually produced on explants *in vitro* as a result of wounding and in response to hormones, either endogenous or supplied in the medium. Explants from almost any plant structure or part, seeds, stems, roots, leaves, can be excised, disinfected, and placed on the surface of the culture medium (Street, 1977) for the production of callus. Continued sub-culture is possible at three to four week intervals by small inocula taken from these callus cultures for long periods.

Although callus tissue culture may appear outwardly to be a uniform mass of cells, in reality the structure is relatively complex with considerable morphological, physiological and genetic variation within the callus. Cell division does not take place throughout the culture mass but is located primarily in a meristematic layer on the outer periphery of cells. The inner part of the callus remains as an undividing mass of older tissue, and in time, may differ physiologically and genetically from the cells of the outer layer. Thus variations in cell age and type may occur within the tissue culture mass.

The induction of new plants begins with de-differentiation of parenchyma cells to produce centre of meristematic activity (meristemoids) (Evens et al., 1981; Thorpe

1979; Torry, 1977). In an early study by Skoog, tobacco callus produced shoots (Skoog and Miller 1957) if a relatively high cytokinine/auxin ratio was supplied. If the ratio was reversed roots tended to be formed. Although the same basic pattern tends to follow with most other plants, an exact formula for optimising conditions for regeneration is needed for each species or cultivar.

1.5. REGENERATION AND GENETIC VARIATION IN PLANT TISSUE CULTURE

Problems related to regeneration and genetic variation in callus tissue culture are commonly a matter of interest and concern to plant geneticists and breeders. Successful application of *in vitro* technology to clonal propagation depends on the induceability of growth and differentiation in tissue and the regeneration of true-to-type viable plants. Great differences exist in organogenesis, and regeneration of plantlets among plant species, varieties and even individuals of the same varieties. Some materials are more easy to regenerate *in vitro* than others. In addition to genetic differences in the morphogenetic potential between species or within species, maturation also seems to play a crucial role in the regeneration process *in vitro*. In general, juvenile tissue is more responsive to *in vitro* conditions.

Plant regenerated from somatic cell culture may exhibit variation which may have a genetic or a non-genetic basis. Genetic variation that occurs in plant tissue culture has been termed somaclonal variation (Larkin and Scowcroft, 1981). For obtaining true to type propagules from a selected genotype, somaclonal variation is undesirable. On the other hand somaclonal variation offers prospects for the recovery of useful mutants in tissue culture. The variation rate and spectrum depend on the genotype, type of culture (micropropagation callus, or cell suspension culture) and environmental conditions. Variation may occur in chromosomes (number or structure), nuclear genes or mitochondrial genes.

The fact that *in vitro* culture can cause genetic variation in regenerated plants aroused much interest and discussion and this type has been frequently reviewed (Evans and Bravo, 1986; Evans and Sharp, 1986; Scowcroft et al., 1986). Larkin and Scowcroft (1981) detailed various sources of variation in regenerated plants. They considered that plants derived from any form of cell and tissue culture should be called somaclones and genetic variation produced in these clones should be called somaclonal variation.

During culture various environmental factors may lead not only to genetic variation but can also influence gene expression or cause variation in certain interactions between genetic material. For instance, changes in gene functions (switch on or off), synthesis of certain proteins, cell differentiation, or protein distribution can cause a phenotypic variation which is called epigenetic variation. Although this type of variation cannot be transmitted to offspring via sexual reproduction, it can be maintained by asexual propagation.

It may of course be that different processes are at work in different species or that a number of processes are operating simultaneously in the one culture. Brown (1989) and Muller et al. (1990) reported that most of somaclonal variations are directly or indirectly related to alteration in the state of DNA methylation. Other mechanisms observed in regenerated plants by many workers are: chromosomal aberration i.e. changes in chromosome numbers (D'Amato, 1977; Orton, 1983), chromosome rearrangments, translocations, reciprocal translocation and ring chromosomes (non-homologous translocations) (Ahloowalia, 1976; Shepard, 1982). Published work also suggests that chromosome deletions, additions, inversion, and crossing-over also occurs in regenerated plants. (Meins, 1983; Evans, 1986; Scowcroft and Ryan, 1986; Grunewaldt and Dunemann, 1991). Cryptic changes can result not only in the loss of genes and their functions but also the expression of genes which have been silent. For example a rearrangement may delete or otherwise switch off a dominant allele allowing the recessive allele to affect the phenotype. In eukaryotes evidence suggests that certain unstable mutants may be explained by transposable elements. The

excision and reinsertion of the genetic element can directly affect the expression of the neighbouring structural gene. Weill and Reynaud (1980) referred to the high mutability and consequent adaptiveness in somatic tissue as 'somatic Darwinism'. Somatic genes rearrangements, amplification and depletion have also been suggested to be involved in somaclonal variation. (Nagl, 1979).

1.6. EVALUATION OF CLONALLY PROPAGATED REGENERANTS

1.6.1. MORPHOLOGICAL MARKERS

The easiest and most commonly used method of screening plants for scientific investigation is morphological analysis. These are the earliest genetic markers employed (Devries, 1912) and they may still be of value for certain plant germplasm management applications e.g. trueness to type. Morphological assays generally require no sophisticated equipment nor preparatory procedures. So their prime advantages are simplicity and lack of expense to score even from preserved specimens e.g. herbarium sheets and in maize (*Zea mays* L.). The main disadvantage of this approach is that possession of a normal phenotype is no guarantee that cryptic changes have not occurred. In addition, many of these changes may be recessive and consequently when in heterozygous forms, do not appear until plants have been selfed and progeny examined (Gobel et al., 1985; Brown et al., 1993). Finally, morphological variation shown by the regenerants may be epigenetic or just physiological and the status cannot be confirmed, until progeny have been examined. This is exacerbated for some species in terms of length of life cycle.

Chromosome numbers and cytomorphological traits have also served as genetic markers, especially in polyploid crop complexes, where these have been important tools for elucidating systematics and evolution (Simmonds, 1976). Two primary types of cytogenetic markers are enlisted in germplasm management, chromosome numbers and chromosome morphology. Chromosome numbers are highly heritable. Chromosome morphological features include size, centromere position,

mitotic configurations, and occurrence of satellites which are observable following staining (Dyer, 1979). All these karyotype features have contributed critical data to plant systematic and evolutionary studies (Bennett, 1984). Karyotypic/cytogenetic observations require specialised equipment (microscopes) but preparative protocols are otherwise relatively simple and inexpensive (Dyer, 1979). Correctly interpreting certain cytomorphological features does require considerable training, experience, and advanced knowledge of cytogenetics.

1.6.2. PROTEINS

Protein and isozyme variants (Weeden and Wendel, 1989) that migrate at different rates under electrophoresis have been the most widely employed molecular genetic markers during the last quarter century. Isozymes are generally fractionated by starch gel electrophoresis whereas, proteins are generally analysed via Polyacrylamide gels (PAGE) in sodium-dodecyle sulphate SDS (Cooke, 1984).

Protein fractionation by SDS-PAGE. is relatively rapid and inexpensive as compared with isozymes and some DNA analysis, especially when conducted with precast mini-gels. Protein electrophoretic migration rate is generally highly heritable and ample polymorphism is available when conducted with appropriate laboratory procedures (Simpson and Withers, 1986). In general however, storage protein electrophoretic profiles are rarely interpretable by locus/allele models.

1.6.3. ISOZYMES

During the last 20 years isozymes revealed through starch gel electrophoresis (SGE) have been the genetic markers most frequently employed in many fruit species for the identification of cultivars as well as characterisation of somaclonal variation. (Bringhurst et al., 1981; Bryne et al., 1988; Weeden et al., 1985; Wolfe, 1976; Denton et al., 1977; Damiano et al., 1995). They are generally but not always governed by single Mendelian genes. They can be assayed from a wide variety of organ and tissues, and analytical procedures are not exceptionally complicated (Murphy et al., 1990; Weeden and Wendel, 1989). In studies of

genetic diversity and divergence, isozymes with similar enzymatic activity and electrophoretic migration rates are presumed to be homologous although this assumption cannot be validated without amino acid sequencing.

1.6.4. GENETIC MARKERS:

The concept of utilising markers in plant breeding as an indirect method of selecting desirable recombinant genotypes is well established. Until relatively recently, the markers employed were morphological characters which are limited in number and often agronomically undesirable. As a means of increasing the number of markers, different strategies have been developed based on existing natural variation present in plant genomes. The significance of this variation is that a large number of genetic markers can be assembled in a single cross. Further more, these markers are inherited in a Mendelian and hence, predictable manner. The availability of such markers provides new opportunities to improve the speed and precision of gene transfer in crop improvement.

The detection and exploitation of polymorphisms in plants and animals represents one of the most significant recent developments in biology. The concept of using genetic markers to identify specific regions of the genome is well established but initially was hampered by the lack of appropriate markers. Technical developments have made possible the development of high resolution genetic linkage maps in a range of organisms. These provide new opportunities to analyse complex phenomena such as comparative genome mapping, genome evolution and studies of gene flow. The greater utility of molecular markers arises from six inherent properties that distinguish them from morphological markers (Powell et al.,1994).

- The phenotype of most morphological markers can only be determined at the whole plant level, whereas molecular loci can be assayed at the whole plant, tissue, and cellular levels.
- Allele frequency tends to be much higher at molecular loci compared with morphological markers.

- In addition, morphological mutants tend to be associated with undesirable phenotypic effects.
- Alleles at morphological loci interact in a dominant-recessive manner that limits the identification of heterozygous genotypes.
- Molecular loci exhibit a co-dominant mode of inheritance that allows the genotypic identification of individuals in a segregating population.
- Fewer epistatic or pleiotropic effects are observed with molecular markers than with morphological markers. Hence a large number of polymorphic markers can be generated and monitored in a single cross.

1.6.4.1. RFLP.

Various DNA polymorphisms are the most highly heritable of all genetic markers. In plants, they can be assayed from three distinct genomes, i.e. nuclear, chloroplast, and mitochondrial which may each evolve according to different modes and tempos (Wolfe et al., 1987).

The emergence and general accessibility of molecular biological techniques has allowed the relatively extensive variation which occurs in the DNA sequence of a given organism to be exploited. The standard method by which this is achieved relies on the ability of certain bacterial enzymes, restriction endonucleases, to recognise and cleave specific DNA sequences within the extremely long DNA molecules which comprise a plant's genome. Cleavage results in the generation of a set of restriction fragments of differing lengths which reflects restriction site changes within a given individual. After electrophoretic separation of the fragments according to their length, and transfer to a solid membrane identification of restriction fragments is achieved by southern DNA:DNA hybridisation with a radioactivity labelled cloned DNA probe and visualised by exposure of the hybridisation membranes to photographic film. The variation which occurs has been termed restriction fragment length polymorphisms (RFLPs). Since the mid-1980s, RFLPs have been used extensively for the construction of genetic linkage maps, and RFLPs linked to many desirable characters have been identified. RFLPs

are also useful for other applications including cultivar identification, evaluating germplasm resources, identifying distantly related parents for inclusion in a breeding programme and for phylogenetic studies.

RFLPs are superior genetic markers, because they are firstly ubiquitous throughout the plant tissue and throughout the plant genomes coding and non-coding sequences and secondly highly heritable, relatively highly polymorphic, and co-dominantly inherited. (Helentntjaris and Burr, 1989).

A major drawback of RFLPs is that their application is technically difficult and in the majority of laboratories the detection methods rely on the use of short-lived radioisotopes. At present, RFLP analysis is relatively slow and labour intensive. It may involve expensive and sometimes radioactive/toxic reagents. (Murray et al., 1988; Helennljaris and Burr, 1989; Bernatzky and Tanksley, 1989). In many cases, these features may inhibit the routine application of RFLPs in plant breeding.

1.6.4.2. AFLP.

The Amplified Fragment Length Polymorphism (AFLP) technique is based on the amplification of subsets of genomic restriction fragments using the polymerase chain reaction (PCR). DNA is cut with a restriction enzyme, and double stranded (ds) adapters are ligated to the ends of the DNA-fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments from a total genomic digest. Selective nucleotides are added to the 3' ends of the primers, that therefore can recognise only a subset of the restriction fragments. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

The restriction fragments for amplification are generated by two restriction enzymes, a rare cutter and a frequent cutter. The AFLP procedure results in the predominant amplification of those restriction fragments, that have a rare cutter site

on one end and a frequent cutter site on the other end. Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. Typically 50-100 restriction fragments are co-amplified and detected in each AFLP reaction. This technique is therefore extremely powerful for the detection of DNA fragments, and hence for the identification of DNA polymorphism. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. The AFLP techniques is robust and reliable because stringent reaction conditions are used for primer annealing: the reliability of RFLP techniques is combined with the power of PCR techniques and of gel analyses of amplified fragments.

The AFLPs like RFLPs are highly heritable, relatively highly polymorphic, apparently selectively neutral, and can be isolated from virtually every type of plant tissue. The AFLP fragments are generally inherited in a simple dominant Mendelian fashion, whereas fragment absence is recessive. In this respect AFLP markers are inferior to co-dominant genetic markers and with expensive DNA polymerase enzymes at a relatively high cost. Nevertheless, AFLP analysis may be amenable to automation, requires very little tissue for analysis, and does not require blotting, probing, probe maintenance in bacteria, or other expensive steps associated with RFLP analysis (Smith and Smith, 1992).

1.6.4.3. RAPD-PCR.

Considerable progress has been made in the application of DNA-based methodologies for the identification of phylogenetic relationships in many crop species. Exciting new opportunities for improving techniques of identification and diagnosis were created by the introduction of the polymerase chain reaction (PCR). However, the advent of PCR has brought the potential of a DNA-based marker system one step closer to being routinely and reliably applied. PCR technology hinges on the availability of DNA polymerase (Taq polymerase) from the thermophilic bacterium *Thermus aquaticus* which retains activity even after

prolonged incubation at temperatures which denature double stranded DNA templates. Defined segments of minute quantities of target DNA can be specifically amplified by supplying Taq polymerase, excess nucleotides and oligonucleotide primers (which are exactly complementary to sequences flanking a target sequence), and repeating a thermal cycle which denatures the DNA, allows the primers to anneal to their complementary sequence and finally activates the DNA polymerase. Taq polymerase will copy the single stranded target DNA unidirectionally from the annealed primers. Any sequence up to a size of approximately 4000 nucleotides, which is flanked by two primer binding sites, can be amplified exponentially by repeating the thermal cycle up to 45 times. The specificity of amplification is determined by the nucleotides of the individual primers. After amplification sufficient product is produced to be visualised directly after electrophoresis by ethidium bromide staining and illumination by UV light.

One of the most important developments occurred in 1990 when a variation of PCR was developed independently by two different laboratories (William et al., 1990; Welsh and McClelland, 1990). The technique concerns the analysis of Random Amplified Polymorphic DNA (RAPDs) and provides a novel and effective method for distinguishing organisms according to the banding patterns of their DNA as well as providing a new means of obtaining genetic markers. RAPD is a modification of PCR technique which requires no prior knowledge of nucleotide sequence and is becoming increasingly attractive as a DNA based marker system. The approach is based on the probability that in the genome of the organism under study, a given single nucleotide sequence will occur in inverse orientation within a distance that is amplifiable by PCR. The primers used are generally only 10 nucleotides in length with their sequence determined arbitrarily. Differences in the sequences amplified from related individuals are caused by either mutation in the primer binding sites or by DNA rearrangements. Differences detected using this technique have been called random amplified polymorphic DNA markers or RAPDs.

The technique is fast, technically easy, and requires few materials. One of the main advantages of RAPD analysis, in contrast to many other PCR-based protocols, is that there is no requirement for a prior knowledge of the molecular biology of the organisms under study; the technology is not dependent upon predetermined nucleic acid sequence data. Traditional PCR-based techniques depends on the availability of DNA or RNA sequence information in order to design primers. Such sequence information is lacking for most organisms and, therefore, systematic studies have tended to be biased towards limited numbers of well suited genes. In these cases it has been possible to construct primers based on relatively highly conserved regions of the genes in question from other species. The unique feature of RAPD analysis is that a single primer of arbitrary nucleotide sequence is added to the PCR. At low stringency, the primer will anneal to a number of complementary sequences within the template DNA. When the single primer binds to the genomic DNA at two sites on opposite strands of the DNA template which are within an amplifiable distance of each other, discrete DNA fragments will be produced through thermocyclic amplification. The multiple products of different sizes which are produced during the reaction from a single genomic DNA template may be resolved by gel electrophoresis. The presence of an amplification product indicates complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotides primers, at each end of the amplified product. Different primers, and the choice is practically unlimited, will initiate the amplification of different parts of the genome. Many markers can readily be identified as a variety of taxonomic levels and in comparison with DNA sequencing, the effort and cost are modest so that many individuals can be assayed. Ideally these arbitrary primers yield at least several but not too many marker bands, that generally are inherited as dominant. Arbitrary primers methods are most useful when analysing closely related germplasm (Smith, 1992).

1.7. OBJECTIVES

The general aim of my study was to develop and use molecular markers to study genetic uniformity of the plants arising through *in vitro* culture and to consider their potential application for blackcurrant cultivar identification

The specific objectives were:

1 • To examine the molecular markers for cultivar discrimination and analysis of their phylogenetic relationships.

2 • To assess genetic uniformity of regenerated plants from micropropagation in soft fruit

3 • To explore the potential of callus culture and regeneration as a means of rapid multiplication in soft fruits

4 • To assess genetic uniformity of the regenerated plants via callus culture followed by regeneration

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL CONDITIONS

The research was conducted in the laboratories and plant growth unit of the Department of Crop Science and Technology, Scottish Agricultural College, Edinburgh. Plants were grown in glasshouses at 22±2°C under 14/10 hours light/dark cycle. The light source used were 400 watt, high pressure, sodium lamps. All *in vitro* cultures were incubated in the culture room at 26°C ± 1°C under 16/8 hour light/dark cycle. In the culture room the light was provided by 70 watt, cool, white fluorescent light ranging from 2000 to 3000 lux.

All *in vitro* culture and preparation was carried out inside a laminar flow cabinet (Bassaire). All the equipment used was sterile and every effort was made to prevent subsequent contamination of cultures. Petri dishes were sealed with a double layer of parafilm M®. Experimental design used in most of the experiments, was a completely randomised design (CRD).

2.2. TISSUE CULTURE

2.2.1. PLANT GROWTH REGULATORS

Plant growth regulators used included the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), indole butyric acid (IBA), and indole acetic acid, (IAA), and the cytokinins used were, kinetin and benzyl aminopurine (BAP). The hormones were dissolved in a few drops of appropriate solvent such as 1M sodium hydroxide (NaOH) or ethanol (EtOH) as recommended by the supplier and made up to 1 mg/ml stock solution with sterile distilled water. Appropriate quantities of hormone stock solution were added. All hormones were added to the

media after autoclaving by micro-pipette through a 0.2 μm micro-filter (flow pure). This was done in order to avoid the degradation of hormones during the autoclaving process. Concentration of the growth regulators used in the different media used in this study is provided in the relevant sections.

2.2.2. PREPARATION OF MEDIA

The composition of the media used in this study consisted of MS media (Murashige and Skoog, 1962) (supplied by Flow Laboratory, Irvine, Scotland) or Gamborgs B5, (Gamborg et al., 1968) (supplied by Sigma, UK) and Nitch and Nitch basal salt mixture described by Nitch and Nitch (1969) and Bourgin and Nitch (1967). The major macro-and micro-nutrients in each media are provided in Table 2.1. The culture media was supplemented with varying concentrations of growth regulators specific to the requirements of each experiment. Sucrose was used as the sole carbon source and was added as a solid at concentration of 3%. The pH of the media was adjusted by adding either 0.1M NaOH or 0.1M HCl, to give a final value in the range of 5.6-5.8. Finally, phytogel (Sigma) was added at a concentration of 0.2% to solidify the medium. The media was sterilised by autoclaving (Drayton Castle model) at 1 kg cm^{-2} pressure and 120°C for 20 min.. The media was kept warm at 50°C until pouring into culture pots. The media was dispensed into sterile pots under sterile conditions in a laminar flow cabinet and allowed to cool and set before being labelled and used.

Table. 2.1. ***Basic ingredients of media used in soft fruit tissue culture and plant regeneration.***

Compound	Concentration (mg/l unless otherwise stated) MS-medium[1]	Gamborg's B5 Medium[2]	Nitsch and Nitsch Basal salt[3]
Macro nutrients:			
NH_4NO_3	1650	—	720
KNO_3	1900	2500.0	950
$CaCl_2.2H_2O$	332.2	113.24	166
$MgSO_4$	180.7	122.09	90.372
KH_2PO_4	170	—	68
$NaH_2PO_4.H_2O$	—	130.5	—
$(NH_4)_2\ SO_4$	—	134	—
$FeSO_4.7H_2O$	27.8	27.8	27.8
$Na_2.EDTA.2H_2O$	37.26	37.26	37.3
Micro nutrients:			
H_3BO_3	6.2	3	10
$MnSO_4.H_2O$	16.9	16.9	18.9
$ZnSO_4.7H_2O$	8.6	2	10
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	—
$Na_2MoO_4\ 2H_2O$	0.25	0.25	—
KI	0.83	0.75	—

Table. 2.1 (Continued)

Compound	MS-medium[1]	B5 Medium[2]	Nitsch and Nitsch[3]
Vitamins:			
Myo-inositol	100.00	____	____
Nicotinic acid	0.5	____	____
Pyridoxine HCl	0.5	____	____
Thiamine HCl	0.1	10.00	____
Glycine (Free base)	2.00	____	____
Phytohormones:			
2,4-dichlorophenoxy acetic acid (2,4-D)	Var [4]	Var	Var
Naphthalene acetic acid (NAA)	Var	Var	Var
Kinetin	Var	Var	Var
Benzylaminopurine (BAP)	Var	Var	Var
Indolebutyric acid (IBA)	Var	Var	Var
Others:			
Sucrose	Var	Var	Var
Phytogel	2g	2g	2g
pH	5.7	5.7	5.7

1. Murashige and Skoog Basal medium (1962).
2. Gamborg et al., (1968).
3. Nitsch and Nitsch, (1969), and Bourgin and Nitch (1967).
4. Variable in different experiments.

2.2.3. EXPLANT SOURCES AND PREPARATION

One blackcurrant cultivar Ben Sark and five raspberry and two strawberry cultivars were supplied by the Department of Horticulture, Scottish Agriculture College at Bush Estate at first or second sub-culture stage. These were further subcultured in the plant growth unit (PGU) of Scottish Agriculture College, Edinburgh. The leaf samples were collected and stored at -80 °C at each sub-culture stage for DNA analysis.

For callus culture two types of explants (leaf discs and petioles) were used in different studies for callus initiation. Explants were taken either from glasshouse grown plants which were supplied either by the Horticulture Department, Scottish Agriculture College, Bush Estate or from local nurseries and were kept in the glasshouse for continuous use or from micropropagated plants.

For the DNA analysis, leaves of blackcurrant cultivars were supplied either by the Scottish Crop Research Institute (SCRI), Dundee or from local nurseries. Leaves of cv. Baldwin were kindly supplied by Smith Kline Beecham, Welsh Fruit Stock Hereford, Brook House Farm, Lincs and Tip Top Farm Essex. The leaves were stored at -80°C for future use

2.2.4. STERILISATION OF LEAVES AND PETIOLES

Newly emerging leaves and petioles were taken from glasshouse grown plants and washed with running tap water. The plant material was submerged in sterile distilled water with two drops of Tween-20 and agitated on a gyratory shaker for 10 min.. After washing, the plant material was again submerged in a 2% sodium hypochlorite solution and agitated on a gyratory shaker for 15 min.. Finally, the plant material was rinsed several times with sterile distilled water to remove traces of sodium hypochlorite. The surface sterilised leaves and petioles were used as explant source for callus initiation.

2.2.5. PREPARATION OF EXPLANT

Surface sterilised leaves were used to punch out leaf discs for use in callus initiation. Discs were produced using a sterile metal cork borer with an internal diameter of 6 mm. Sterile petioles of about equal thickness were separated from leaves after sterilisation and were placed into 90 mm diameter Petri dishes. The cut ends of petioles, which were directly exposed to sodium hypocholorite during the sterilising process became brown and were removed with the help of a fine sharp scalpel. The rest of petioles were cut into approximately 10 mm long segments and used as explants for callus initiation.

2.3. CALLUS CULTURE AND MAINTENANCE

2.3.1. CALLUS INITIATION

All type of explants were cultured on B5 medium for callus initiation. Petiole segments were placed horizontally on the surface of the culture medium and pressed slightly to ensure contact with the medium. Three pieces of explants were placed in each Petri dish (50 mm diameter).

The leaf discs were carefully placed on the surface of the culture medium, keeping the abaxial surface up the discs were pressed slightly to make contact with the medium and adaxial surface of the leaf discs. Three leaf discs were placed in each Petri dish of 50 mm diameter. Petri dishes were sealed with a double layer of parafilm. All the Petri dishes were placed upside down to avoid condensation and inhibit light penetration. All the Petri dishes were placed randomly in the culture room and incubated for six weeks before scoring callus growth.

2.3.2. SUB-CULTURE AND MAINTENANCE

Sub-culturing becomes imperative in order to maintain the culture or to increase the volume of callus. Continuous growth of cells in a limited environment leads eventually to the accumulation of toxic biotics and the exhaustion or drying-out of

the medium. Therefore, callus requires to be transferred to a fresh nutrient medium for continuity and enhanced callus growth. The length of sub-culture period depends upon the rate of cell growth and genotype in use. Once well established, normal callus culture lines will require regular sub-culture at approximately four weekly intervals (Dixon, 1985). In the case of *in vitro* culture of soft fruit species, different workers subcultured callus with different intervals ranging from 4 to 10 weeks (e.g. Nehra et al., 1990: 8, 4 weeks. Toyoda, et. al., 1990: 30 to 40 days). For this project it was decided that callus would be scored 6 weeks after initiation and then subsequently subcultured for another four weeks before transfer to regeneration media.

All the cultures were removed from the Petri dishes and only healthy-looking callus pieces were sub-cultured on to the new media. Callus pieces were divided into small pieces if they were large enough and transferred to the surface of fresh media in 50 mm diameter Petri dishes. All the cultures were incubated in the culture room with the conditions given above. (Section 2.1).

2.3.3. MEASURING CALLUS GROWTH

Growth of callus culture has been measured using a number of approaches such as callus fresh weight (Turner and Dickinson, 1993); % increase in callus fresh weight (Chandler et. al., 1986; Jain et al., 1990); callus dry weight (Bressan et al., 1985); automated image analysis (Nyange, 1994) and callus score based on an area covered (Dietert et al., 1982; Ogihara and Tsunewaki, 1979). When selecting appropriate methods emphasis was given to non-destructive and reliable techniques. Therefore a callus score methodology was selected as a measure of growth. All the explants were scored after 6 weeks of callus initiation. Callus growth was visually classified into the five classes given in Table 2.2 depending upon the growth area covered.

Table 2.2. Scoring based on ranking (0 to 5) callus growth

Scores.	**Growth on the basis of areas covered.**
0	No growth.
1	Growth started and covered only just margins.
2	Poor growth covered 1/4th area of explant.
3	Fair growth cover 1/2 area of the explant.
4	Good growth covered 2/3 area of explant.
5	Vigorous growth covered all the explant.

The reasons for selecting a growth scoring method was that the same callus was used for regeneration and further studies and this method provided maximum security to avoid the risk of contamination, and also this method is very quick and easy.

2.3.4. REGENERATION FROM CALLUS

To regenerate plants from callus pieces, 10 week old callus pieces were transferred to a regeneration media in 50 mm Petri dishes and incubated for 4 weeks in a growth room. After four weeks calli were transferred to fresh media for another 4 weeks. After a total incubation of 8 weeks on regeneration media bud and shoot formation was noted. Regenerated shoots were transferred to the rooting media in 100 ml plastic vials and closed with plastic screw caps, and incubated in the culture room until sufficient roots were developed. Shoots produced at 10 week old callus on callus initiation media were directly transferred to the rooting media and incubated until sufficient roots were developed.

2.3.5. TRANSFER TO SOIL AND ACCLIMATISATION

Epicuticular wax that helps to control transpiration is lacking or present in reduced quantities in the plantlets regenerated *in vitro*. Consequently, the regenerated plantlets frequently show symptoms of severe water stress when they are transferred to an open bench in a glasshouse (Grout and Aston, 1977). Control of the humidity around the plantlets following their transfer from the high humidity atmosphere of the culture tubes is critical for survival and special treatments were needed to acclimatise the regenerated plants to the new environment.

Regenerated shoots with sufficient roots were removed from the vials. Roots were washed thoroughly in running tap water to remove traces of agar, and handled very gently. Regenerated plants with washed roots were transferred to the mixture of 50:50 perlite and compost in disposable plastic pots. Pots were placed in plastic trays and flooded with water. Plants were covered with propagators for hardening off and the trays were placed in the glasshouse. Plants were kept in the hardening condition for four weeks and then transferred to 9 cm plastic pots containing compost and watered regularly.

2.4. BIOCHEMICAL MARKER ANALYSIS

2.4.1. PROTEIN ELECTROPHORESIS

Of the many methods of electrophoresis available the dissociating buffer system (SDS-PAGE) is most widely used to distinguish cultivars (Hames, 1981). In this system polypeptides are fully denatured by reduction with Dithiothreitol (DTT), and saturated with SDS, which eliminates charge variability between polypeptides, giving them all the same charge to mass ratio and forcing them into a rod-like shape. Separation in SDS-PAGE is based on molecular weight, eliminating conformational and charge density variabilities. Electrophoretic migration is then proportional to the effective molecular weight of sub-unit protein.

2.4.2. SAMPLE PREPARATION

Mature fully expanded leaves (weight 0.2 g) were collected either from greenhouse grown plants or from stored material at -80°C in a freezer and were ground in 800 μl of extraction buffer with an appropriate amount of fine purified acid-washed sand (mesh+70). The extraction buffer employed was Tris-HCl containing 0.5M Tris-HCl pH 7.5, plus 3% SDS and 0.25% Dithiothreitol (DTT). After grinding, sample solutions were placed into 1.5 ml Eppendorf tubes and were labelled. The samples were then centrifuged at 10000 x *g* for 10 min. to separate solid particles from the soluble protein. The supernatant was decanted into 1.5 ml Eppendorf centrifuge tubes and labelled.

2.4.3. GEL PREPARATION

Excel Gel TM SDS, gradient 8-18 preprepared gels and precast anode and cathode SDS buffer strips were employed. Electrophoresis was carried out on a horizontal Pharmacia LKB 117 Multiphore II electrophoresis unit. The plate of LKB 117 was stabilised at 10°C by switching on cool circulating water 20 min. prior to electrophoresis to avoid overheating at higher voltages. Approximately 1 ml of 0.1% of "Triton x" was applied on the plate to assure complete contact for thermal energy exchange to prevent gel from overheating. The slab gels were placed on the cooling plate with great care to avoid any bubbles between gel and plate. Cathode and anode buffer strips were applied to the respective sides of the gel by applying the narrowest sides of the strip on to the gel surface. A white sample loading well-strip was placed on the gel close to the cathodec buffer strip.

2.4.4. SAMPLE APPLICATION

A duplicate sample volume of 10μl was applied with an auto pipette directly onto the white well strip one by one in alternate wells to avoid diffusion. After applying all the samples electrophoresis was carried out under the following conditions:-

Voltage.	600 V.
Current.	50 mA.
Power.	30 W
Time.	70 min.

A tracking Bromophenol blue dye was also loaded in the first and the last sample for easier monitoring of the electrophoretic run. Electrophoresis was stopped when the Bromophenol blue front reached the anodic buffer strip.

2.4.5. STAINING, DESTAINING AND PRESERVING OF GELS

Immediately after electrophoresis the gel was immersed in Coomassie Blue staining solution (Phast Gel® Blue R 1 tablet and made up to 400 ml with destaining solution), and left in the solution overnight. After staining, differential destaining was undertaken by frequent changes of destaining solution (Ethanol 250 ml, acetic acid 80 ml and made up to 1000 ml with distilled water) usually at least 2 to 3 changes of destaining solution were necessary before the background became clear The gels were then transferred to preserving solution (Glycerol 87%w/v 30 ml and make up to 250 ml with destaining solution) and remained there until assessed.

2.4.6. GEL EVALUATION

Gels for variety description, discrimination and identification were evaluated by eye using a light box (Hancocks Co.). For relief during determination gels were rinsed several times and stored in distilled water. The distance that protein bands migrated were measured and used to calculate Rf values (relative mobility of protein subunits). In addition, the intensity of each band was recorded. Electrophoretograms were created by means of an IBM personal computer and Harvard Graphic/Draw partner programme (Vers.3.0). Photographs of the gels were taken using an Olympus OM-2N camera, tripod, and light box, with a Kodak 200 ASA colour print film. Gels were preserved in a preserving solution.

2.5. MOLECULAR MARKER ANALYSIS

2.5.1. SAMPLE STORAGE FOR DNA EXTRACTION

For molecular analysis plant leaves were collected from different culture conditions. Collected samples were stored in 1.5 ml Eppendorf tubes after submerging them in liquid nitrogen for a few seconds and then in a -80°C freezer until required for analysis. Different samples were stored in different coloured Eppendorf tubes which were clearly marked on the top and side showing types and generation /sub-culture of the sample. For cultivar identification and regenerated plants freshly grown top leaves were collected from glasshouse grown plants.

2.5.2. DNA ISOLATION

Extraction of DNA is the first step in molecular analysis. Plant DNA isolation methodology has evolved rapidly in the last decade. From cumbersome, messy, and often inefficient large scale phenol procedures, DNA technology moved to more efficient methods involving ultra-centrifugation in caesium chloride gradients. Still, such procedures are slow and expensive, and not well suited for assaying large number of samples. More recently a number of "mini-prep" procedures have been developed that permit rapid isolation of DNA using small amounts of tissue. There are a number of different protocols available for extraction of DNA from small samples, which is fortunate because it appears that different procedures work best for different plant groups, as might be expected considering the great diversity of plant secondary compounds that in many cases may interfere with a particular method of DNA isolation."DNA extraction kits" such as "Pure gene" and "Elu-quick" were assessed and found unsuitable for soft fruit species because a large amount of other secondary chemicals (possibly phenolic substances) also came along with extracted DNA, which inhibited the RAPD-PCR reaction. The CTAB DNA isolation method was found to be very easy, quick and economical giving isolated DNA of high quality with minimum smearing. The CTAB method described by Doyle and Doyle (1987) was found best with the slight modifications described below:

0.5 g of leaf material was ground in liquid nitrogen with a pinch of sand, and 3 ml of CTAB solution (100 mM Tris pH 8, 1.4M sodium chloride, 20 mM EDTA, 1% DTT, 2% CTAB and 2% PvP-40) was added towards the end of grinding. This was incubated at 60°C for 30 min. 2.5 ml of chloroform / isoamyl alcohol (24:1) was then added and mixed well. The mixture was spun at 16000x *g* for 5 min., and the resulting supernatant was decanted into another 1.5 ml Eppendorf tube containing an equal volume of ice cold isopropanol and mixed gently to precipitate the DNA. In most cases there was very clear precipitation of DNA. In those cases where DNA was not precipitated, DNA was pelleted by spinning at 16000 x *g* for one min. The pellet was rinsed with 1 ml 76% ethanol. The pellet was left under sterile conditions in a laminar flow cabinet for 2 to 3 hours in order to dry completely. After drying, the pellet was re-suspended in 500 µl of T E buffer (10 mM Tris-HCl, (pH 7.5), 1 mM EDTA). 5 µl of RNase A (10mg/ml) was added to the DNA and incubated at 65°C for 15 min. Any insoluble material was removed by centrifugation for 5 min. at 16000*g* and the supernatant removed to clean sterile labelled tubes and stored at 4°C until required for PCR amplification.

2.5.3. ESTIMATION OF QUANTITY, QUALITY AND PURITY OF DNA

The DNA extracted from different samples was run on agarose gel directly as described in Section 2.6.1. The samples showed a single, sharp band of about 23 kb (Figure 2.1). A single sharp band of high molecular weight without smearing, indicates a good quality DNA with uniform segment size and less damage during the extraction process. When the extracted DNA samples were tested for PCR work, the amplification was successful. Therefore the quality of the extracted DNA, using CTAB methods was considered satisfactory for amplification work.

Quantification and purity of the DNA was based on the spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the nucleotide bases, as described by Sambrook et al. (1989). A DU®-65 spectrophotometer was used for taking the readings. Silica (Quartz) Ultra Micro Spectrophotometer cuvert (Sigma) was used for holding samples and T.E. buffer (10 mM Tris HCl, (pH 7.5),

1 mM EDTA) solution was used for calibrations of the spectrophotometer at 260 nm and 280 nm. Readings were noted for each DNA sample at wavelengths of 260 nm and 280 nm. The reading at 260 nm was used to calculate the DNA concentration in the sample. An optical density value 1.0 corresponds to approximately 50μg /ml for double stranded DNA (Maniatis et al., 1982). The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provide an estimate of the purity of DNA. Pure preparation of DNA have OD_{260}/OD_{280} value of 1.8. If there is contamination of protein or phenols the ratio will be significantly lower than 1.8, and higher ratios correspond to contamination by RNA.

2.5.4. DNA AMPLIFICATIONS

PCR were carried out for amplification of DNA. The techniques of William et al. (1990) were adopted with slight modifications for DNA amplification of the samples. Amplification reactions were carried out in volume of 50 μl of reaction mixture in Ultraflux™ PCR tubes (Scotlab). The reaction mixture consisted of 10 mM HCl pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 200 μM each of dATP, dGTP, dCTP, dTTP, 1.25 units of Stoffel fragment Taq DNA polymerase (Perkin Elmer Cetus, USA) and of Genomic DNA template were present at a concentration of 30-40 ng per reaction based on dilutions calculated from spectrophotometer readings at 260 nm. and 0.2 μM of 10-base primers (Pharmacia Biotech, Uppsala, Sweden and Oswel, Edinburgh, Scotland).

DNA amplification was carried out in Gene E (Techne) thermocycler, using a step cycle, programmed for 45 cycles of 1 min. at 92°C for DNA denaturation, 2 min. at 35°C for annealing, and 3 min. at 72°C for primer extension followed by a final primer extension step at 72°C for 10 min.. A positive control comprising reaction mix with λ-DNA replacing genomic template DNA and two λ specific primers were often included to ensure the reaction components were working properly, and a negative control, comprising the reaction mix minus DNA template was always included to avoid contamination.

After amplification reaction 10 µl of loading buffer was added to the amplified products in order to stop any further reaction. 8-10 µl depending on the gel used was loaded. Amplified DNA products were visualised by electrophoretic separation using the same conditions as used for the visualisation of source DNA. A 100 base pair DNA ladder (GIBCOBRL, Life Technologies™) was always loaded and used to check the fragment length of the PCR products.

2.5.5. STANDARDISATION OF RAPD ANALYSIS

For best PCR results, optimisation of different reagents used in PCR reaction mixture and other PCR conditions is a pre-requisite. These include, quantification of target DNA, polymerase enzyme, primer and $MgCl_2$ concentration, temperature and duration of each cycle and the total number of cycles for amplifications. In particular the concentration of $MgCl_2$ can have the profound effect on the specificity of yield of an amplification (Saiki,1989).The magnesium ion (Mg^{++}) concentration may affect all of the following, primer annealing, product specificity, strand dissociation temperature of both template and PCR product, formation of primer-dimer artefacts and enzyme activity and fidelity (Innis and Gelfand, 1990). Muralidharan and Wakeland (1993) compared profiles from amplification which differ in concentration of template and primer and found that apart from some quantitative differences there were significant qualitative differences. Some of the bands observed with lower concentration of template and primers were absent in profiles from higher concentration of template and primer. Dramatic alteration in RAPDs profile were produced by minute variation in the concentration of magnesium below a level of 2mM threshold (Ellsworth et al., 1993). Therefore, it is essential to optimise the magnesium ion concentration and amount of DNA template for any PCR programme.

In this study nine different concentrations of magnesium ion (i.e. 0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mM) were examined using one primer 5' CACTGCAGTC 3'. DNA of the parent plant was used as the template and all

other conditions were the same for all $MgCl_2$ treatments. The amplifications were carried out in two replications. The PCR protocol followed was as described under Section 2.5.4.

The results obtained were quite clear (Figure 2.2). At 1 mM $MgCl_2$ concentration only one band was visible whereas, at 1.5-2.0 mM at least three bands were visible and at 2.5-3.5mM concentration seven bands. At higher concentrations the number of visible bands reduced. Figure 2.1 shows that at the concentration of the 2.5-3.5mM the number of bands are similar, but at the lower concentration resolution is very sharp and an increase in concentration results in a loss of resolution. Therefore it was decided that 2.5 mM $MgCl_2$ would be used for PCR reaction in this study.

The DNA concentration was also optimised by using 10, 20, 30, 40 and 50 ng in each reaction and it was found that to be 40 ng in each reaction gave good results and this concentration was used in this study. The optimum DNA concentration was found to be 40 ng in each reaction. Different workers used different DNA concentration in PCR reaction Graham et al. (1996) 15 ng, Graham et al. (1994) 20 ng, Lanham et al. (1995) 50 ng, and Parent (1993) 20 ng, Hancock et al. (1994) 50 ng. The reason for these differences might be the quality of extracted DNA, the accuracy of DNA quantification methods used to estimate the DNA concentration, and the PCR conditions employed.

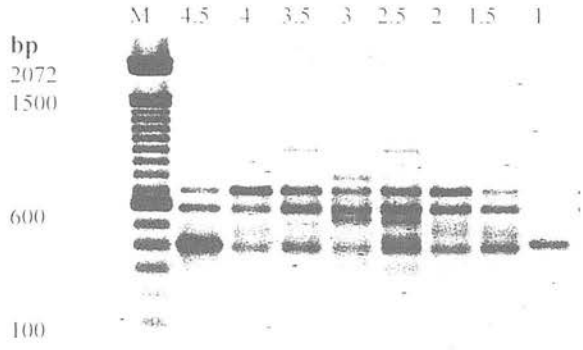


Figure 2.2. Effect of different concentrations of (Mg++) ion on the amplification of DNA in RAPDs. Number of each lane represents the mM of $MgCl_2$ used in this reaction.

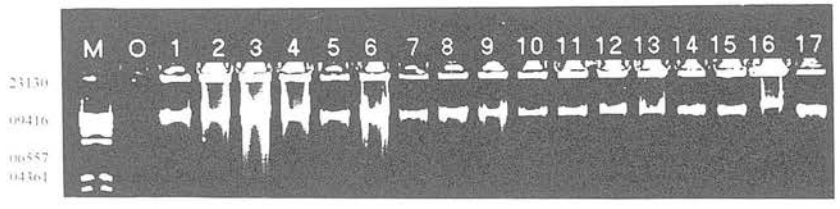


Figure.2.1. DNA extraction through CTAB method. DNA was run 1.4% agarose gel in TBE-buffer for one hour and thirty minutes, visualised after staining in ethidium bromide solution. M is high moleculer weight marker and bp for segment size of the DNA marker in base pairs. Lane 0 is blank and lanes 1-17 contained the isolated sample DNA.

2.6. ELECTROPHORESIS OF DNA

2.6.1. AGAROSE GEL ELECTROPHORESIS

To confirm the size and successful extraction, isolated DNA and for analysis PCR amplified DNA products were visualised with ethidium bromide after electrophoresis on a 1.4% agarose gel, by adopting the standard protocol of Sambrook et al. (1989). Gels were prepared in 1X TBE (89 mM Tris-HCl (pH 8.3) 89 mM boric acid, 5 mM EDTA) buffer. The required amount of agarose (Bio-Rad) was added to the TBE buffer and microwaved until the agarose dissolved completely and the solution became clear. Once cooled to 60°C the solution was poured into the appropriate gel tray containing a suitable comb. The agarose gel was allowed to set. After 30 min. the comb was removed. The gel was then sub-merged into a running buffer 0.5X TBE. DNA samples were mixed with loading buffer (0.05 % w/v bromophenol blue + 40 % w/v sucrose + 0.1 % w/v EDTA pH 8.0 and 0.5 % w/v SDS) in 1:5 ratio. 8µl of this solution was loaded into each of the wells created by the combs. Lambda Hind III digested DNA markers (Sigma) or 100 base pair ladder (Life Technologies$_{TM}$) were also loaded on each gel. Electrophoresis was carried out by using Mini Sub™ or wide DNA Cell (Bio-Rad) at 55 Volts for one hour and thirty min. The gels were stained for twenty min. in ethidium bromide solution (0.5 µg / ml) and visualised on a UV transilluminator at 302 nm.

2.6.2. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was used for silver staining of amplified product in order to observe more DNA fragments as compared with agarose gel electrophoresis. Excel gel® (12.5%) 48S, for DNA of 250 x 110 x 0.5 mm size and precast anode and cathode SDS buffer strips were employed. Electrophoresis was carried out on a horizontal Pharmacia LKB 117 Multiphore II electrophoresis unit. The plate of LKB 117 was stabilised by connecting and setting the temprature at 15 °C and switching on cool circulating water 20 min. before starting electrophoresis in order to avoid overheating at higher voltage. Approximately 1 ml of 0.1% of insulating fluid "Triton x" was applied on the plate to ensure complete contact for

thermal energy exchange and to prevent gel over heating. The slab gels were placed on cooling plate with great care to avoid any bubbles between gel and plate. Cathode and anode buffer strips were applied to the respective sides of the gel by applying the narrowest sides of the strip on to the gel surface.

After amplification 5 μl of amplified product were mixed thoroughly with 2 μl of loading buffer (10mM Tris 1mM EDTA 0.025% Xylene cynol solution 40% of bromophenol blue pH 7.5) and sample volume of 7μl was applied with an auto pipette directly into the precast wells one by one. After applying all the samples electrophoresis was carried out under the following conditions:- Voltage 600 V, Current 50 mA, Power 30W and Time 90 min. or until the bromophenol blue reached the anodic buffer strip.

2.6.3. VISUALISATION OF DNA FRAGMENTS IN GELS

2.6.3.1. ETHIDIUM BROMIDE STAINING

The fluorescent dye, ethidium bromide was used to visualise DNA following agarose gel electrophoresis. The compound intercalates within DNA molecules which consequently fluorescence under ultraviolet radiation. Once electrophoresis was complete, gels were immersed in ethidium bromide solution for 20 min. and destained for 5 min.. DNA was visualised under ultraviolet radiation by placing the stained gel over a UV transilluminator (302nm). Photography of gels was carried out using a UVP Image Store 5000 ultraviolet product computer system.

2.6.3.2. SILVER STAINING

The Pharmacia Biotech protocol included in the silver staining kit (Catalogue No. 71-7178-00) edition AB was used, immediately after electrophoresis was complete. Briefly, the gel was removed from the tank and placed in a container with 250 ml of fixative solution (Glacial acetic acid 25 ml made up to 250 ml with distilled water) for 30 min. The fixative solution was removed, and 300 ml distilled water were added and washed three times, 2 min. each time. Silver solution [(silver nitrate

solution (1.0%w/v) 25 ml, Formaldehyde (37%w/v) 0.25 ml made up to 250 ml with distilled water)] were than added to the containers for 20 min., and washed with 300 ml of distilled deionised water for 0.5 min. Developing solution [(sodium carbonate 6.25 g. Formaldehyde (37%w/v) 0.25 ml Sodium thiosulphate solution (2%w/v) 0.25 ml) made up to 250 ml with distilled deionised water] was added to the container for two to five min. Once the bands became very clear the gel development was then stopped by adding 250 ml of a 1.46 % EDTA-$Na_2.2H_2O$ solution for 10 min. The gel was then washed three times in tap water and then photographed.

2.7. STATISTICAL ANALYSIS

2.7.1. TISSUE CULTURE DATA ANALYSIS

The scored data was transformed using $\sqrt{Y+0.5}$ before analysis The results of each test were evaluated using analysis of variance (ANOVA). Minitab 11.1 for windows statistical package on Scottish Agriculture College computer network was used to analyse the results of tissue culture. Microsoft Excel version 5.0 was used to create graphs and charts.

2.7.2. GENETIC SIMILARITY ESTIMATE

Each of the clear, RAPD bands across all the samples was assigned a number (1, 2, 3, 4....n). Each band was treated as a unit character, and the samples were scored for the presence or absence of a band and coded as 1 or 0 respectively. Genetic similarity was calculated between the parent and regenerated plant / tissue cultured material, This can be referred to as "shared fragments" this coefficient is defined as the number of shared bands in the two samples divided by the total number of bands produced by two samples (Nei and Li, 1979). The similarity between samples can be used to generate a matrix, according to the following formula

$$\textbf{Similarity} = (2 \times N_{ab}) / (N_a + N_b)$$

When

N_{ab} = number of shared fragments between individual "a" and "b"

N_a= number of scored fragments of individual "a" and

N_b = number of scored fragments of individual "b"

Because similarity was estimated between different samples and the parent plant , therefore sample "a" in all cases was parent plant

While the variation was calculated as

$$\textbf{Variation(\%)} = \textbf{(1-Similarity)x100}$$

2.7.3. LINKAGE ANALYSIS

Cluster analysis of measured data is a standard method for analysing the relatedness of individuals (and hence grouping them). Data can include RAPD-PCR, RFLP or sequence information and can be used to estimate genetic diversity and phylogenetic relationships in many crops.

Cluster analysis was performed to evaluate the relationship by using the distance-matrix method. Pairwise distances were calculated based on Nei and Li similarity index performed by the programme Package 'RAPDistance'. A neighbour joining tree (Saitou & Nei, 1987) was then constructed from the matrix of paired distance using Neighbour Programme in the RAPDistance Package. Phylogenetic trees were produced with the help of Dr. Frank Wright at BioSS (Biomathematics and Statistics Scotland, University of Edinburgh) and Dr. Michael Möller from the Royal Botanical Garden, Edinburgh.

CHAPTER 3.

3.1. IDENTIFICATION OF BLACKCURRANT CULTIVARS AND THEIR PHYLOGENETIC RELATIONSHIP USING RAPD-PCR.

3.1.1. INTRODUCTION

Although not grown extensively blackcurrant has high economic value. The composition of its fruits is very high in vitamin C, being four times that of orange and forty times that of apple (Huang, 1985). The genus *Ribes* comprises about 150 species of spiny and non-spiny shrubs, all diploid in nature $2n=2x=16$ distributed mainly in the temperate regions of Europe, Asia and America (Keep, 1995).

The essentials of successful blackcurrant breeding involve hybridisation among the best available cultivars and/or selections, careful evaluation of the seedlings and selection of the best, testing them at representative locations, followed by careful controlled clonal propagation finally resulting in naming, release, and distribution through commercial nurserymen. Theoretically, individual plants of a given cultivar are genetically identical, and it is important to have effective means of monitoring varietal purity.

As blackcurrant plants are vegetatively propagated, each cultivar is essentially a clone. The identification of a variety is essential for breeding and germplasm maintenance and has an important role in the protection of plant breeders' rights. Cultivar registration is usually based on morphological distinctness evaluated over a period of time and involves comparison with standard or existing cultivars under the same growing conditions. Such characterisation of cultivars requires a large set of phenotypic data, much of which is often difficult to assess and sometimes variable due to environmental influences. As a result this can give way to subjectivity in the observers, and lead to identification mistakes (Cousineau and

Donnelly, 1992). Furthermore, these methods involve a lengthy survey of plant growth that is costly, labour intensive and vulnerable to environmental conditions.

A number of laboratory based methods have been successfully developed and have been used to characterise vegetatively propagated cultivars, such as protein and isozyme electrophoresis and paper chromatography; strawberry (Arulsekar et al., 1981; Nehra et al., 1990; Bringhurst et al., 1981); raspberry (Cousineau and Donnelly, 1992, 1989; Couineau et al., 1993 ; Haskell and Garrie 1966) and in potato (Douches and Ludlam, 1991). As isozymes and proteins are gene products, differential expression by environment, tissue-specificity and / or internal factors such as age and nutrient conditions, is inevitable (Stegemann et al., 1973; Hosaka et al., 1985). Thus, reliability of isozyme or protein bands depends on how uniform in terms of age and growth stage is the plant material. In addition isozymes must be enzymatically active for detection, extraction must be from fresh tissue and that is not always possible.

Molecular markers are playing an increasingly important role in genetic studies of horticultural and agricultural crops. Restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA's (RAPDs) have a wide array of uses including genetic mapping, tagging genes of interest, pedigree verification, cultivar identification, and measuring levels of genetic diversity (O'Brien, 1990). Isozymes and RFLPs have been the most employed genetic markers, but interest in RAPDs has grown rapidly as they are often highly polymorphic and their visualisation requires no radioactivity (Grattapaglia et al., 1992).

Random amplified polymorphic DNA profiles represent base differences or chromosomal rearrangements that are uncovered after polymerase chain reaction (PCR) amplification using random oligonucleotides primers (Williams et al., 1990). Random amplified polymorphic DNA markers are generally expressed as a dominant trait, because amplification of a product proceeds with the presence of a pair of sequences homologous chromosomes to that of primers on either one or

both homologous chromosomes. If one of the alleles at RAPD site is un-amplifiable, then marker / marker homozygotes cannot be distinguished from marker/null heterozygotes. Random amplified polymorphic DNAs have been used to distinguish genotypes of raspberry, (Grahm et al., 1994), strawberry (Gidoni et al., 1994; Hancock et al.) asparagus, beans, peppers, watermelons (Weeden et al., 1992), broccoli, cauliflower (Hu and Quiros, 1991), lettuce (Paran et al., 1992), potato (Mori et al., 1993).

Isozymes have been used to characterise soft fruit strawberry cultivars. However, only 60% of the genotypes from the University of California breeding programme could be distinguished using three enzyme systems (Arulsekar et al., 1981; Bringhurst et al., 1981). This lack of resolution limits the utility of isozymes in cultivar identification and breeding. In contrast, random amplified DNA polymorphisms DNA (RAPDs), have been developed recently (William et al., 1990), and these RAPDs markers are distributed throughout the genome. Procedures for their detection is rapid, thus potentially allowing the use of detection kits in the field or in packing houses (Gidoni et al., 1994).

The objective of this study was to examine and compare protein SDS-PAGE electrophoresis with the possibility of producing cultivars-specific RAPD markers for distinguishing commercial blackcurrant cultivars. In particular, the ability of the methodology to distinguish between closely related cultivars will influence the use of such technology for evaluating the purity of the plants regenerated via tissue culture of a single cultivars. In addition such information would allow a comparison of phylogenetic relatedness of commonly used varieties. This in turn would allow evaluation of the RAPD methodology by comparison with known pedigree relatedness.

3.1.2. MATERIALS AND METHODS.

3.1.2.1. PLANT MATERIALS

Ten blackcurrant cultivars. were used for this study. Ben Sark, Ben Lomond, Ben More, and Malling Jet were bought from local nurseries and maintained in the plant growth unit of Scottish Agriculture College for future use. Black Smith, Ben Nevis, Cotswold, Baldwin, Ben Alder and Black Reward cultivar leaves were supplied by SCRI, Dundee.

3.1.2.2. PLANT DNA ISOLATION AND AMPLIFICATION

Total genomic DNA was isolated either from fully expanded leaves of the greenhouse grown plants or from frozen leaves using a modification of CTAB procedure (Doyle and Doyle, 1987), as described in section (2.5.2). After successful extraction, the DNA was quantified by spectrophotometer as described in section (2.5.3) and was diluted to desired concentration i.e. 10 ng /µl

The DNA was amplified in 50 µl volumes. All the RAPD amplification analyses were performed as described in section (2.5.4). After amplification, the electrophoresis of amplified DNA was carried out as described in Section (2.6.1). The amplified DNA was visualised followed by staining with ethidium bromide as described in section (2.6.3.1). RAPD-PCR patterns can include weak or minor bands that are especially vulnerable to variation in protocol. It is widely accepted that procedure optimisation and consistent reaction conditions are important elements in reducing these fluctuations and ensuring reproducible results (Rafalski and Tingey, 1993). Penner et al. (1993) tested the reproducibility of RAPDs by comparing the amplifications products of the same primer/template combinations produced in different laboratories. They demonstrated the general reliability of the method, observing that some laboratories generated different size ranges of DNA fragments, variations attributed principally to differences in thermocyclers. The selection of polymorphic bands for inclusion in current data set was based on both

band characteristics and reliability. Reliability was directly confirmed by replicated runs. All the amplification reactions were repeated at least twice with fresh DNA extraction.

A total of twenty nine ten-base random primers were used initially with two cultivars Ben Sark and Malling Jet, from which twelve primers were selected for use in this experiment Table 3.1.1.

Table 3.1.1. Primers and their base sequence used for the detection of polymorphism in blackcurrant cultivars

	Primer	Sequence.
A	OPH-01	5' GGTCGGAGAA 3'
B	OPH-03	5' AGACGTCCAC 3'
C	OPH-06	5' ACGCATCGCA 3'
D	87729803	5' GACCGCCACC 3'
E	87729804	5' TTCAGGGGTC 3'
F	87729805	5' GGACTAGTGG 3'
G	87729806	5' CCAATCCGTG 3'
H	87729808	5' GGAGCTTGCC 3'
I	87729810	5' TCACGGCACC 3'
J	86370601	5' GACCGCCACC 3'
K	86370607	5' GGTCGGAGAA 3'
L	86370611	5' GGAGCTTGAC 3'

3.1.2.3. SDS-PAGE ELECTROPHORETIC PROCEDURE.

Sample and gel preparation methods, sample applications, staining and destaining of the gels were exactly similar to the procedure described in Section (2.4).

3.1.2.4. DATA ANALYSIS

SDS PAGE protein gels were evaluated by eye using a light box and electrophoretograms were created by means of an IBM personal Computer and Harvard Graphic / Draw partner programme as described in the section (2.4.6).

In RAPD-PCR analysis the index proposed by Nei and Li (1979) was used to calculate similarities by using the computer programme RAPDistance. A second measure of similarity was derived from the pedigree data, using the proportion of genetic material from common founding clones, as described by Lanham et al. (1995). For example, the pedigrees for Ben Sark, Ben More and Ben Alder are as follows 'Ben Sark'['Goliath' (50%) x'öjebyn' (50%)]; 'Ben More' [Unknown (50%) x{'Goliath'(25%) x 'öjebyn' (25%)]} and 'Ben Alder' ['Ben Lomond'(50%) x 'Ben More' (50%)]. Lanham et al. calculated the maximum similarities between Ben Sark and Ben More as 50% and between Ben More and Ben Alder 50% and between Ben Alder and Ben Sark as 25%. (öjebyn' an accession from the Swedish wild population of *Ribes nigrum* constitutes 50% of Ben Sark 25% Ben More and 12.5% for Ben Alder). The rankings of these similarities are more important than their absolute value because of incomplete blackcurrant pedigree. The average linkage cluster analysis was performed to produce dendrogram using the RAPDistance programme Neighbour Joining Tree as described in section (2.7.3).

3.1.3. RESULTS

3.1.3.1. CULTIVARS IDENTIFICATIONS BY SDS PAGE

Banding patterns obtained using SDS-PAGE on blackcurrant leaves showed that cultivars contained largely common bands and there were few polymorphic bands that could be used to discriminate the cultivars tested (Figure 3.1.1). Banding patterns were divided arbitrarily into three regions. Region 'A' contained four bands, more or less deeply stained. This region is common in all the cultivars

except Ben Sark which lacked band 3. Region 'B' contained the majority of the polymorphic bands between cultivars. This region consisted of one thick comparatively darkly stained subunit common to all cultivars and a variable number of thinner bands, not always clearly distinguished. Region 'C' contained the high molecular weight proteins which stained very weakly and were not easy to distinguish in some gels. This region contained two bands one very thick and dark stained in common in all cultivars and a second band which was either very weak or was absent as in the case of Malling Jet. Ben Sark lacks band 3A and 4B, Malling Jet lacks 3B and 2C, Ben Nevis lacks 2B and 4B while Black Reward lacks only 4B. The rest of the six cultivars shared all bands in common and could not be separated. Thus only four cultivars Ben Sark, Ben Nevis Black Reward and Malling Jet could be separated easily using protein banding patterns.

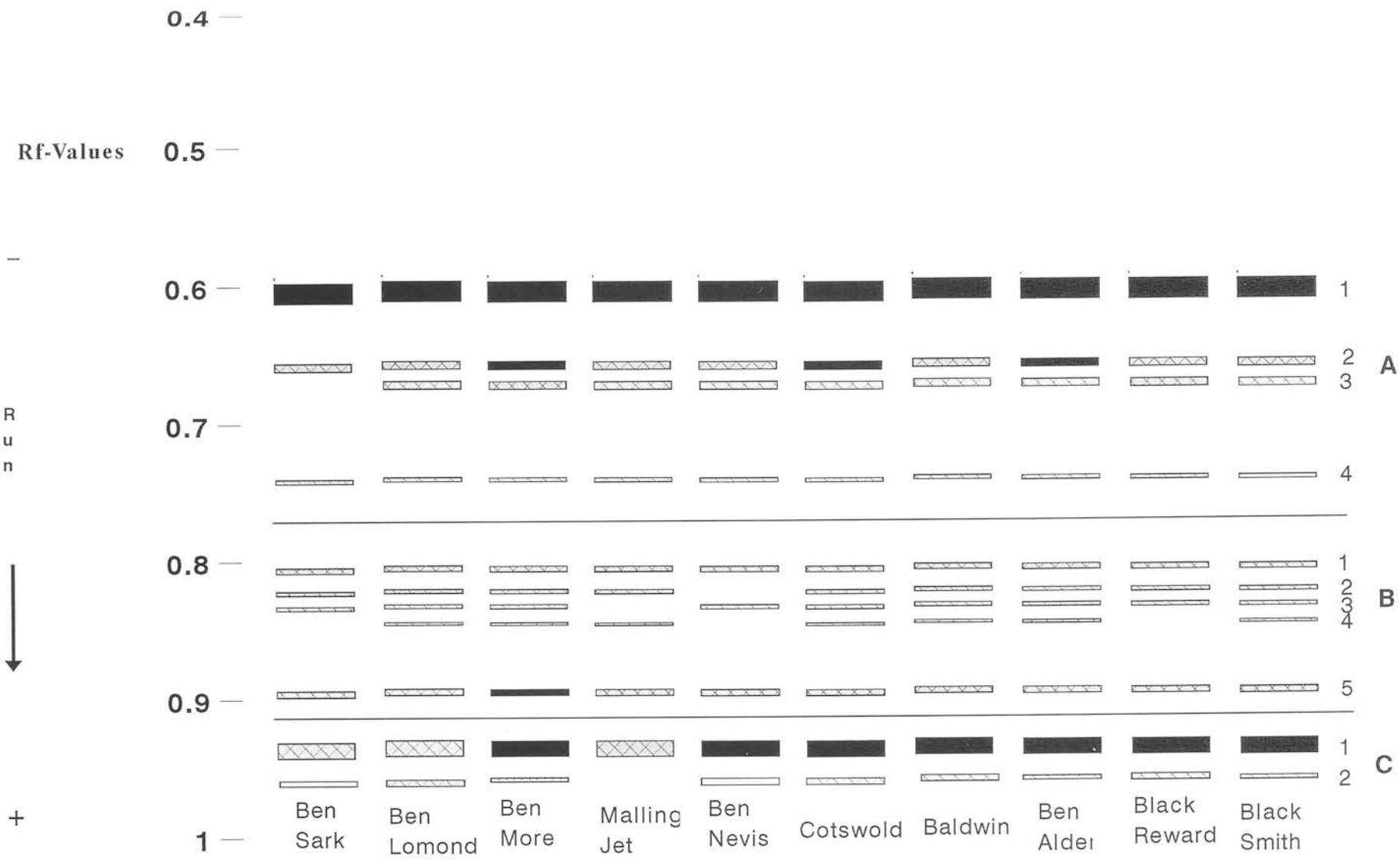


Figure 3.1.1 Protein electrophoretograms of ten blackcurrant cultivars

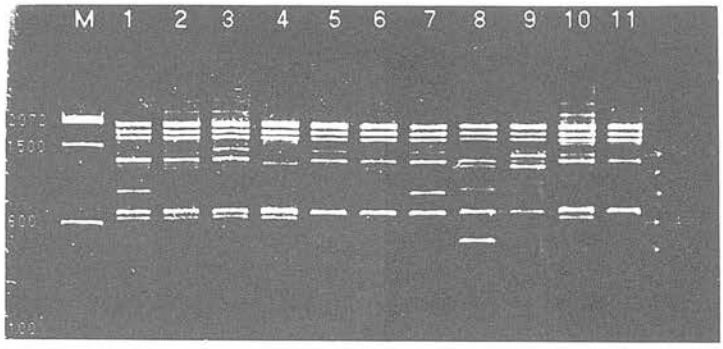
3.1.3.2. CULTIVARS IDENTIFICATION USING RAPD-PCR MARKERS

From an initial group of 29 primers, sixteen of them were useful in distinguishing among the eleven cultivars. Of the other primers 11 lacked polymorphisms in these samples and 2 failed to produce polymorphic amplification profiles. Of the sixteen primers that produced polymorphic amplification product profiles, the 12 with the greatest level of polymorphisms and/or clarity of bands separation were used to further evaluate the power of this technology for discriminating among genotypes and examining relationships. Out of these twelve, only three primers were needed for cultivar discriminations. Primer A was chosen on the basis of its ability to separate the maximum number of cultivars while Primer B was chosen on its ability to further separate the remaining cultivars. Primers C was used to separate all the cultivars by 4-8 bands.

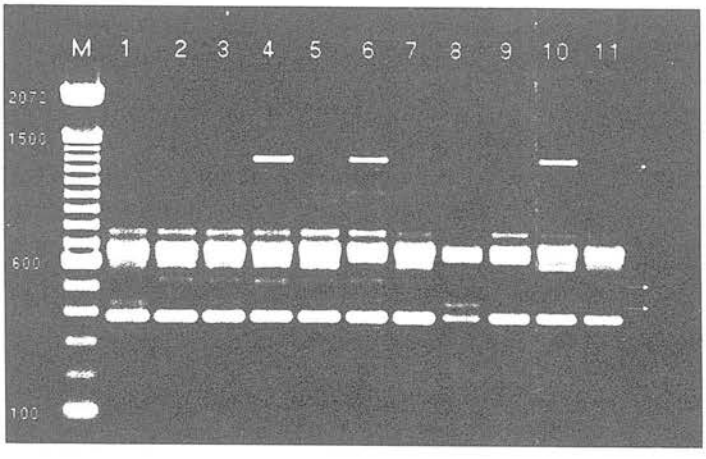
Table 3.1.2. Cultivar-specific RAPD pattern derived from Figure 3.1.2. which distinguish each of the eleven cultivars tested, individually. 1=Presence of PCR product; 0=absence of PCR product

	RAPDS									
Cultivar	Primer A					Primer B			Primer C	
	1A	2A	3A	4A	5A	1B	2B	3B	1C	2C
Ben Sark	0	1	1	1	0	0	0	1	1	0
Ben Lomond	0	1	0	1	0	0	1	0	0	0
Ben More	1	0	0	1	1	0	1	1	1	1
Malling Jet	0	1	1	1	0	1	1	0	0	0
Amos Black	1	0	0	0	0	0	1	1	1	1
Ben Nevis	0	1	0	1	0	1	1	1	1	1
Cotswold	1	0	1	1	0	0	1	0	0	1
Baldwin	0	1	1	0	1	0	0	1	1	0
Ben Alder	1	1	1	0	1	0	1	0	1	1
Black Reward	1	1	0	1	1	1	1	1	0	0
Black Smith	0	1	0	1	1	0	1	1	1	0

A



B



C

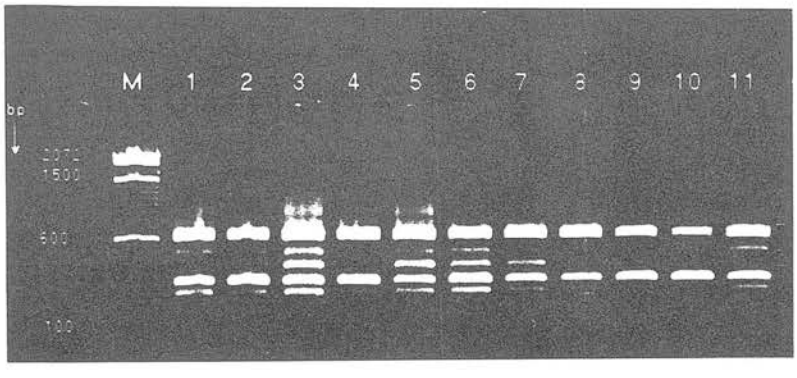


Figure 3.1.2. Amplification products derived from blackcurrant cultivars using 10 base primer A, B and C. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. From 1 to 11 are blackcurrant cultivars.

Primer A produced 13 scoreable markers, five of them were polymorphic among the eleven blackcurrant cultivars as shown in the figure 3.1.2-a. Most of the blackcurrant cultivars could be separated by specific profiles resulting from primer A amplification with the exception of Ben Sark and Malling Jet which share identical profiles and Ben Lomond and Ben Nevis which were also identical to each other (Table 3.1.2). Primer B produced nine bands (Figure 3.1.2-b), three of which were polymorphic. All the remaining cultivars were separated by three polymorphic bands produced by this primer (Table 3.1.2). At this stage cultivars were separated by a minimum of two marker bands. By adding the two polymorphic amplification fragments of primer C, each and every cultivar could be separated by 4 to 8 band differences from each other (Table 3.1.2).

3.1.3.3. RELATIONSHIP AMONG CULTIVARS.

All the 12 primers used gave 5 to 11 bands each. The amplifications of products of primer F and H are shown in Figure 3.1.3 a and 3.1.3 b respectively. For evaluation purposes dendrogram based on cluster analyses were produced both using only three primers (A, B and C) as well as by all 12 primers (Figure 3.1.4, 3.1.5). The analysis based on 3 primers divides the cultivars initially into two groups, Ben More, Cotswold, and Ben Nevis into one and rest of the cultivars into the second group. This group was again further divided into two groups leaving Baldwin isolated as shown in Figure 3.1.4. Ben More and Cotswold, Black Smith and Black Reward are more closely related respectively as compared to other cultivars. The data from using all 12 primers, grouped the *Ribes nigrum* cultivars into two main clusters (Figure 3.1.5); Ben Sark, Malling Jet and Ben Lomond into one group while rest formed a second group. This second group was further divided into two sub-groups each of three cultivars leaving Black Smith isolated. This does reflect the different background of this cultivar, of unknown origin, raised by the Laxton brothers and introduced in 1916.

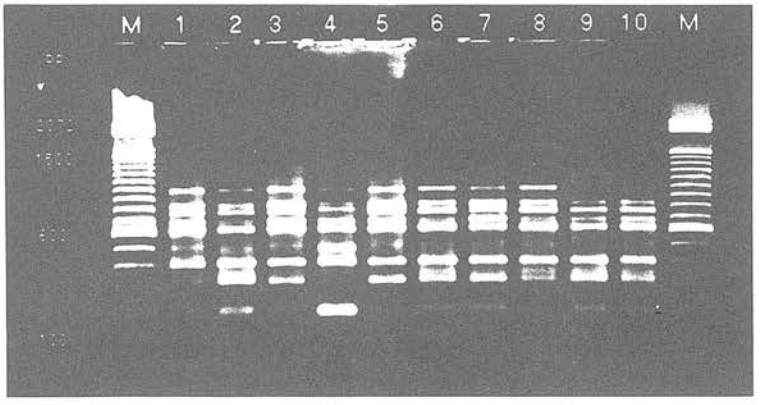


Figure 3.1.3.a. Amplification products derived from ten blackcurrant cultivars using 10 base primer F. From both ends M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. From 1-10 are black currant cultivars.

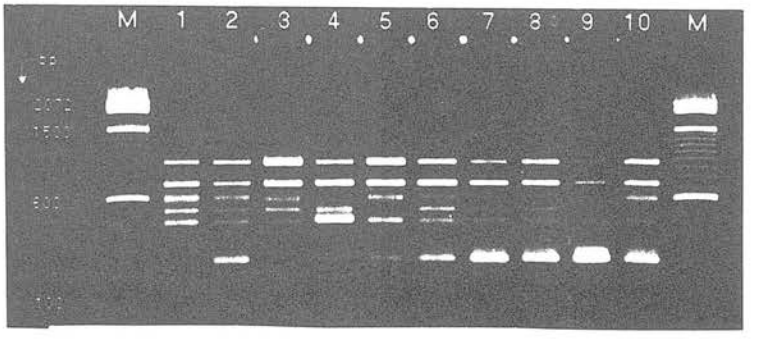


Figure 3.1.3 b .Amplification products derived from ten blackcurrant cultivars using 10 base primer H. From both ends M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. From 1-10 are black currant cultivars.

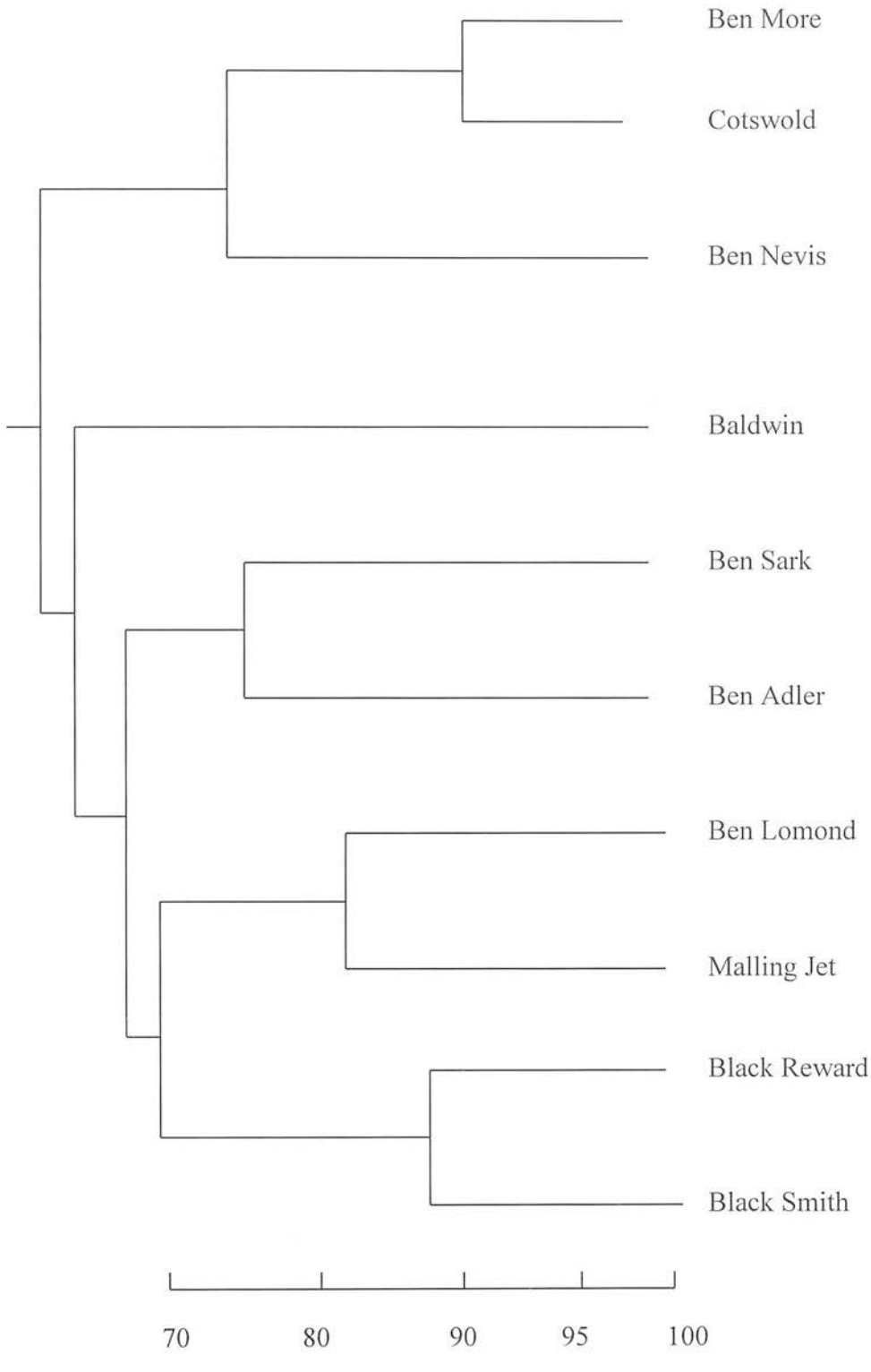


Figure 3.1.4. Dendrogram. of ten blackcurrant Ribes nigrum cultivars based on data generated by three RAPDs primers

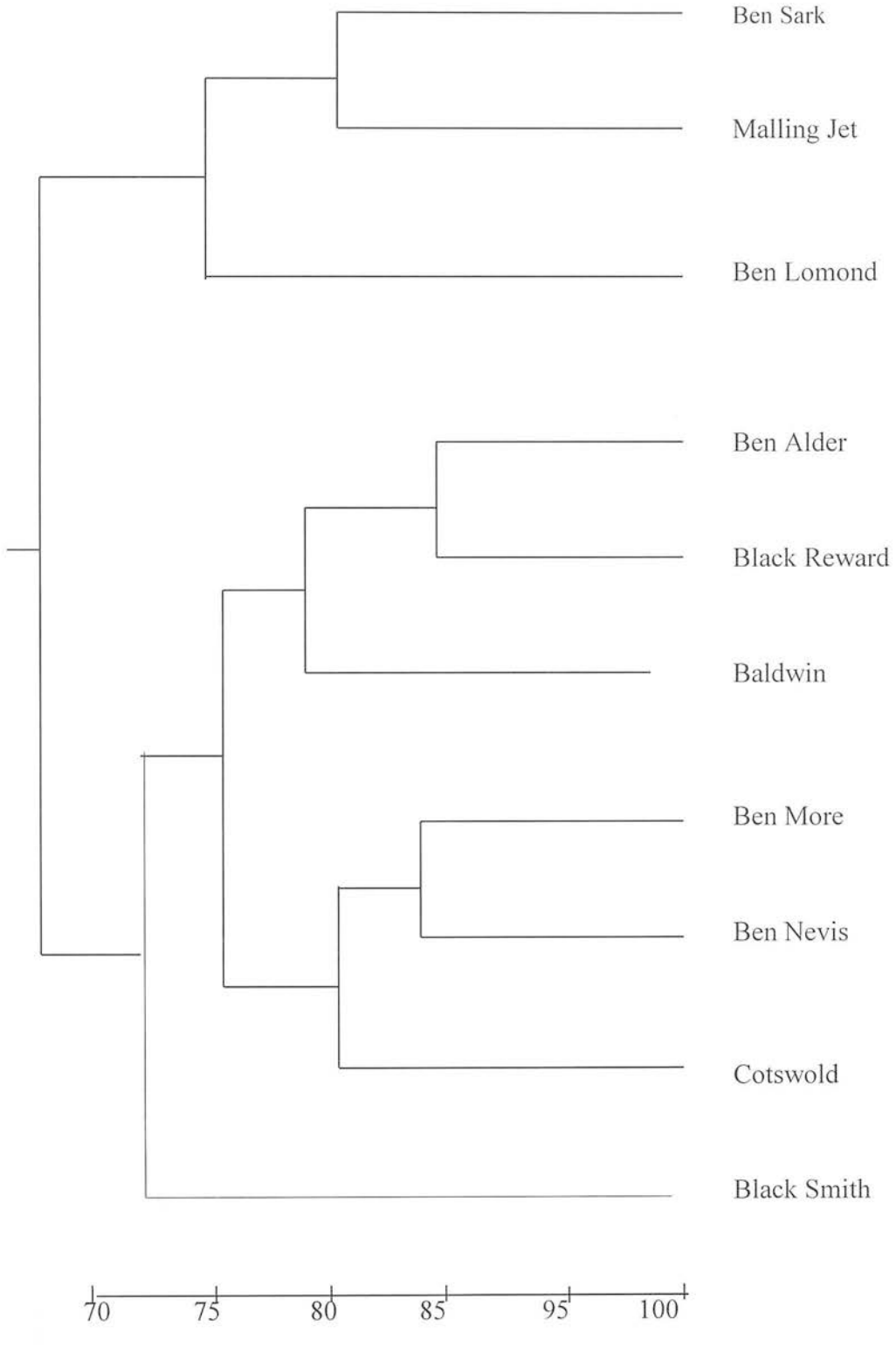


Figure 3.1.5. Dendrogram. of ten blackcurrant Ribes nigrum cultivars based on data generated using twelve primers in RAPD-PCR

Table 3.1.3. Similarity matrix based on the number of shared fragments of RAPD-PCR data using twelve primers.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1.	Ben Sark	0.0									
2.	Ben Lomond	0.714	0.000								
3.	Ben More	0.752	0.714	0.000							
4.	Malling Jet	0.819	0.781	0.667	0.000						
5.	Ben Nevis	0.686	0.762	0.819	0.676	0.000					
6.	Cotswold	0.705	0.705	0.819	0.638	0.790	0.000				
7.	Baldwin	0.747	0.629	0.723	0.638	0.695	0.752	0.000			
8.	Ben Alder	0.695	0.676	0.695	0.590	0.686	0.762	0.743	0.000		
9.	Black Reward	0.667	0.724	0.724	0.657	0.676	0.734	0.752	0.800	0.000	
10.	Black Smith	0.752	0.695	0.714	0.647	0.667	0.647	0.724	0.676	0.743	0.000

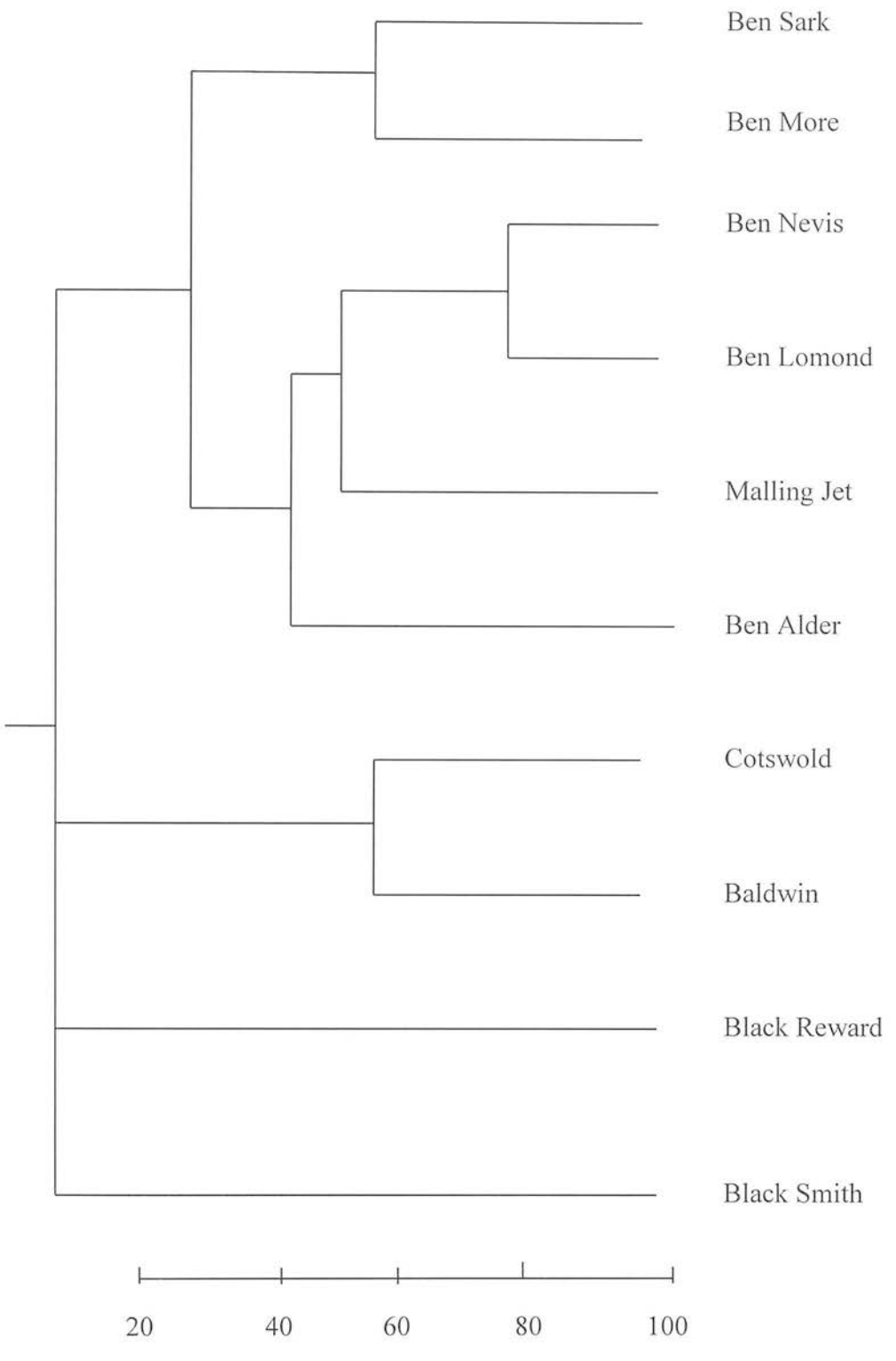


Figure 3.1.6. Dendrogram. of ten blackcurrant Ribes nigrum cultivars based on data generated using parentage

Table 3.1.4. Similarity matrix based on the pedigree analysis

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1.	Ben Sark	100									
2.	Ben Lomond	19	100								
3.	Ben More	50	19	100							
4.	Malling Jet	0	25	0	100						
5.	Ben Nevis	35	100	19	25	100					
6.	Cotswold	0	0	0	0	0	100				
7.	Baldwin	0	0	0	0	0	50	100			
8.	Ben Alder	25	50	50	25	50	0	0	100		
9.	Black Reward	0	0	0	0	0	0	0	0	100	
10.	Black Smith	0	0	0	0	0	0	0	0	0	100

Cluster analysis based on pedigree is presented in Figure 3.1.6. While there are similarities between Figure 3.1.5 and Figure 3.1.6, there are also some very obvious differences. Cultivar Black Smith appears reasonably separated from other cultivars in both types of analysis. On the other hand Ben Lomond and Ben Nevis are linked closely together by pedigree but this is not obvious using the data generated by RAPD analysis

Overall, grouping based on pedigree analyses is different from those based on RAPDs. Cultivar pair Ben Nevis and Ben Lomond are identical by pedigree (100%) this in practice means that genetic commonality between them lies between 50% and 100% but is estimated by RAPDs (76%). This latter observation demonstrates the power of such markers in the detection of subtle genetic differences between closely related cultivars.

A similarity matrix of the *R. nigrum* cultivars based on all RAPD primers scores is presented in Table 3.1.3. Similarity ranged from 59% (Ben Alder : Malling Jet) to 82% (Malling Jet : Ben Sark, Ben Lomond : Ben Nevis and Ben Lomond : Cotswold). In the majority of close groupings some relationship in term of parentage exists. Ben Nevis and Ben Lomond are sister seedlings while Ben Lomond and Ben More are the parents of Ben Alder. (Table 3.1.3).

A similarity matrix based on parentage is presented in Table 3.1.4. Similarity ranged from 100% (Ben Lomond : Ben Nevis) to 0% (Many examples). As in the case of cluster analysis (Figure 3.1.5 and Figure 3.1.6) certain anomalies were apparent in the similarities detected using RAPDs and those that would be expected from the known parentages, e.g. Ben Sark and Cotswold and Ben Sark and Baldwin have 0% similarities when compared on the basis of pedigree, whereas they have 74% and 69% similarity based on RAPDs scores respectively. This discrepancy reflects the ability of RAPDs markers to detect not only subtle differences between cultivars but also unexpected similarities. The RAPDs results

are more realistic in that any two *Ribes nigrum* cultivars which have nothing in common in their pedigree and would thus score 0% similarity would be very unlikely to have nothing in common at the molecular genetic level; the group overall having such a narrow genetic background.

3.1.4. DISCUSSION

Using SDS-PAGE, the results expected were bands that could be used to discriminate and identify cultivars. Differences observed between cultivars should form the basis of a system for characterising them and the protein sub units dissociated during electrophoresis should form a fingerprint. Electrophoresis is well recognised as a potential technique for cultivar identification. There are many advantages such as small sample size and short testing period, the opposite of morphological analysis, but there are constraints too. One of them is the need for extreme standardisation of the technique. A successful electrophoresis system depends on following the methodology exactly. The final results obtained in these experiments do not give much encouragement to the development of SDS-PAGE for clonally regenerated plants of the same cultivars because very few differences were observed between cultivars. Out of the ten cultivars, six possessed similar banding pattern with no polymorphic bands and only four cultivars could be separated by some minor bands.

The extensive polymorphisms were detected using RAPD-PCR among the cultivars tested and could be used for cultivar identification. All blackcurrant cultivars tested were distinguishable from each other with as few as two primers. (Table 3.1.2). RAPD-PCR has been successfully used for cultivar identification of other clonally propagated soft fruit species such as raspberry Parent et al., 1993 and Graham et al., 1994. Thus, for a closely related group of cultivars, from a narrow genetic base, RAPD-PCR offers considerable potential for distinguishing individual genotypes. Therefore RAPD-PCR appears to offer a great potential in order to evaluate/examine the purity of plants regenerated via tissue culture of a single

cultivar and this will be examined further to characterise the micropropagated plants/clones as well as plants regenerated through callus culture and regeneration.

The RAPD-PCR amplification product generated can be used to examine and establish systematic relationships (Hadrys et al., 1992). Initially, the data generated using three primers was used to examine the relatedness and then all 12 primers, as shown in the Figures 3.1.4 and 3.1.5 respectively. Both the figures grouped Ben More, Ben Nevis and Cotswold into one group, while Ben Nevis and Ben More and Ben More and Cotswold were related more closely using 12- and 3- primers respectively. All other cultivars were placed in different groups using both three and twelve primers based dendrogram (Figure 3.1.4 and 3.1.5). These results show that the level of relatedness calculated by RAPD-PCR does not give the absolute relationship because the dendrogram can be influenced both by the number of primers used and the quality of primers in term of level of polymorphism generated by a particular primer. Therefore, caution must be exercised when using the results of RAPD-PCR to examine relationships among different genotypes. Ideally to test the genetic relationship of genotypes within a particular species with RAPDs, requires a saturated linkage map using a large set of data generated by a large number of primers.

The dendrogram and similarity matrices calculated from both the cultivar parentage and banding patterns of all 12 primers, show certain differences but the dendrogram based on parental information show only general agreement at 40% similarity level. The sister seedling pair Ben Nevis and Ben Lomond showed the expected high degree of similarity in the RAPD-PCR analysis, at 76%. This method is able to distinguish between sibs while reflecting the close relationship. This technique may have potential applications for the horticulture industry and for germplasm collections throughout the World in differentiating between sister seedlings/cultivars. The cultivar Cotswold was 50% Baldwin and 50% Victoria (which in turn was thought to be selected from Baldwin). This was supported by RAPD-PCR analysis that gives 75% similarity to Baldwin. The degree of similarity

calculated from the parentage was generally lower than that calculated from banding patterns and the former should be regarded as the lowest level of the similarity since it ignores the fact that many early cultivars were nothing more than a supposedly superior selection from wild species. It is likely that many founding clones, although given different cultivar names, were in fact very similar in terms of their genetic contribution. It would be expected that similarity values obtained from the banding pattern would be higher in value than those obtained from parental contribution and this is confirmed in this study. Therefore values obtained from banding analysis are likely to be a more accurate reflection of the genetic relationship of the ten cultivars and this could be of value as a predictor for future breeding methods.

Nevertheless similarity matrices and dendrogram created by the 12 primers can add useful information on the relationship of existing cultivars and help to substitute for a lack of information in pedigrees. Indeed the use of pedigree to create relationship has certain weaknesses because parentage for some of the genotype are not as descriptive as desired, for example Baldwin is over 150 years old and of unknown derivation, and several of the parental type such as Victoria are merely thought to be selections from Baldwin. The exact parentage is therefore impossible to define beyond this. Also some of the older types are described as open pollinated seedlings from a particular genotype, so the male lineage is effectively lost. In addition to this the parentage of cultivars Black Smith and Black Reward is completely unknown. Therefore in order to gain clear understanding of the relationship between cultivars it is better to use a combination of methodology both pedigree and RAPDs.

3.2. AN ASSESSMENT OF GENETIC INTEGRITY OF BALDWIN CULTIVARS MULTIPLIED AT DIFFERENT NURSERIES

3.2.1. INTRODUCTION.

Until recently, the blackcurrant industry in the United Kingdom relied almost exclusively upon the cultivar, Baldwin, which has been grown since the nineteenth century. In 1920, Hatton classified 26 blackcurrant cultivars all of pure *Ribes nigrum* decent, into four main groups of similar varieties (as represented by French Black, Boskoop Giant, Goliath and Baldwin). The Baldwin group was described as the green bud group with buds that are long, rather large, conical and pointed; the bushes of such a cultivars have a small spreading habit; fruits have tough skin and an acid flavour. This cultivar has been preferred by processors because of superior flavour. The bulk of the blackcurrant crop is used for juice production, and so special attention in breeding is given to selection for juice quality, notably flavour, colour and ascorbic acid content. As the cultivated blackcurrant has been developed from a single species, *Ribes nigrum* (Hedrick, 1938), and subsequent breeding has depended upon two cultivars, Baldwin and Boskoop Giant, to develop a further group of cultivars, released in the early 20th century (Lanham et al.,1995) the genetic base of present day blackcurrant cultivars is probably very narrow.

Traditionally blackcurrant cultivars are propagated by vegetative means such as hard wood cuttings (MAFF, 1981). Since the cultivar Baldwin has been multiplied vegetatively and grown for over a century there are chances of some changes due to mutation and / or chimera formation as well as by human mistake such as wrong identification.

The profitable production of blackcurrant is largely determined by the varieties grown and therefore the utmost care is needed in their multiplication and trueness to type. The identification of a cultivar is essential for breeding and germplasm

maintenance. The system for cultivar registration has been primarily described in Section 3.1.1. As blackcurrant plants are vegetatively propagated, each cultivar is essentially a clone. The use of morphological and other biochemical methods for examining the variations within clones has limited potential. However, examination of the DNA genome provides a significantly more powerful source of genetic polymorphism (Beckmann and Soller, 1986). The most detailed approach uses restriction fragment length polymorphisms (RFLP) and has been exploited in various crops species (Tanksley et al., 1989). However, this has limitations as discussed in Section 1.6.4.1. Therefore in this study the purity of a single cultivar of blackcurrant was evaluated by RAPD-PCR analysis.

The objective of this experiment was to study the power of RAPD-PCR polymorphisms to evaluate the purity of the oldest blackcurrant cultivar propagated and maintained at different areas by vegetative means.

3.2.2. MATERIALS AND METHODS

3.2.2.1. PLANT MATERIALS

Samples of the cultivars Baldwin, were sought from seventeen nurseries and institutes, of these only six provided leaf material. The samples which were analysed are described in Table 3.2.1.

Table.3.2.1. Samples and their sources of blackcurrant cultivar Baldwin used to analysed the purity using RAPD-PCR

Samples	Institutes and nurseries who provided the samples
A	SCRI Dundee
B	John Hargreaves and Sons Brook House Farm Gedney Dyke, Spalding, Lincs.
C	Hill Top Baldwin from Welsh Fruit Stocks, Bryngwine, Kington ,Hereford
D	Tip Top Lamberts Farm, Earls Colne, Essex
D1	Tip Top Lamberts Farm, Earls Colne, Essex
E	SmithKline Beecham S.B house, Brentford, Middlesex
F	Whitehouse, Newcastle, Monmouth, Gwent

Welsh Fruit Stocks Bryngwine Kington Hereford held sample C since 1962 and this originated from Hill Top Farm Nursery, Hereford. Tip Top Lamberts Farm, Earls Colne, Essex provided two samples from different sources D, a Baldwin source from the West of England and kept at Essex for the past eight to 15 years; D1 Baldwin taken from NSA Plants Ltd. and maintained there for eight years. F was provided by Whitehouse, Newcastle, Monmouth, Gwent this was originally from Hugh Price of Wellington, Somerset in 1969 and was probably the Hilltop sport of Baldwin which was originally propagated at Hill Top Farm Herefordshire.

3.2.2.2. DNA ISOLATION AND RAPD-PCR METHODS

Total genomic DNA was isolated from the frozen leaves of the Baldwin samples by CTAB method of Doyle and Doyle (1987) with slight modifications as described in Section (2.5.2). The DNA was quantified by using spectrophotometer as described in Section (2.5.3), and diluted to the desired concentration of 10 ng/µl.

Amplification reactions were performed in the total volume of 50 µl of reaction mixture consisting of 20 ng of genomic DNA. The primers and their base sequences used are given in the table 3.2.2. All the other parameters and reaction components were performed as described in Section 2.5.4. After amplification the DNA was separated by electrophoresis on agarose gel as described in Section 2.6.1 and DNA was visualised by staining with ethidium bromide as described in Section 2.6.3.1. In addition to agarose gel, the amplification products of seven primers were separated on polyacrylamide gel electrophoresis as described in Section 2.6.2, and the DNA was visualised after silver staining as described in Section 2.6.2.3.

Table 3.2.2 Primers and their base sequence used for the detection of polymorphism in blackcurrant cultivar Baldwin

NO.	Primer	Sequence.
1	87729801	5' CACTGCAGTC 3'
2	87729803	5' GACCGCCACC 3'
3	87729805	5' GGACTAGTGG 3'
4	87729807	5' GGAGCTTGAC 3'
5	87729808	5' GGAGCTTGCC 3'
6	87729809	5' CGGTGAGGCT 3'
7	87729810	5' TCACGGCACC 3'
8	OPH-06	5' ACGCATCGCA 3'
9	OPH-03	5' AGACGTCCAC 3'
10	OPH-01	5' GGTCGGAGAA 3'

3.2.3. RESULTS.

3.2.3.1. AGAROSE GEL ELECTROPHORESIS AND ETHIDIUM BROMIDE STAINING.

The primers evaluated generated between 4-8 major bands along with a number of bands of lesser intensity. A total of 69 fragments were amplified and 20 (28%) of these showed polymorphism. Figure 3.2.1 a, b and c shows primer 2, 4 and 5 amplification products respectively separated by agarose gel electrophoresis followed by ethidium bromide staining. Figure 3.2.1 a shows separation of samples A, B, C, in one group and D, E, F into another group of similar samples differing by one band between the groups. Figure 3.2.1 b shows separation of samples C and D from rest of group by one band while C and D differ by two bands from each other. In figure 3.2.1 c sample D lacks four and three bands as compared to sample A, B, C and E, F respectively, Both groups are similar within group but E, and F lacks one band as compared to A, B, and C. From overall picture of this figure it is clear that sample D is the most distant from rest of the samples. Ultimately all the data from all ten primers separated on agarose gel were used to calculate a similarity matrix and to construct a dendrogram. Table 3.2.3 shows the pair-wise similarity coefficients, which ranged from 0.884 to 0.992. The result of pair-wise comparisons indicated that sample D collected from Tip Top Essex was the least similar to all other genotypes (less than 0.91 similarity coefficient). Sample B and C are highly associated as indicated by the large value of the similarity coefficient (0.992). Sample F is also highly associated with sample B and C as indicated by the large values of similarity coefficients 0.969 and 0.961 respectively. A dendrogram displaying hierarchical relationships between accessions is given in Figure 3.2.2. This cluster analysis reflects clearly that B, C, F A and E are more closely related as compared to D which is farthest from all other group while B is very close to C. The DNA extracted from one of them D1 (NSA plant taken and maintained since eight years at Tip Top Essex) did not amplified even after repeated extraction and reprecipitation so it was discarded from analysis.

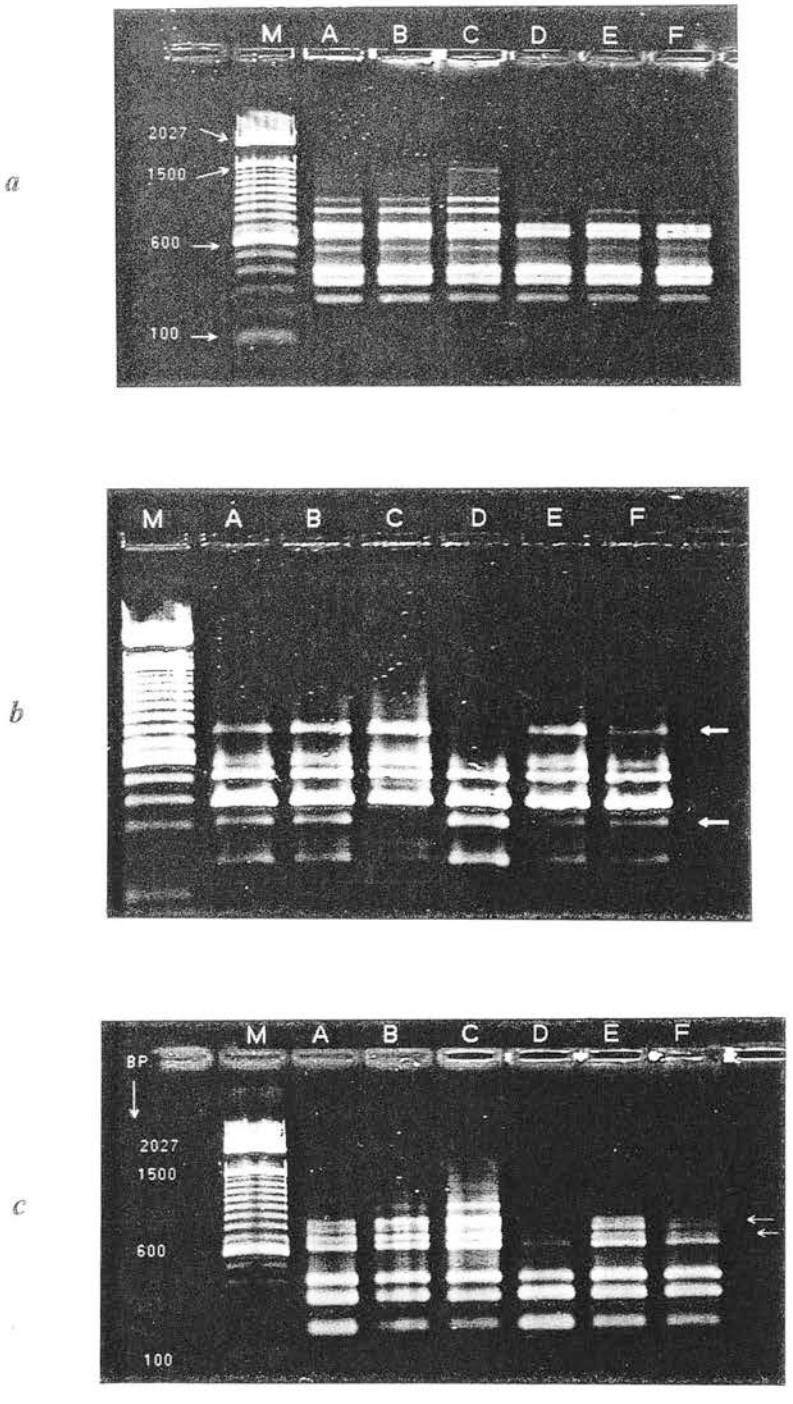


Figure 3.2.1. Amplification products derived from six samples of Blackcurrant cultivar Baldwin separated by agarose gel electrophoresis followed by ethidium bromide a represents primer 2, b primer 4 and c primer 5.(Table 3.2.2). From left to right M represents nucleic acid markers i.e. 100 base pair, followed by six samples (Table 3.2.1).

	A	B	C	D	E	F
A	0.000					
B	0.961	0.000				
C	0.953	0.992	0.000			
D	0.884	0.896	0.888	0.000		
E	0.94	0.954	0.945	0.909	0.000	
F	0.928	0.969	0.961	0.893	0.936	0.000

Table 3.2.3. ***Similarity matrix based on the number of shared products A, B, C, D, E, F, are samples of cultivar Baldwin collected from different sources and analysed using data derived from RAPDs run on agarose gel followed by staining with ethidium bromide.***

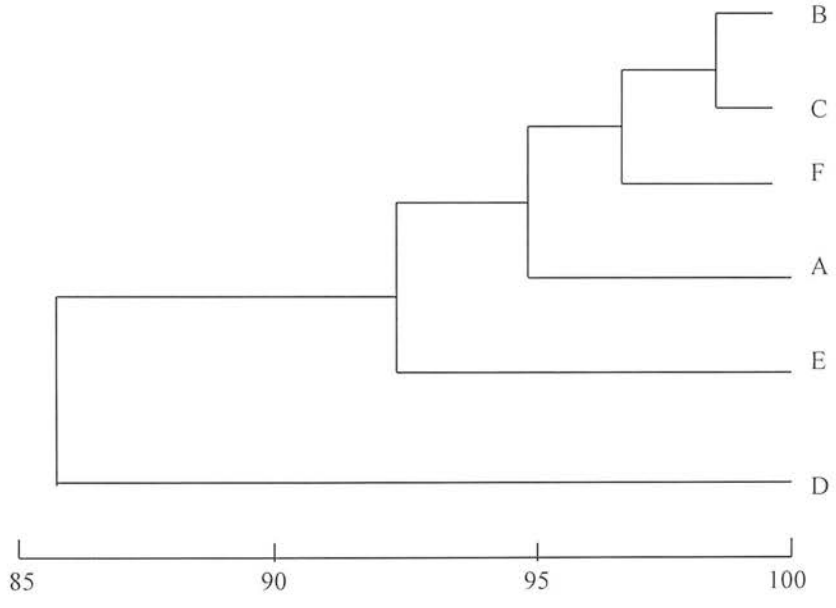


Figure 3.2.2. ***Dendrogram of six samples of Baldwin cultivar collected from different sources and analysed using data derived from RAPD markers run on agarose gel electrophoresis using ten random primers.***

3.2.3.2. POLYACRYLAMIDE GEL AND SILVER STAINING ANALYSIS

The amplified products of primers were separated on polyacrylamide gels. Each primer gave 20-31 bands. Figure 3.2.3 shows the amplification products of five primers separated by polyacrylamide gel followed by silver staining. The data generated by seven primers was used to calculate the similarity matrix. Table 3.2.3 shows the pair-wise similarity coefficients which ranged from 0.785 to 0.939. The results from the pair-wise comparison again show that sample D was least similar to all other genotypes (less than 0.855 similarity coefficient except sample E). Sample E and F are more closely related to each other as compared with other samples (similarity coefficient 0.939). A phylogeny analysis was performed with the data of 164 RAPDs bands of Baldwin samples. The phylogeny tree Figure 3.2.4 depicts the relationship between the samples used. Excluding sample D, the remaining samples were grouped into two. Initially group one contained B and C while the other consisted of A, E and F. Sample E and F were more closely than to sample A.

	A	B	C	D	E	F
A	0.000					
B	0.859	0.000				
C	0.828	0.859	0.000			
D	0.810	0.853	0.785	0.000		
E	0.896	0.902	0.859	0.865	0.000	
F	0.871	0.902	0.834	0.841	0.939	0.0

*Table 3.2.4. **Similarity matrix based on the number of shared products A, B, C, D, E, F, are samples of cultivar Baldwin collected from different sources and analysed using data derived from RAPDs run on polyacrylamide gel followed by silver staining***

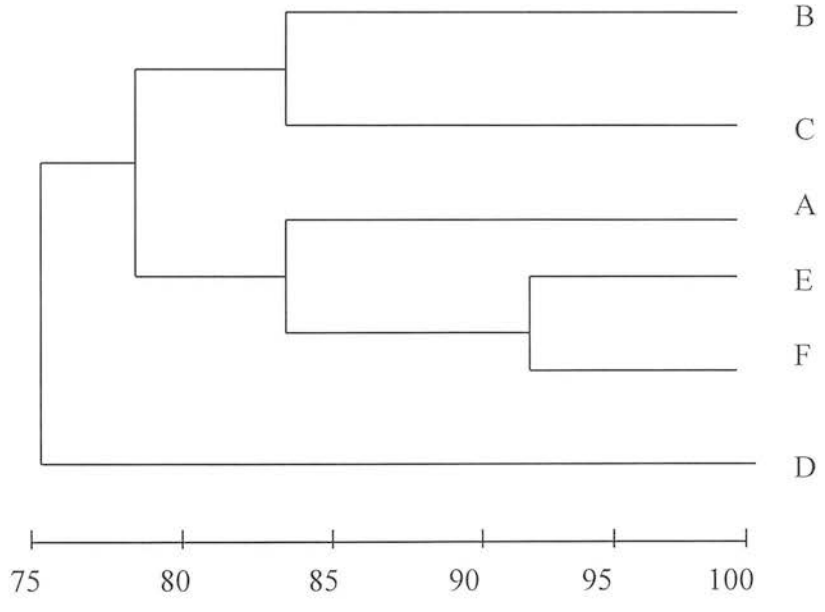


*Figure 3.2.4. **Dendrogram of six blackcurrant cultivar Baldwin collected from different sources analysed using data derived from RAPD-PCR run on polyacrylamide gel electrophoresis followed by silver staining***

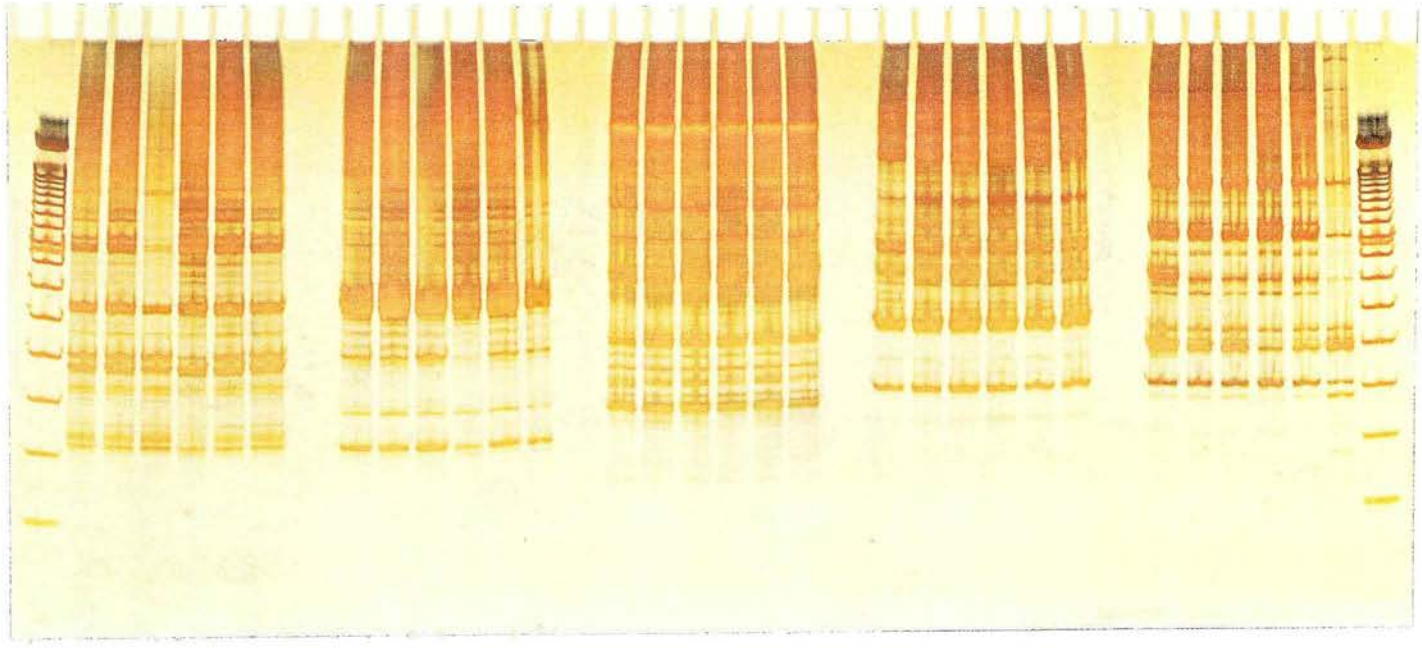


Figure 3.2.5. Amplification products derived from six samples of Black currant cultivar Baldwin separated on polyacryalamide gel electrophoresis followed by silver staining. Both ends represent nucleic acid markers i.e. 100 base pair. From left to right group of six represents the six samples amplified by using primer 1 to primer 5, Table 3.2.2

3.2.4. DISCUSSION

Polymorphisms were easily detected with RAPD markers using ten primers on agarose gel electrophoresis. Sample D was the most distant in the group (0.893) while B and C were the closest (0.992). The dendrogram (Figure 3.2.2) represents the simple relationship among the samples and indicated that B, C, F are more closely similar to A as compared to E and D while D is the most dissimilar one. Results obtained from the polyacrylamide gel electrophoresis and silver staining experiment showed a reduction in similarity between the groups, as indicated by similarity coefficients (down to 0.785. between C and D; and between B and C to 0.859). The dendrogram figure 3.2.4 also presents a different degree of relatedness between E and F contradictory to agarose gel electrophoresis. Indeed, no two samples showed an identical banding pattern over the range of primers used. This was unexpected and surprising since the material originated from a common ancestor and the cultivar was maintained by vegetative means and therefore each sample should be a sub-sample of a common clone. Some of the variations may well be due to some silent or minor mutation which occurred in the genome of that sample and either did not express itself in the phenotype of the plant or resulted in minimum changes in the phenotypic characters crucial for morphological identification. Variation may have resulted from a mix-up of material occurring prior to arrival at different nurseries. Another possible reason could be the development of a chimera or bud sport which might have been used as propagating material at some stage of the propagation system and which leads to the different genetic make up of regenerated plants

Comparison of the methodology reveals differences as a result of gel separation and staining. Of the two approaches, it would seem that the agarose methodology gave results more in keeping with the known history of the sources of each of the samples. The data from the agarose system supported the close relatedness of sample B, C and F which are known to be from a common source (Hill Top selection). The close relatedness was confirmed from the higher value of similarity coefficient using agarose gel electrophoresis and ethidium bromide staining. The

two methods revealed different levels of relatedness for two samples (E and F). This is the result of scoring a large number of bands as revealed by polyacrylamide gel and silver staining than with agarose gel. One possible reason for this deviation of the pattern could be that in RAPD-PCR, primers can bind (with reduced efficiency) to targets containing one or two mismatches (Bachmann, 1994). This will lead to an imperfect primer attachment site. These bands show as very weak bands on agarose gel and can be recognised and discarded easily but in polyacrylamide gels, these weak and unreliable bands are difficult to recognise and have a greater chance to be included in the analysis. This may affect the overall interpretation. Although by disregarding the weak bands incorrect results can be avoided and reliability of assay increased this may be at the expense of additional information which may be lost as it might not be due to mismatch. Therefore there may be a sacrifice of polymorphisms, as revealed by a particular primer in favour of more robust results.

Although the variation was detected in sub samples of a cultivar using RAPD-PCR methodology, this does not necessarily mean that those samples were actually different in the field because RAPD-PCR picks up the differences evenly along all the genome. In the total DNA a large part of it is simple repetitive thus any changes occurring in this DNA does not express it self in plant morphology. The morphological characters are the result of interaction between active gene and environment. The methodology of cultivar registration and evaluation should not be based on one technique. It should be a combination of suitable morphological and molecular techniques to make the results more reliable and to reduce the chance of mistakes

CHAPTER 4.

MICROPROPAGATION OF SOFT FRUITS

4.1. GENERAL INTRODUCTION.

Traditionally, soft fruits are propagated by vegetative means. The most readily identifiable weaknesses of conventional or traditional propagation are the slow rate of multiplication and the need for specialised conditions in order to keep disease and virus free. This can be costly and can be unreliable. As a result of the introduction of new cultivars and the limited supply of virus-free clones of existing cultivars there is a need for a more economical, efficient and practical method of propagation (Wainwright et al., 1986). One method that has been used is micropropagation which involves the production of plants from very small plant parts (meristem) grown aseptically *in vitro*, where environment and nutrition can be rigidly controlled. This methodology is attractive in comparison to traditional methods of propagation. For certification in the UK, of all three species *Ribes, Rubus* and *Fragaria* are required to have a 'soil-less' phase of culture (SOAEFD, 1997). This is in response to perceived needs for preventing virus build-up and /or fungal pathogens being transmitted via contaminated soil. Certified stocks derived from micropropagated materials are inspected for health and vigour only as no guarantee can be given at this stage on the trueness-to-type of such stocks. These stocks are identified by having the suffix 'M' included in the certificate number. Where appropriate the suffix 'M' can be removed once the material has been fruited and the trueness to type checks are carried out by suitably qualified personnel. The general view is that the use of micropropagation methodology for multiplication is secure. However, most systems do not rigorously test, this assumption (Hussey and Stacey, 1981) and when testing is undertaken tends to rely on morphological assay. The standard system of meristem culture within the potential propagation system for all three species of soft fruit *Ribes, Rubus* and *Fragaria* allows for up to 10 cycles of sub-cultures followed by re-establishment of

mature plant material *in-vivo* (along with necessary testing). Subsequent meristem culture should utilise original source plant material (SOAEFD, 1997). In practice, agreement has been sought and granted from the Government Inspectorate for exceptions to this standard procedure when difficulties have arisen with renewal of source planting material. (D. Mitchell, personal communication). The UK standard approach of the use of micropropagation follows the norm accepted within most EU countries i.e. 10 sub-culture cycles followed by re-establishment. There is considerable anecdotal evidence that variants are produced via meristem culture (D. Mitchell, personal communication). Usually these are removed following morphological assessment prior to sending on to the next phase of multiplication. This study aims to assess RAPD-PCR as a means of establishing levels of genetic uniformity within meristem propagating material of soft fruit species and further to investigate whether the number of sub-culture cycles of such cultivars can influence the level of variability produced.

4.2. USE OF RAPD TO ESTIMATE GENETIC VARIABILITY IN BLACKCURRANTS REGENERATED VIA MICROPROPAGATION.

4.2.1. INTRODUCTION

blackcurrants are traditionally propagated by hard wood cuttings 20 cm in length which are directly planted in the field in the Autumn season or early Winter (MAFF, 1981). With the introduction of new cultivars and limited supply of virus-free clones of existing cultivars, there has been a need for a method of propagation that is more economical in the use of plant materials (Wainwright et al., 1986). The potential of micropropagation was explored and found to be very successful by several workers such as Brennan et al. (1989); Flegmann and Wainwright. (1981); Wainwright et al. (1984, 1985, 1986); Orlikowska. (1984)., Orlikowska et al. (1991), Brennan et al. (1990) and Huang et al. (1985). However, the genetic integrity of regenerated plants via micropropagation was assumed rather than tested

rigidly. It has been established now that tissue culture regimes may have an effect on the genetic constitution of plants. Therefore there is a need to assess the genetic integrity of micropropagated plants in order to have more knowledge about the genetic stability of regenerated plants.

4.2.2. MATERIALS AND METHODS

4.2.2.1. PLANT MATERIAL

Plant material of blackcurrant cultivar Ben Sark at the first sub-culture stage was supplied by the Department of Horticulture Scottish Agriculture College, Bush Estate, and were subcultured in the M S medium described in Table 2.1. supplemented by 30 g\l. sucrose and 1.4 mg\l of the hormone BAP. Three meristem shoots were placed, under aseptic conditions, into each culture jar. These were incubated in the tissue culture room (26 °C $\pm$ 1°C) for a four week period. After four weeks these plants were further sub-cultured and at this time three samplesof leaves were collected by pooling within each bunch of plantlets originating from the single shoot pieces. These leaf samples were used for fresh DNA extraction in replicated runs. Samples were collected only from those plantlets which were further subcultured to store at -80°C for analysis. Material was sub-cultured every four week and leaf samples taken for sixteen generations.

4.2.2.2. DNA EXTRACTION

Total genomic DNA was isolated from the frozen leaf material stored at -80C using a modification of CTAB procedure (Doyle and Doyle, 1987), as described in Section 2.5.2. To confirm the successful extraction the extracted DNA was visualised after electrophoresis in 1.4% agarose gel and subsequently stained in ethidium bromide (Section 2.6.3.1). Extracted DNA showed a single sharp band of greater than 23k bp. DNA of any sample showing smearing or the absence of sharp bands was discarded and the extraction was repeated. After successful extraction, the DNA samples were quantified by using spectrophotometry (Section 2.5.3). All

samples were diluted accordingly to make the final concentration 10 ng/μl. 4μl of the DNA solution was used in each 50 μl (total volume of PCR reaction mixture), as template DNA.

In the present study, RAPD markers were used to detect DNA polymorphism and genetic similarity among the generations of micropropagated plants. The protocol of Williams et al. (1990) was used with a slight modification as described in section 2.5.4. In each PCR, DNA from parent mother plant from which the meristem was taken to initiate the micropropagation was also included and each sample was compared with the parent plant for differences. Each reaction was repeated twice with the same conditions with fresh DNA extractions and only the reproducible bands were included in the analysis. Weak bands were not scored because they were rarely reproducible to ensure the reliability of the assay. A negative control without target DNA was always run in each PCR amplification to detect any contamination in the reaction mixture.

4.2.2.3. PRIMER SCREENING.

A total of 45 primers were screened. Primers were selected on the principle that primers able to amplify more DNA fragments gave more information. Therefore primers were tested using the DNA from the parent plant as template. The number of random fragments amplified by each primer was counted and primers not showing satisfactory amplification were discarded from the programme. The primers showing many bands with good resolution and separation were selected for use. A total of 12 primers produced good amplifications and were included in this study (Table 4.2.1).

Table 4.2.1. Primers and their base sequence used for the detection of polymorphism in regenerants of blackcurrant cultivar Ben Sark

NO.	Primer	Sequence.
1	87729801	5' CACTGCAGTC 3'
2	87729804	5' TTCAGGGGTC 3'
3	87729805	5' GGACTAGTGG 3'
4	87729806	5' CCAATCCGTG 3'
5	87729807	5' GGAGCTTGAC 3'
6	87729808	5' GGAGCTTGCC 3'
7	87729809	5' CGGTGAGGCT 3'
8	87729810	5' TCACGGCACC 3'
9	87729811	5' TGCCAGTGGA 3'
10	PO298	5' CAGTTCGAGG 3'
11	OPH-01	5' GGTCGGAGAA 3'
12	OPH-03	5' AGACGTCCAC 3'

4.2.2.4. DATA ANALYSIS.

Amplification products of each DNA fragment were represented by a band on a photograph. Each band represented a set of DNA segments/fragments of almost equal length. (The words segment fragment and band have been used synonymously, representing a single set of DNA segments of equal length). The number of bands produced by each primer for each sample was scored. The total number of bands produced by all the primers used for a single sample was counted and compared with the total number of bands produced by the same primer for the parent. Data generated from detection of the fragments were analysed employing the equation of Nei and Li (1979) described in Section 2.6.2 and similarity between parents and individual sample was calculated. Variation between parents and each sample was calculated by the equation described in Section 2.6.2.

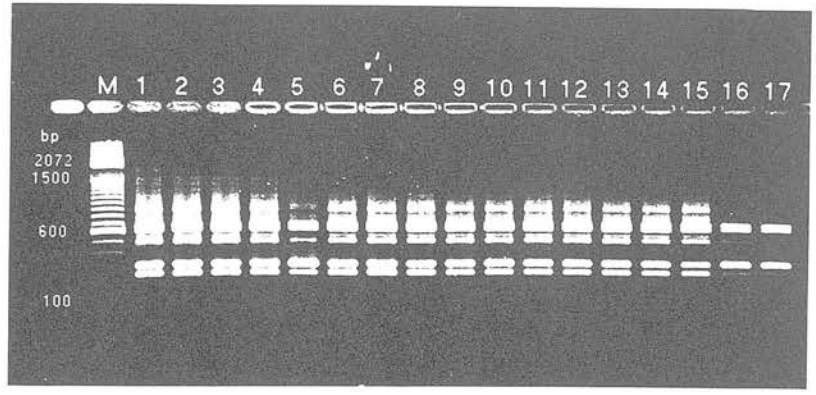


Figure 4.2.1. Amplification products derived from micropropagation of 16 subculture/generation cycle of blackcurrant cultivar Ben Sark using random 10 base primer A. From left to right: M represents nucleic acid markers. (Fragment sizes of the DNA markers are given in base pairs). The DNA samples consists of; No. 1 parent DNA, those from 2-17 are successive sub-cultures.

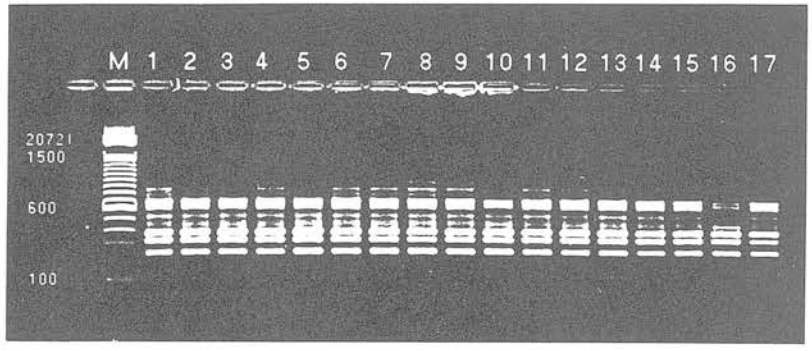


Figure 4.2.2. Amplification products derived from micropropagation of 16 subculture/generation cycle of blackcurrant cultivar Ben Sark using random 10 base primer A. From left to right: M represents nucleic acid markers. (Fragment sizes of the DNA markers are given in base pairs). The DNA samples consists of; No. 1 parent DNA, those from 2-17 are successive sub-cultures.

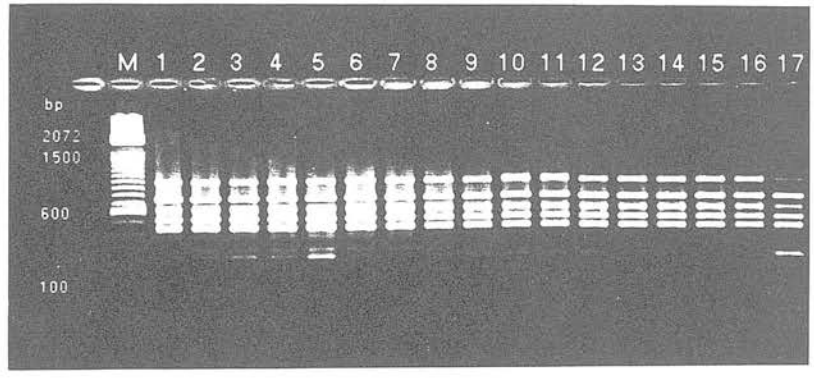


Figure 4.2.3. Amplification products derived from micropropagation of 16 subculture/generation cycle of blackcurrant cultivar Ben Sark using random 10 base primer A. From left to right: M represents nucleic acid markers. (Fragment sizes of the DNA markers are given in base pairs). The DNA samples consists of; No. 1 parent DNA, those from 2-17 are successive sub-cultures.

4.1.3. RESULTS.

Twelve primers (Table 4.2.1) were used to screen the 16 subcultures of the blackcurrant cultivar Ben Sark. All primers used in the analysis gave between 3 to 9 DNA bands with an average of 6.4 band per primer. A total of 77 DNA fragments were amplified which means 1540 bases were explored in this experiment. All DNA fragments produced were monomorphic as shown in Figures 4.2.3, 4.2.4 and 4.2.5 showing 100% similarity to the parent and with each other except the sub-culture number 15 and 16 as shown in the Table 4.2.2. In sub-culture 15 a total of nine fragments were detected as polymorphic which leads to the similarity of 93.7% and variation of 6.2% as shown in Table 4.2.2. while in sub-culture number 16 twelve fragments were polymorphic and shows similarity of 86% with a variation of 13.4% as shown in the Table 4.2.2.

Table 4.2.2. Results of analysis of micropropagated plants of blackcurrant cultivar Ben Sark estimated by RAPD-PCR amplification by using 12 primers.

Sub-culture	Similarity	Variation
Parent	1	0
1	1	0
2	1	0
3	1	0
4	1	0
5	1	0
6	1	0
7	1	0
8	1	0
9	1	0
10	1	0
11	1	0
12	1	0
13	1	0
14	1	0
15	0.94	6.20
16	0.86	13.37

4.1.4. DISCUSSION.

The results obtained from the RAPD-PCR analysis of the regenerated micropropagated plants of the blackcurrant cultivar Ben Sark revealed that plants regenerated up to the 14th generation cycle or after 56 weeks in tissue culture environment showed no variation. The evidence appears to suggest that the more recently a cell has been derived from the apical meristem the less likely it is to display variation. The cells in the tips of the apex are maintained in a continuing embryonic state so that the derivatives have the potential to differentiate into many

different tissues within the plant body. It is possible that more efficient DNA repair mechanisms operate in these cells but this would be difficult to demonstrate with present techniques (Hussey, 1986). These results support the rule of SOAEFD Certification of Stocks of soft fruit according to which the maximum time any material may be maintained in culture is one year (52 weeks) or not more than 10 sub-cultures whichever is sooner. After the 14th generation considerable variation was noticed in the 15th sub-culture which increased in the 16th sub-culture. This variation is of considerable importance as it is tissue culture induced and needs to be further explored. It can in no way be confused with epigenetic effects which may appear by the first *in vitro* cycle. (Sibi, 1986)

No report in the literature was found about the use of RAPD-PCR in blackcurrant species for the evaluation of similarity or variation of plants regenerated via micropropagation. Therefore direct comparison of the RAPDs results obtained here with those of other workers was not possible. However, some workers have reported the existence of somaclonal variation detected using morphological techniques in related soft fruit species such as strawberry. Off-types were reported by Damiano (1980). These included some temporary off-type characters (possibly epigenetic) which disappeared after 1-2 years of cultivation while other variation was permanent. One of the first research studies on phenotypic stability in strawberry (Swartz et al., 1981) reported three off-type variants in meristem culture, namely: white-streaked leaf chlorosis, an irregular sectorial yellow chlorosis, and dwarf-type plants. The dwarf-type plants were characterised by reduced leaf and petiole size, lack runnering, lower yields, and smaller size of fruits. Other tissue culture variants included compact trusses as well as runnerless and female-sterile plants in strawberries. Off-type plants regenerated from meristem culture are induced by medium composition, time of culture, and number of sub-culture (Shaeffer et al. 1980). It has been suggested by these workers that the frequency of off-type plants increases with the duration of culture. In the present study results generally support this idea.

The dominant nature of RAPDs markers does not allow one to distinguish the dominant homozygote from the heterozygote from the banding patterns. Therefore, for a dominant homozygote at a particular locus, mutations affecting only one of the two alleles would remain undetected. The results presented in this study are indicative of the level of variation and not absolute, because sub-samples were used in this study in terms of selective sub-culture of plants in each sub-culture cycle to reduce the size of the experiment.

The genome is most probably randomly sampled by RAPDs markers. This method appeared simple and the results were reproducible. Because only micro-amount of materials are necessary, this approach can be used to assess each stage of *in vitro* culture. Large sample size can be treated rapidly and the technique lends itself to automation (Williams et al., 1990; Welsh et al., 1991; Hedrick, 1992).

This study supports the use of RAPD-PCR to give rapid indication of the level of genetic stability of *in vitro* culture regenerated plants. It needs minute amounts of plant material and therefore it could be used at any stage of micropropagation to get the indicative reflection of the genetic stability of the plants. Ideally to test the genetic integrity of plants regenerated clonal material for a particular species with RAPDs a number of markers equal to or larger than the one required to obtain a saturated linkage map would be desirable. For conifers, this number has been estimated to be larger than 200-300 (Carlson et al., 1991; Neale and Williams, 1991). Once a diagnosis of stability has been obtained with such a number of markers for every targeted species and set of production procedures, genetic stability of *in vitro* propagated plants could proceed with higher confidence at an industrial scale.

4.3. USE OF RAPD TO ESTIMATE GENETIC VARIABILITY IN RED RASPBERRIES REGENERATED VIA MICROPROPAGATION.

4.3.1 INTRODUCTION

Rubus species are of world-wide economic importance for fresh and frozen fruits and a number of processed items. Raspberries grow wild in temperate parts of Europe and especially in eastern Europe. The plant has perennial roots and biennial canes which bear fruits on the previous year's growth. The fruits are small berries (2-5g), which are collected and sold as fresh fruits, as well as processed. Raspberry fruits are known especially for their pleasant flavour. Their main uses are as fresh and frozen fruits and as raw material for syrups, confectionery, wines, sweets, ice cream and dairy products.

Plant tissue culture has gradually developed greater prominence in this industry. In an effort to control virus infections spread through traditional propagation procedure, growers have been encouraged to use only certified material to established new plantings (Stace-Smith, 1984). The limited number of virus-tested plants that form the basis of certification programmes can be increased most rapidly through micropropagation. Furthermore, rapid increase of advantageous new selections developed by plant breeders can expedite evaluation trials, shortening the intervals to cultivar release, and distribution to the growers.

Red raspberry (*Rubus idaeus* L.) has been micropropagated successfully for a number of years (Hulth, 1979; Anderson, 1980; Welander, 1985). Micropropagation of red raspberry can produce healthy plants far more quickly than conventional means and the operation can be carried out year round (Donnelly and Daubeny, 1986). Despite the advantages, the uniformity of plants propagated by this technique has been assumed rather than tested rigidly. This study was aimed at evaluating the effect of the tissue culture regime and time of culture/number of sub-culture on the genetic uniformity of regenerated plants.

4.3.2. MATERIAL AND METHODS

4.3.2.1. PLANT MATERIALS

Raspberry cultivar, Autumn Bliss was supplied by the Department of Horticulture, Scottish Agriculture College at Bush Estate, at second sub-culture stage. These were further subcultured in the plant growth unit (PGU) of Scottish Agriculture College, Edinburgh. The culture media used for this investigation were based on MS medium (Murashige and Skoog, 1962) described in Table 2.1. The MS media was further supplemented by 30 g\l. of sugar 150 mg\l of Na Fe EDTA. The growth regulators added were 3 mg\l of BAP and 0.3 mg\l of IBA. After autoclaving the media was dispensed in sterile jam glass jars which were used for micropropagation. For sub-culture plantlets were pulled out and placed into a 90 mm diameter Petri dish. The leaves were removed and the stem cut into pieces and inserted into the fresh jar containing media and were labelled. The sub-culture cycle was repeated every 6 weeks until 14th generations. Leaves were collected from individual plant which were further subcultured. Where plantlets did not survive on subculture leaf samples were collected from parallel sibling meristem cultures and placed in an Eppendorf and stored at -80°C as described in Section 2.5.1 until required for DNA isolation and further analysis. The stems were cut into pieces and subcultured onto fresh medium to provide the next generation.

4.3.2.2 DNA EXTRACTION

The CTAB DNA isolation methods as described by Doyle and Doyle (1987), with slight modifications as described in Section (2.5.2), was used in this study. The CTAB DNA isolation method was found to be very easy, quick and economical and isolated DNA was of high quality with minimum smearing. To confirm the successful isolation the DNA was evaluated by electrophoresis in 1.4% agarose gel and visualised after staining in ethidium bromide as described in Section (2.6.1 and 2.6.3.1). Extracted DNA showed a single sharp band of high molecular weight was used for amplification reactions. DNA of any samples showing smearing or no sharp band was discarded and the extraction was repeated. After the isolation the

DNA samples were quantified using a spectrophotometer (Section 2.5.3). All the samples were diluted accordingly to make the final concentration of 10 ng/µl. Two µl of the DNA solution were used in each 50 µl of total PCR reaction mixture as template DNA.

4.3.2.3 PRIMER SCREENING AND SELECTION.

A total of 35 primers were tested using the DNA from the parent plant as template. The primers were chosen on the principle that primers able to amplify larger number of DNA fragments will provide more information. The number of random fragments amplified by each primer was counted and primers not showing satisfactory amplification were discarded from the programme. Finally 8 primers showing more bands with good resolution and separation were selected for use. Table 4.3.1.

4.3.2.4 REPRODUCIBILITY OF RAPDs

The reproducibility of RAPDs markers is affected by several factors, including the genomic DNA concentration, the temperature profile of the thermocycler, the magnesium ion concentration, and the choice of thermostable polymerase. It is extremely important to optimise the amount of genomic DNA used in PCR amplifications. The addition of too much genomic DNA can result in smeary patterns, while the use of too little leads to a lack of reproducibility. (Rafalski et al., 1996). Genomic DNA concentration of 20 ng/50 µl reaction was found optimum for this study.

Table 4.3.1. Primers and their base sequence used for the detection of polymorphism in red raspberry regenerants.

NO.	Primer	Sequence.
1	87729802	5' CCCGTAGAGA 3'
2	87729808	5' GGAGCTTGCC 3'
3	87729809	5' CGGTGAGGCT 3'
4	86370603	5' CTGCATCGTG 3'
5	86370609	5' CCAATCCGTG 3'
6	86370611	5' CTGACCAGCC 3'
7	OPH 1	5' GGTCGGAGAA 3'
8	PO2 98	5' CAGTTCGAGG 3'

4.3.2.5 RAPD-PCR ANALYSIS

The protocol of Williams et al, (1990) was used with slight modification as described in Section 2.5.4. In each RAPD-PCR reaction mother plant DNA was included and each sample was compared with the parent plant for any visible differences on the gels. Each reaction was repeated with the same conditions and only the reproducible bands were included in the analysis. Weak bands were not scored because they were rarely reproducible to ensure the reliability of the assay. A negative control without target DNA was always run in each PCR amplification to detect any contamination in the reaction mixture.

Amplification products of each DNA fragment were represented by a band on a photograph. Each band represented a set of DNA segments/fragments of almost equal length. The number of bands produced by each primer for each sample was scored. Total number of bands produced by all the primers used for a single sample were scored and compared with total number of bands produced by the

same primer for the parent. The parent plants were those from which micropropagation was initiated at the start of the experiment. Data was analysed employing the equation of Nei and Li, (1979) described in section (2.7.2) and similarity between parents and each individual sample was calculated. Variation between parents and each samples was calculated as by the equation described in Section 2.7.2.

4.3.3 RESULTS

A total of eight primers were used for amplifications of DNA template isolated from 14 sub-culture and one parent plant. All the primers used gave 6 to 10 bands (Figures 4.3.1-4.3.6). A total of 62 DNA profiles were produced giving an average of 7.7 bands for each primer used indicating that 1240 bases of plant genome were explored in this experiment. Therefore variations among 1240 bases have a chance to be detected using these primers.

Similarity values ranged from 100% to 74% (Table 4.3.2). The maximum variability was observed in the 7th sub-culture and started appearing from the 5th sub-culture. Primers five and six were monomorphic for all the sub-culture analysed, (Figure 4.3.1 and 4.3.2) while the rest gave some polymorphic bands at different sub-cultures.

Table 4.3.2 ***Results of analysis of Micropropagated plants of Red Raspberry, estimated by RAPD-PCR amplification by using 10 primers***

Sub-culture	Similarity	Variation
Parent	1.00	0
2	1.00	0
3	1.00	0
4	1.00	0
5	0.875	12.50
6	0.9841	1.64
7	0.74	26
8	0.96	3.27
9	0.968	3.27
10	0.958	4.20
11	0.932	6.78
12	0.95	5.00
13	0.959	4.06
14	0.885	11.5
15	0.844	15.50

No polymorphic bands were detected until the 4th sub-culture using all 8 primers giving them a similarity of 100% to their parent plant. Variation to parent, shown by different samples was not following any principle of increasing or decreasing in relation to the number of sub-culture \ generation cycles (Table 4.3.2).

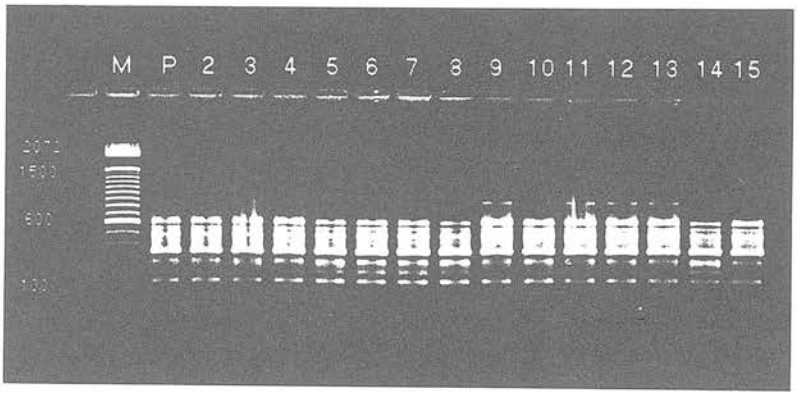


Figure 4.3.1. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autum«Bliss using random 10 base primer No.5. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

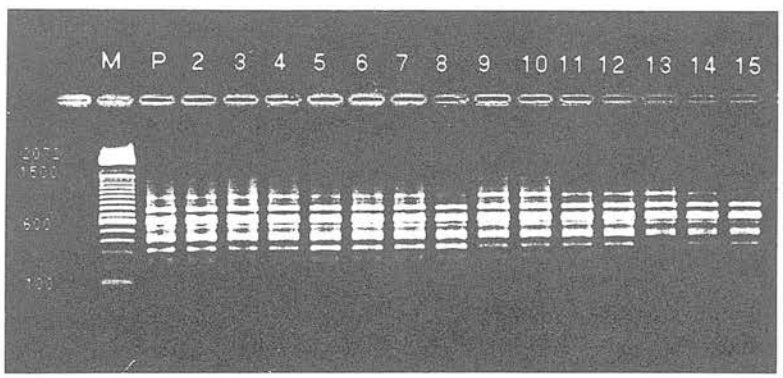


Figure 4.3.2. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autum«Bliss using random 10 base primer No. 6. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

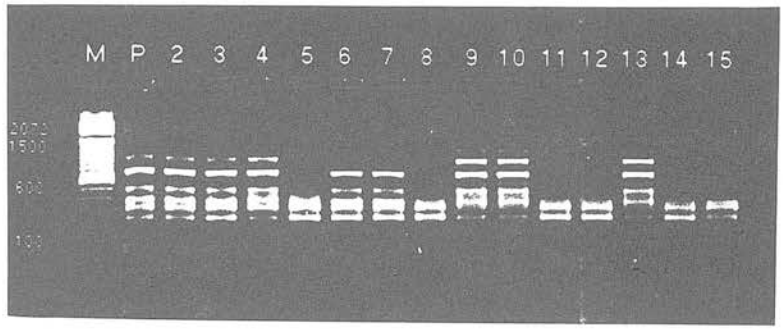


Figure 4.3.3. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autumn Bliss using random 10 base primer No.4. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

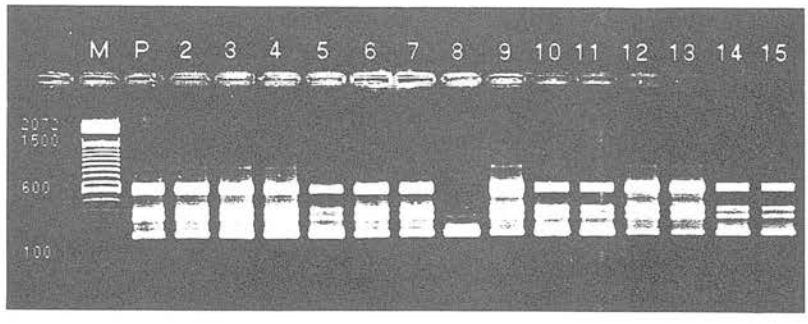


Figure 4.3.4. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autumn Bliss using random 10 base primer No. 3. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

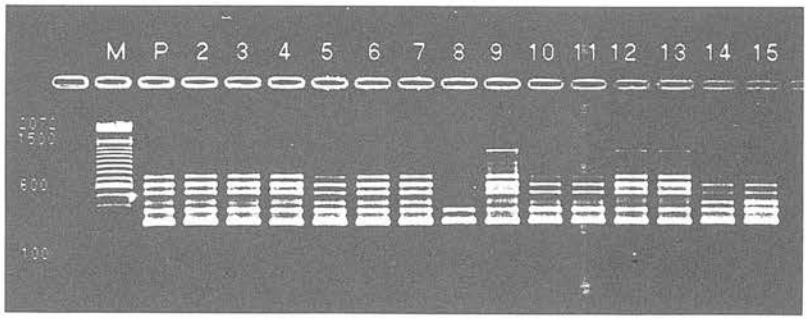


Figure 4.3.5. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autum™Bliss using random 10 base primer No.1. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

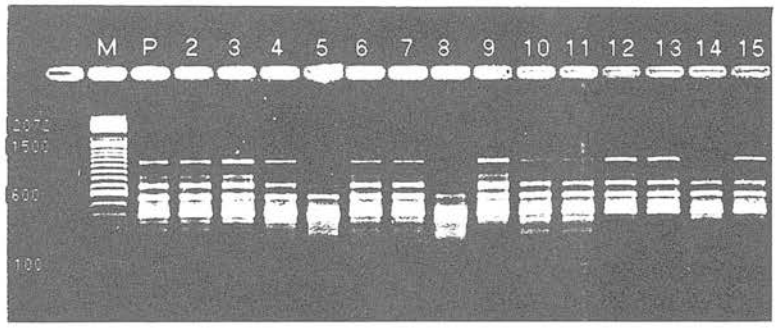


Figure 4.3.6. Amplification products derived from micropropagation of 14 generation of Red raspberries cultivar Autum™Bliss using random 10 base primer No. 2. From left to right M represents nucleic acid markers i.e. 100 base pair, bp represents fragments sizes of the DNA marker in base pairs. The DNA samples consist of P parent DNA, from 2-15 are successive subcultures.

Maximum variation was observed in the 7th sub-culture while it decreased up to 3.2% to 4.2% in successive 8th and 9th sub-cultures respectively, and rose up to 15.5% in the 14th sub-culture. The interesting phenomenon observed in this study was that some DNA profiles which disappeared in one generation reappear in the next or 2nd next generations as shown in Figures 4.3.3 4.3.4, 4.3.5 and 4.3.6. Overall variation was haphazard and not related to the number of sub-culture/generation cycles.

4.3.4 DISCUSSION

The genetic stability of micropropagated plants of the red raspberry cultivar Autumn Bliss was evaluated in relation to the effect of sub-culture /generation cycles in this study using RAPD-PCR analysis. Plants regenerated up to the 4th sub-culture seem genetically stable and true to type as all the primers used showed no polymorphism (Figure 4.3.1 to 4.3.6). However, in the 5th sub-culture considerable polymorphism was revealed (12.5% variation with the parent, Table 4.3.2). This variability appeared to decline in the 6th sub-culture (1.64%). Variation reappeared in the 7th sub-culture material (maximum level of 26% Table 4.3.2). These results do not support the principle of the SOAEFD 1997 Certification of Stocks of Soft Fruit (D. Mitchell, Personal communication), which advocates a maximum time in culture of one year or not more than 10 sub-culture cycles.

Variation in meristem culture in *Rubus* species was also reported by Feucht and co-workers (Feucht et al., 1985) who examined 4000 plants of *Rubus* cultivar Schoenemann which had been propagated *in vitro* from side-shoot meristems and which were in the third year of production. They found 24 plants (0.68 %) with small deformed fruits. This variation was identified by morphological screening and was found to be transferable to the offspring. It may be that variation detected at the level of DNA bases, may be much higher than that detected by morphological observation.

The nature of the variation and its appearance and disappearance in subsequent generations was surprising. There are several possible explanations why individual PCR generated bands disappeared and subsequently reappeared.

Mutation is a single cell event where only one tissue layer at a time is usually affected. This inevitably leads to the formation of chimerical shoots, most of which are unstable and confine the mutant tissue to limited areas in developing organs. Occasionally, stable periclinal chimeras are formed when a complete layer of mutant tissue is established (Derman, 1947). A wholly mutant shoot can result from the establishment of a sectoral chimera. An additional possibility is that of disruptions occurring in the layering of an established periclinal chimera, either spontaneously or as a result of the immersion of the shoot apex in the agar media. This chimera either pre- existing or tissue culture induced may give a genetically variable population, some mutant and other normal. The sampling methodology and the effect of chimera on the presence / absence of individual PCR bands may account for the appearance / disappearance of such bands. Sub-samples were used in this study at sub-culture of the plants in each sub-culture cycle in order to reduce the size of the experiment. At any one sub-culture regenerants may have been a mutation and from which the leaf samples were collected. These may not survive in the next sub-culture, in which case leaf samples would have been collected from their normal sibs. These may have normal genotypes as revealed by RAPD-PCR and those plantlets were used for further multiplication. This might lead to haphazard variation detected by RAPD-PCR.

Another possible explanation for the unstable expression of variation could be the involvement of transposable elements. Transposable genetic elements are stretches of DNA which can move from one locus in the genome to another independently of extensive sequence homology (Calos and Miller, 1980). This phenomenon of unstable expression of variation is a hallmark of transposon-induced changes and has been reported in a number of cases such as wheat (Ahloowala and Sherington, 1985), tobacco (Lorzh and Scowcroft, 1983), and in alfalfa (Groose and Bringham,

1984). Another reason for relating transposon to somaclonal variation is the known effect of environmental stress resulting in a burst of transposable element activity. Wide intra- and inter-specific crosses, together with some viral infections, stimulate activity of previously silent transposons (Peterson, 1985; Doring and Starlinger, 1986). *In vitro* culture is indeed an environmental stress and may stimuate the previously silent transposon.

More circumstantial evidence of tissue culture as a possible trigger of a transposition burst comes from experiments performed in alfalfa (Reisch and Bingham, 1981). An unstable mutant affecting the flower colour has been reported by Groose and Bingham (1985). This arose initially as a white-flowered somaclonal mutant from culture of a purple-pigmented genotype. When the white mutant was re-cultured and plants regenerated, 32% of 1356 regenerated plants had purple flowers. The unstable mutant, is transmitted to progeny which revert when tissue cultured.

The appearance of polymorphisms in the 5th sub-culture also coincided with the breakdown of power supply of the cooling system in Plant Growth Unit at SAC Edinburgh over a weekend. This resulted in an (undesirable) high temperature (43°C) for 36 hours and resulted in the death of most of the micropropagated plant material. A few were rescued and used as source material for further multiplication/sub-culture. This might have any effect on the genetic base of micropropagated plants of *Rubus*. On the other hand *Ribes* material was also simultaneously affected by this undesirable heat treatment/stress but no variation in relation to this incident was found in *Ribes* regenerants. It may be that *Ribes* genotypes have a more stable genetic make-up as compared with *Rubus* genotypes.

Finally the results in this study do not gave an absolute measure of level of variation but confirm the presence of culture induced variation in micropropagated plants. These results suggest the need for a future study of genetic stability of the regenerated plants via micropropagation in *Rubus*. Such a study would include all

the plants regenerated at each cycle with its subsequent mother plant. This can be achieved easily in *Rubus* as number of plants arising from each mother plant are not as high as in *Ribes* and *Fragaria* which give clusters of plantlets. The results produced from such experiments would provide a useful insight into the genetic variability induced by micropropagation.

4.4. USE OF RAPD-PCR TO ESTIMATE GENETIC UNIFORMITY IN STRAWBERRY CULTIVAR TANGO REGENERATED VIA MICROPROPAGATION.

4.4.1. INTRODUCTION

Strawberry belongs to the first group of fruits that are available after the winter. Their general nutritive value (Mazhorov, 1977) make the high labour intensity of strawberries growing and harvesting reasonable. The adaptation of tissue culture proliferation techniques to mass propagation of strawberry has been shown to be a viable alternative to traditional runner propagation (Boxus et al., 1974). The rapid proliferation obtained in tissue culture allows nurseries to meet an unexpected demand for a particular cultivar; a million or more plants can be produced in a year from a single meristem tip (Boxus et al., 1974, 1977). The introduction of new cultivars, which include time for production of significant nursery stock, can thus be advanced by 1 to 2 years. Additionally, strawberry plants can be stored *in vitro* in refrigerators for several years (Mullin, 1976). Storage in tissue culture demands less space and energy than traditional runner plant storage, and *in vitro* cultures can be initiated any time during the production cycle or year. Another advantage of micropropagation is the elimination of pest and pathogen pressure during the production cycle, assuming that the initial stock plant is free of diseases. This is particularly critical for the certified or registered pathogen-free nursery stock programmes.

In spite of these advantages, some phenotypic changes in both appearance and performance have been noticed in tissue culture produced strawberry plants (Schaefer et al., 1980) and these have raised concerns about the value of this method. These off-types were evaluated using morphological methods. This study aimed to examined the level of genetic uniformity at DNA level in the strawberry plants produced by micropropagation using RAPD-PCR technology.

4.4.2. MATERIALS AND METHODS.

4.4.2.1. PLANT MATERIALS

A total of 34 micropropagated strawberry plants was analysed in this study. These plants were at the 3rd sub-culture/generation cycle and were kindly supplied by the Department of Horticulture, Scottish Agriculture College, Bush Estate. These plants were propagated on MS medium described in Table 2.1 supplemented by 40mg\l of Na Fe ETDA, 30 g\l sucrose and BAP and IBA at 1mg\l each.

4.4.2.2. DNA EXTRACTION

Total genomic DNA was isolated from leaves of individual plants using a modification of CTAB procedure (Doyal and Doyal, 1987), as described in (Section 2.5.2). To confirm the successful extraction the extracted DNA was visualised after electrophoresis in 1.4% agarose gel and subsequently stained in ethidium bromide Section 2.6.1. and 2.6.3.1. Extracted DNA showed a single sharp band of greater than 23k.bp. DNA of any sample showing smearing or the absence of sharp bands was discarded and the extraction was repeated. After successful extraction, the DNA samples were quantified by using spectrophotometers (Section 2.5.3). All samples were diluted to make the final concentration 10 ng/µl. 4µl of the DNA solution was used in each 50 µl (total volume of PCR reaction mixture), as template DNA

In the present study, RAPD-PCR markers were used to detect DNA polymorphisms and genetic similarity among the generations of micropropagated plants. The protocol of Williams et al. (1990) was used with a slight modification as described in (Section 2.5.4). In each PCR, DNA from parent plant was also included and each sample was compared with the parent plant for differences. Each reaction was repeated with the same conditions and only the reproducible bands were included in the analysis. Six primers were used in this study to amplify the template DNA (Table 4.3.1).

Table 4.4.1. Primers and their base sequence used for the detection of polymorphism in regenerants of strawberry cultivar Tango

NO.	Primer	Sequence.
1	8637060	5' CTGCATCGTG 3'
2	87729810	5' TCACGGCACC 3'
3.	8637060F	5' CCACCGCCAG 3'
4	86370607	5' GGTCGGAGAA 3'
5	87729806	5' CCAATCCGTG 3'
6	86370611	5' CTGACCAGCC 3'

The number of bands produced by each primer for each sample was scored. The total number of bands produced by all the primers used for a single sample was counted and compared with the total number of bands produced by the same primer for the mother plant. A plant at subculture one was used as the mother plant because the original plant from which the micropropagation was initiated was not available. Data generated from detection of the fragments were analysed employing the equation of Nei and Li (1979) described in Section 2.7.2 and similarity between the parent and individual sample was calculated. Variation between parents and each sample was calculated by the equation described in Section 2.7.2.

4.4.3. RESULTS

Six primers (Table 4.4.1) were used to amplify and screen the 34 plants regenerated via micropropagation of the Strawberry cultivar Tango. All primers used in the analysis gave 3-7 DNA bands with an average of 5.1 bands per primer. All the reactions were repeated at least twice. A total of 31 DNA fragments were amplified and thus 620 DNA bases were explored in this investigation. Of the 34 plants analysed only two of them showed 100% similarity to the parent plant (Table 4.4.2). The variation ranges from (0.00) to 31.91%. Five plants (14.7%) showed more than 10% variation to the parent while 14 of the total plants (41%)

showed less than 5% variation and 18 plants (52%) showed more than 5% variation to the parent plant source (Table 4.4.2).

Table 4.4.2. Results of genetic uniformity of strawberry cultivar Tango plants regenerated via micropropagation, estimated from RAPD-PCR data generated using 6 primers

Samples No.	Similarity	Variations(%)
Parent	1.00	0.00
1	0.935	6.45
2	0.984	1.64
3	1.00	0.00
4	1.00	0.00
5	0.912	8.77
6	0.68	31.91
7	0.893	10.714
8	0.949	5.08
9	0.949	5.08
10	0.931	6.896
11	0.931	6.897
12	0.808	19.23
13	0.949	5.08
14	0.984	1.639
15	0.966	3.334
16	0.984	1.639
17	0.931	6.89
18	0.807	19.23
19	0.912	8.77
20	0.984	1.58
21	0.83	16.93

Table 4.4.2 continued.

samples No.	Similarity	Variations (%)
22	0.949	5.08
23	0.966	3.334
24	0.852	14.81
25	0.966	3.334
26	0.966	3.333
27	0.966	3.333
28	0.784	21.56
29	0.984	1.64
30	0.931	6.897
31	0.984	1.639
32	0.931	6.896
33	0.966	3.333
34	0.984	1.639

4.4.4. DISCUSSION

The results produced from the analysis of micropropagated plants of strawberry cultivar Tango using RAPD-PCR showed a large number of variants. Similar results were also observed by other workers in strawberry micropropagation using morphological studies of the regenerated plants. For example Swartz et al., (1981), observed chlorotic leaf type variants less than 0.1%, while increased runnering 48% and number of crowns per square 62%. They also observed a mean increase (58%), in late runner production in micropropagated plants as compared to standard runner plant. Further reports note an increase of fruiting, linked to enhanced crown proliferation and to flower-bud differentiation, and a marked fruit quality decline in comparison to standard propagated plants from the same clone (Dijkstra, 1987; Hennerty et al., 1987; Swartz et al., 1987). The frequency of

producing genetically uniform plants via micropropagation is probably genotype dependent. Sansavini et al. (1990) found that different variants produced by micropropagation were sexually transmissible to the next generation and production of variants from micropropagation is genotypically dependent. Their findings also confirm the already reported influence of the genotype on the production of genetically uniform plants.

This study is unable to determine the cause of variation. However, other researchers (Shaeffer et al., 1980) describing the off-types produced by micropropagation infer that it may be influenced by medium composition especially high concentration of BAP, time of culture, temperature and light intensity. The higher rate of variation observed in this study may be due to the fact that RAPD-PCR most probably randomly sampled the whole genome under study and some mutations occurring in non coding DNA not express in terms of morphological characters.

The results obtained in this study did not support the rule of SOAEFD certification of stock of soft fruit (Appendix 4.1), according to which the maximum time any material may be maintained in culture is one year or not more than 10 sub-cultures.

The use of *in vitro* propagation for foundation plant production will necessitate more rigorous testing using molecular markers followed by field inspection and roguing operation since variant plant production is increased. Certain types of variants e.g. sectorial chlorotic plants, dwarf and leaf spot susceptible are easily recognised and rouged. The occurrence of white-streak and female-sterile variant can only be expressed in the year after planting. The results of this study recommend that a combination of both approaches molecular and morphological testing should be used in order to avoid the risk of variant multiplication.

These results are based on one study with one genotype. In order to investigate how general this behaviour could be needs to be further tested. So additional study

will be required to investigate genotype and improved methodology developed for investigating variation arising down through sub-culture generations. This may involve either following through an entire production system with complete testing of every part of a system or of cross checking a single generation and the follow through to next.

CHAPTER 5

5.1. PLANT REGENERATION OF CALLUS TISSUES INDUCED FROM LEAF EXPLANTS OF STRAWBERRY

5.1.1. INTRODUCTION

With the introduction of new cultivars and the limited supply of virus-free clones of existing cultivars, there is a need for an economical, efficient and practical method of propagation using available plant material. One method that has been used is micropropagation (Boux, 1974; Boux et al., 1977; Damiano, 1980). Generally this has been considered relatively safe, although occasional morphological variants have been found (Swartz et al., 1981). This methodology although attractive in comparison to traditional runner production can be unresponsive to needs. An alternative system based on the exploitation of callus and regeneration has been suggested by Nishi and Oosawa (1973). Callus is a coherent and amorphous tissue, produced on explants *in-vitro* as a result of wounding and in response to hormones, either endogenous or supplied in the medium. Most workers do not advocate the use of callus as a means of regeneration / multiplication because of the problem of variability. Indeed some phenotypic variation has been reported recently among callus culture regenerants (Jones et al., 1988; Nehra et al., 1990). However some species appear to produce stable callus (Hussey, 1983, 1986). For others it is thought that environmental variables during culture may influence the level of variation. However to exploit this approach will require the production of genetically stable callus. A number of variables during culture such as medium composition, number of sub-culture / generation cycle may influence the production of genetically stable callus. (Shaeffer et al., 1980). The main aim of this study was to evaluate the effect of different culture variables on the genetic uniformity of plants regenerated through callus culture and regeneration in strawberry (*Fragaria ananassa*).

5.1.2. MATERIAL AND METHODS

5.1.2.1. PLANT MATERIAL

Young leaves newly developing from glasshouse and *in vitro* grown strawberry plants of cultivar Tango were harvested and surface sterilised as described in Section 2.2.4. These leaves were used to cut the leaf discs with the help of a sterile metal cork borer with internal diameter of 6mm (Section 2.2.5).

5.1.2.2. CULTURE MEDIUM

The basal medium consists of Murashige and Skoog's (1962) mineral salt medium (Table 2.1). This was supplemented with 3% sucrose, 0.2% phytogel and adjusted to pH. 5.7 with NaOH before autoclaving. Six media (Table 5.1.1) differing only in plant growth regulator type and concentration were investigated in this study as described in table 5.1.1.

Table 5.1.1. Combinations and concentration of different plant growth regulators added to culture media.

Media	Cytokinin	Concentration.	Auxin	Concentration.
Media-3	BAP	1.12 mg\l	2,4-D	1.1 mg\l
Media-5	BAP	2.25 mg\l	2,4-D	0.22 mg\l
Media-10	BAP	1.12 mg\l	NAA	0.186 mg\l
Media-13	BAP	2.25 mg\l	NAA	0.186 mg\l
Media-28	BAP	2.25 mg\l	IBA	0.2 mg\l
Media-29	BAP	2.25 mg\l	IBA	1.0 mg\l

5.1.2.3. EXPERIMENTAL PROCEDURE

Calli were produced by planting three sterile leaf discs in each Petri dish with 5 replications on callus initiation media (Table 5.1.1) for an initial period of 6 weeks. All the leaf discs were marked 1, 2 and 3 on the Petri dish randomly. After scoring the callus growth, calli were transferred to fresh media for another 4 weeks. After remaining a total of 10 weeks on callus initiation media, shoots produced on each leaf disc were counted under the following five categories as described in Table 5.1.2

Table 5.1.2. Category and their descriptions used in scoring and analysing the regenerated shoots

Category	Descriptions.
1. Small shoots	1 to 2 cm
2. Medium shoots	2 to 3 cm
3. Large shoots	More than 3 cm
4. Abnormal shoots.	Shoots had translucent stems and leaves that were thickened, turgid and brittle
5. Plants survived.	Shoots that develop roots to became independent plants.

Regenerated plants with sufficient roots were transferred to the mixture of 50:50 perlite and compost. Plants were kept in hardening conditions for four weeks as described in Section 2.3.4 and then transferred to 9 cm plastic pots containing compost and watered regularly.

The number of each category of shoots in each leaf disc were counted and data produced were subjected to analysis to investigate the statistical differences with respect to regeneration ability for the different types of media under study.

5.1.3. REGENERATION FROM *IN VITRO* CULTURE LEAF EXPLANTS.

Analysis of variance revealed highly significant differences (Appendix 5.1.1 $P<0.01$ and 5.1.2 $P<0.001$) among the media for total shoot production and number of plants survived respectively. Figure 5.1.1 shows that calli on media 3 and 5 did not produce any shoots while the maximum number of shoots were produced on medium 29 containing BAP/IBA 2.25/1 mg\l. There was good shoot production on medium 28 but this medium produced 64% abnormal shoots as well (Table 5.1.1 and 5.1.2). Medium 29 produced the maximum number of plants that survived. There was apparently no difference in media 10, 13 and 28 on plant survival (Figure 5.1.2). Medium 28 produced more total number of shoots as compared with media 10 and 13, but abnormal shoots on medium 28 failed to survive when transferred to rooting media.

5.1.4. REGENERATION FROM GLASSHOUSE-GROWN LEAF EXPLANTS.

The leaf discs were taken from leaves harvested from glasshouse grown plants. All the other experimental conditions and methodology were exactly similar to the previous experiment described above. Data was collected after 10 weeks and subjected to analysis of variance to investigate the statistical difference for regeneration ability for the different types of media (Table 5.1.1) under study.

Statistical analysis (Appendix 5.1.3 and 5.1.4) revealed highly significant differences among the media for total shoot production and number of plant survived for both ($P<0.01$). Figure 5.1.4 indicates that that maximum number of shoots were produced on medium 13 whereas medium 10 produced all abnormal shoots (Figure 5.1.3). It was also indicated (Figure 5.1.4) that the maximum number of plants that survived were found on medium 13, while medium 29 produces second highest.

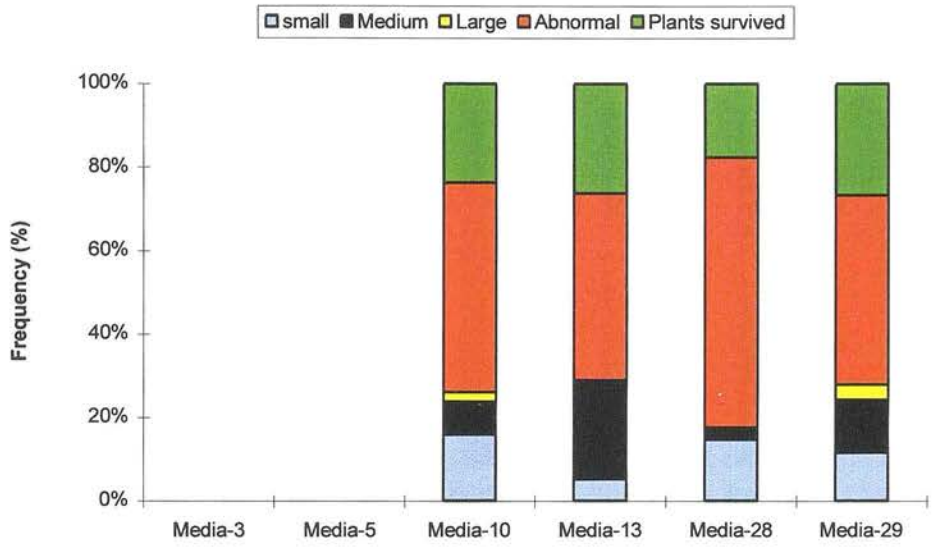


Figure 5.1.1. Relative frequency of different categories of shoots produced from each leaf disc derived from in vitro-grown leaf explant. N=15

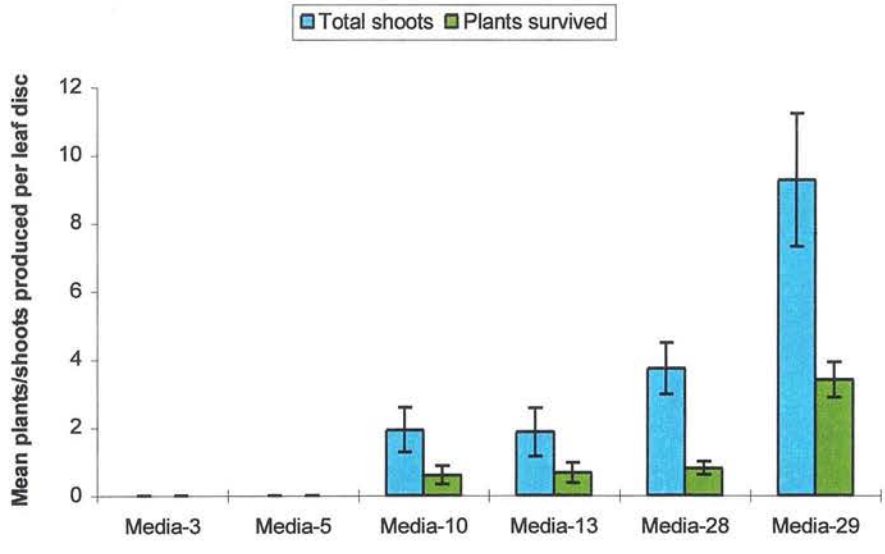


Figure 5.1.2. Mean number of shoots / plants survived produced from each leaf disc derived from in vitro-grown leaf explant on media differing in concentration and types of cytokinin and auxin (Table 5.1.1).

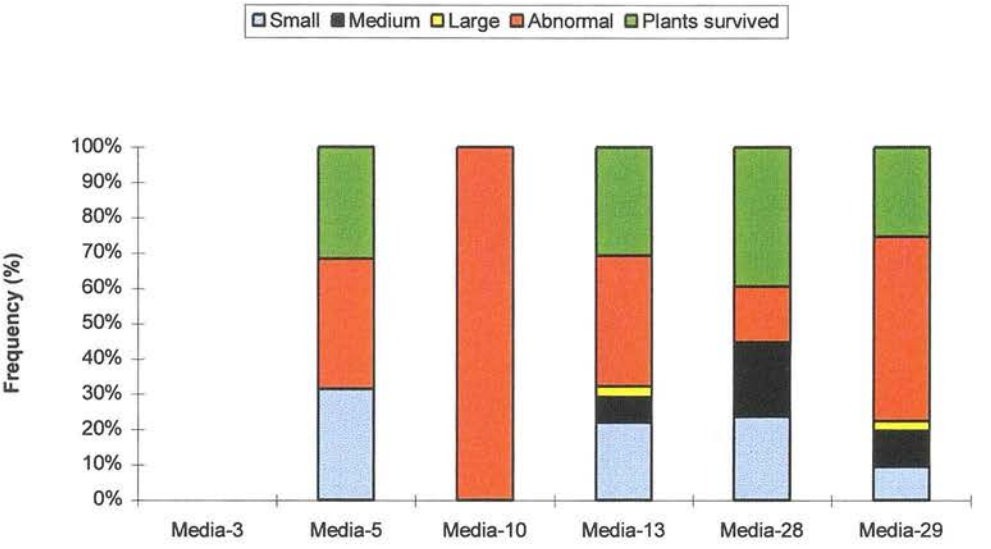


Figure 5.1.3. Relative frequency of different categories of shoots produced from each leaf disc derived from glasshouse-grown leaf explant. N=15

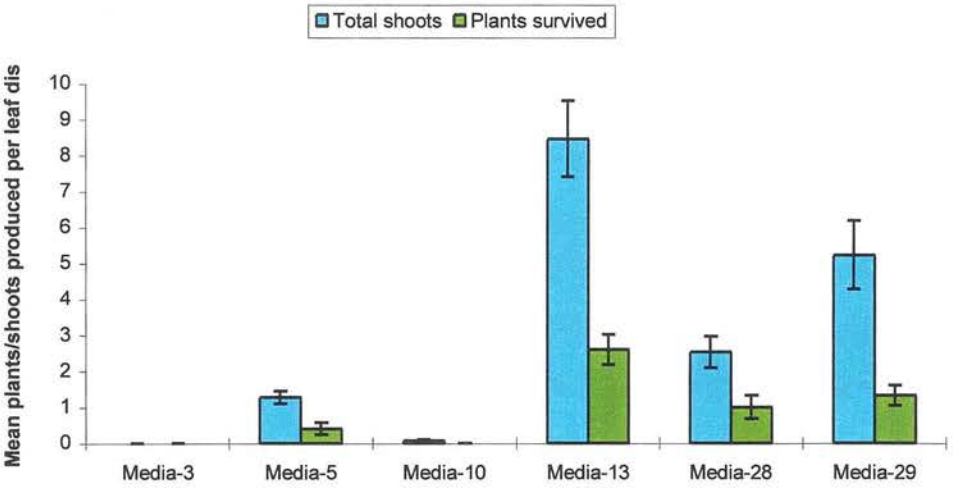


Figure 5.1.4. Mean number of shoots / plants survived produced from each leaf disc derived from glasshouse-grown leaf explant on media differing in concentration and types of cytokinin and auxin (Table 5.1.1).

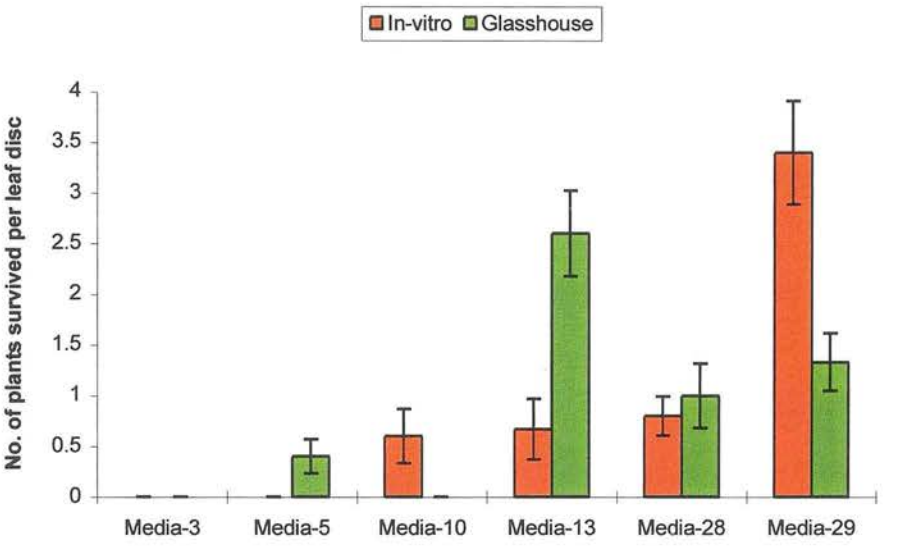


Figure 5.1.5. Effect of leaf explant source environment on the production of number of plants on different media (Table 5.2.1)

Figure 5.1.5. compares the two types of explant sources, indicating the influence on the same genotype \ cultivar of different environment growth conditions. Leaf discs taken from *in vitro* culture explants source produced maximum plants on medium 29 whereas leaf discs taken from glasshouse grown plants produced maximum plants on medium 13. In contrast to leaf discs taken from glasshouse grown plants, the leaf discs taken from *in-vitro* grown explant did not produce any plants on medium 5 but it produced some plants on medium 10 (Figure 5.1.5).

5.1.5. EVALUATION OF REGENERATION MEDIA.

From the previous two experiments maximum number of shoots and plants were produced on media 13 and 29 using glasshouse and *in vitro* explant sources respectively. No shoot initiation was observed on medium 3 containing BAP/2, 4-D 1.12/1.1 which produced a large amount of callus only. The aim of this study

was to investigate the two step plant production system producing callus on one media and transfer it to regeneration media to switch on shoot initiation from callus. The media 13 and 29 (Table 5.1.1) were used as shoot initiation media and the concentration of MS basal salt mixture was also investigated in this experiment. For this study the effect of four different media described in Table 5.1.3 on shoot initiation on callus produced using medium 3 (Table 5.1.1) were evaluated.

Table 5.1.3. Regeneration media and their composition used to regenerate shoots from callus

Media	Cytokinin	Concentration.	Auxin	Concentration.	Media concentration
R1	BAP	2.25	NAA	0.186	Full strength
R2	BAP	2.25	NAA	0.186	1/2 strength
R3	BAP	2.25	IBA	1.0	Full strength
R4	BAP	2.25	IBA	1.0	1/2 strength

The young expanded surface sterilised leaves of glasshouse grown strawberry cultivar Tango were used to cut the leaf discs as described in Section 5.1.2.1. Calli were produced by planting sterile leaf discs on initiation medium3 (Table 5.1.1) for 6 weeks followed by another 4 week period on the same fresh medium. After 10 weeks, calli were harvested and four small pieces of callus randomly placed in each Petri dish with five replications for four weeks. This was followed by another four weeks on the same fresh regeneration medium. After a total 8 weeks remaining on regeneration medium, buds and shoot formation were noted and shoots were transferred to the rooting medium (containing no plant growth regulators) for 6 weeks or until sufficient roots were formed. Regenerated plants with sufficient roots were transferred to the mixture of 50:50 perlite and compost. Plants were kept in hardening condition for four weeks as described in section (2.3.4) and then transferred to 9 cm plastic pots containing compost and watered regularly. A

factorial combination of treatments in a completely randomised design was used. Shoots produced were noted as described in section 5.1.2.3.

Statistical analysis (ANOVA) for total shoots produced and total number of plants that survived revealed highly significant difference (Appendix 5.1.5 $p<0.001$and 5.1.6 $p<0.01$ respectively) among the regeneration media. Figure 5.1.6. describes the relative frequency of the different types of shoots produced on each media. Maximum abnormal shoots (47%) were produced on R1 medium with R4 medium producing the 2nd largest number of abnormal shoots (42%). There was no difference in the total number of shoots produced on media R3 and R4 (Figure 5.1.7). However, medium R3 produced maximum the number of plants that survived possibly because the frequency of abnormal shoots production on this media was lower (38%) in contrast to medium R4 (42%) (Figure 5.1.6).

The shoots regenerated at different periods in culture, 10 and 18 weeks, were easily rooted on hormone-free MS basal medium. The rooted plantlets were transplanted to the glasshouse as described in Section 2.3.5 with 90-100% survival. The regenerants were evaluated morphologically under glasshouse conditions for evidence of uniformity. In addition RAPD-PCR analysis of the regenerants was carried out to investigate the genetic uniformity of the regenerated plants.

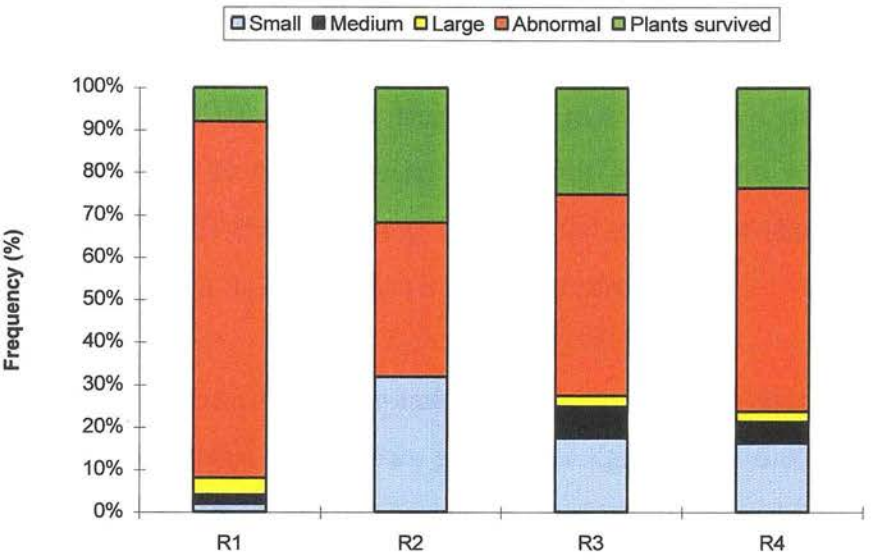


Figure 5.1.6. Relative frequency of different categories of shoots produced on regeneration media (Table 5.1.4) from each calli piece initiated on media-3 Table 5.1.1)

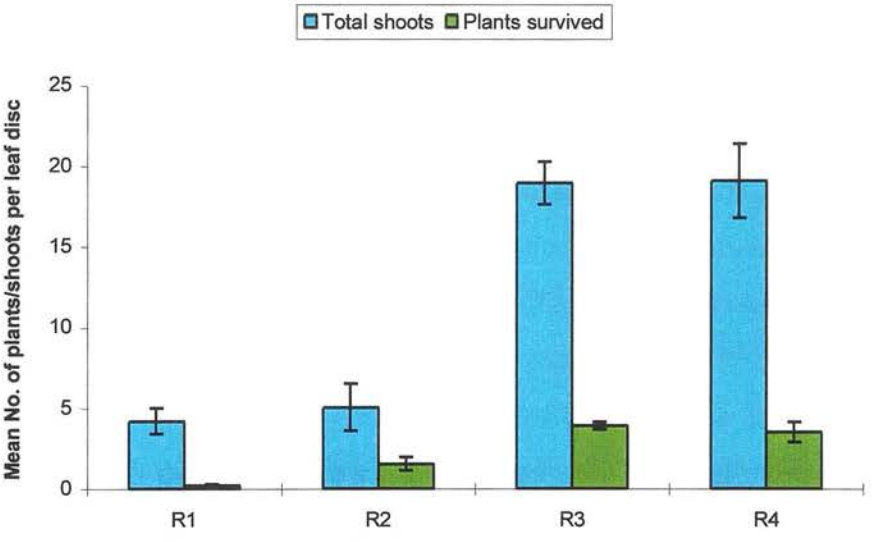


Figure 5.1.7. Mean number of shoots / plants survived on different regeneration media (Table 5.1.4) produced from each piece of calli initiated on media-3 Table 5.1.1.

5.1.6. DISCUSSION

Various combinations of cytokinin (BAP) and auxins (2, 4-D, NAA, IBA) were tested in preliminary experiments. BAP 2.25 mg\l in combination with 0.18 and 1.0 mg\l with NAA and IBA respectively gave maximum shoots regeneration. Media containing 2, 4-D produced only callus at 1/1 ratio with BAP while at higher BAP/2, 4-D ratio produced a substantial number of shoots in glasshouse-grown leaf explants only (less than 0.5 per leaf disc).

The organogenic potential of explants from *in vitro* culture shoots compared with those taken from glasshouse-grown plants was significantly different for the same media. In general, the calli from *in vitro* leaf explants exhibited higher regeneration frequency than those induced from glasshouse-grown. These results are similar to those obtained in various other studies (Liu and Sanford, 1988; Jones et al., 1988, for strawberry and Cousineau et al., 1991, in raspberry). These workers found that different concentrations of growth regulators were necessary to optimise regeneration from both types of explants. In the *in vitro* explants the high regeneration frequency may be due to their adaptation to *in vitro* environments. Maximum shoot regeneration from *in vitro* was found on regeneration media-29 containing BAP/IBA 2.25/1.0 mg\l whereas, glasshouse-grown explants gave maximum shoots on medium 13 containing BAP/NAA 2.25/0.1 mg\l. The *in vitro* shoots in this study were maintained on BAP/IBA 1.0/1.0 mg\l prior to explant preparation. Thus, the poor shoot regeneration on leaf explant taken from *in vitro* shoots may possibly be due to a hormonal effect between NAA and IBA interaction caused by accumulation of IBA in the leaf tissue.

No significant differences were found between full and half strength MS basal salt mixture for regeneration whereas incorporation of auxins produced significant differences. Media R3 and R4 containing BAP/IBA (Table5.1.3) showed good shoot regeneration from the callus produced from glasshouse grown leaf explants on medium 3.

5.2. ANALYSIS OF GENETIC STABILITY OF PLANTS REGENERATED FROM LEAF DISC CALLUS OF STRAWBERRY BY USING RAPD-PCR TECHNOLOGY.

5.2 1. INTRODUCTION

Traditionally, the genetic stability of regenerants has been evaluated by morphological means. The first disadvantage of this approach is that the possession of a normal phenotype is no guarantee that more cryptic changes have not occurred. Secondly, many of changes may be recessive and consequently do not appear until plants have been selfed and progeny examined (Gobel et al., 1985), not always possible to achieve with some species. The third difficulty is the length of time required to discover whether phenotypic changes develop i.e. from small explant to fully matured flowering plant. Depending on the species, karyological analysis reveals only significant chromosomal changes such as alterations in ploidy level as well as structural rearrangements (Karp and Bright, 1985; Karp, 1991). However, chromosomal examination cannot reveal alteration in individual genes. Isozyme, protein electrophoresis and high performance liquid chromatography of protein can provide a relatively convenient method for examining biochemical changes but these techniques are limited in the amount of polymorphism detectable among closely related genotypes.

Restriction fragment length polymorphism (RFLP) markers are useful for sampling various regions. However, they are time consuming, costly, require large amount of plant tissue and involve the use of radioactive hazardous chemicals. Also the result of such an analysis is limited only to the gene sequence used as probe. As no particular sequence has yet been identified as being directly involved with somaclonal variations, the relevance of this technique is severely restricted (Brown et al., 1993). The development of the polymerase chain reaction is one of the major technical advances in molecular biology in recent years The polymerase chain reaction (PCR) can amplify polymorphic DNA in conjunction with random

ten base primers (Williams et al., 1990). This technique allows differences at the DNA level to be detected by using a small amount of template DNA for amplification, under a specific set of conditions to generate RAPDs markers. This study aims to evaluate the level of genetic variability of plants regenerated on one combination of medium, against a parental norm.

5.2.2. MATERIALS AND METHODS.

5.2.2.1 PLANT MATERIAL

Five groups of regenerated plants were produced to evaluate the different environmental influences on genetic uniformity of regenerated plants. Group 5 consisted of 34 micropropagated plants all at 3rd subculture stage to be used as a control to compare with the plants regenerated via callus phase Section 4.4. Groups 1 and 2 were both produced on medium 29 (Table 5.1.1) initially for 6 weeks followed by another four weeks on the same fresh medium. The shoots appearing on leaf discs were transferred to rooting media (hormone-free MS basal salt mixture). The only difference between group 1 and 2 was their explant origin. In group 1 callus was initiated from micropropagated leaf explant at 3rd subculture stage and group 2 leaf explant source was glasshouse-grown plants. Groups 3 and 4 were derived from callus initiated from glasshouse grown plants (6 weeks on initiation media followed by another 4 weeks on the same fresh medium and transferred to regeneration medium R3 (Table 5.1.3). In group 3 callus was initiated on medium 3 which produced only callus on initiation medium. Medium 29 (Table 5.1.1) showed callus and shoot regeneration on initiation media which was used to initiate callus for group 4. All the other conditions including regeneration medium were the same. Leaf explants were taken as described in Section (2.2.4). Due to time constraints it was only possible to examine variability in the control genotypes and from group 4. All the 24 plants analysed in group 4 were siblings regenerants and were not the successive subculture/generations.

5.2.2.2. DNA EXTRACTION:

DNA was extracted by the methods described Doyle and Doyle (1987), with a slight modification as described in Section (2.5.2). To confirm the successful extraction, the DNA was visualised after electrophoresis and subsequently stained in ethidium bromide (Section 2.6.2). DNA of any sample showing smearing or unclear band was discarded and the extraction was repeated. After the extraction DNA samples were quantified using spectrophotometers (Section 2.5.3). All the samples were diluted to make the final concentration 10 ng / µl. Four µl of the DNA solution were used in each 50 µl of a total volume of PCR reaction, as template DNA.

5.2.3. RAPD (PCR) CONDITIONS

A total of 28, 10-base random primers were screened using parent DNA. Of these, 8 primers (Table 5.2.1) were chosen on the basis of providing good sharp results as well as their ability to amplify more DNA fragments. Primers that amplify more DNA fragments have more annealing sites that result in more chances of detecting polymorphism.

Table 5.2.1. Primers and their base sequence used for the evaluation of genetic uniformity of plants regenerated via callus culture and regeneration in strawberry cultivar Tango

No.	Primer	Sequence
1.	86370602	5-AGACGTCCAC-3
2.	86370603	5-CTGCATCGTG-3
3.	86370605	5-TCACGGCACC-3
4.	8637060F	5-CCACCGCCAG-3
5.	86370607	5-GGTCGGAGAA-3
6.	86370609	5-CCAATCCGTG-3
7.	8637061J	5-GGAGCTTGAC-3
8	86370611	5-CTGACCAGCC-3

PCRs were carried out for amplification of DNA. The techniques of William et al. (1990) were adopted with slight modifications for PCR amplification of the DNA samples (Section 2.6.6). All other amplification procedures were exactly the same.

5.2.4. RESULTS

The callus produced on this media was greenish with pink areas as shown in Figure 5.2.1 and 5.2.2. Some buds and shoots were formed at 6 week old callus and those of reasonable size were directly transferred to the rooting medium, small shoots and buds were left and transferred to the fresh medium. When callus was transferred to the regeneration media all the buds and shoots were removed and only the callus was transferred. Other plants transferred directly to the rooting medium after 6 weeks and 10 weeks were not used in this study. The plants produced from callus culture and regeneration that were used in this analysis looked morphologically very similar to each other as shown in the Figure 5.2.1.

Parent explant source and 24 regenerated plants were screened using 8 primers as described above. Each primer yielded a wide array of strong and weak bands (Figure 5.2.4) Only reproducible fragments with intense bands were scored in this study, which generates a total of 78 bands. Thus 1560 bases were explored in this experiment and any possible changes in 1560 nucleotides will have a chance to be detected. Similarity and variations observed in the regenerants are shown in Table 5.2.2., which shows the range of similarity 0.99 to 0.77 and variation 0.64 to 22.83. Four regenerated plants (16%) showed more than 10% variation and 10 plants (40%) more than 5% while 14 plants (56%) showed less than 5% variation to the explant source.

Figure 5.3:1 Strawberry plants regenerated via callus culture from which DNA was extracted for RAPD-PCR analysis shows no morphological difference

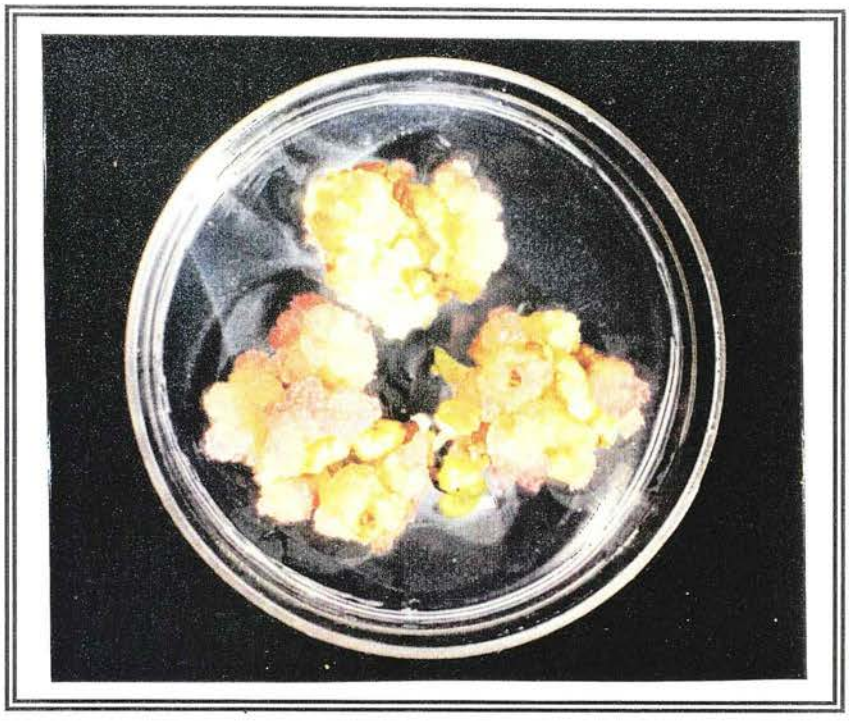


Figure 5.2:2 Callus produced from leaf disc of strawberry plants CV. Tango

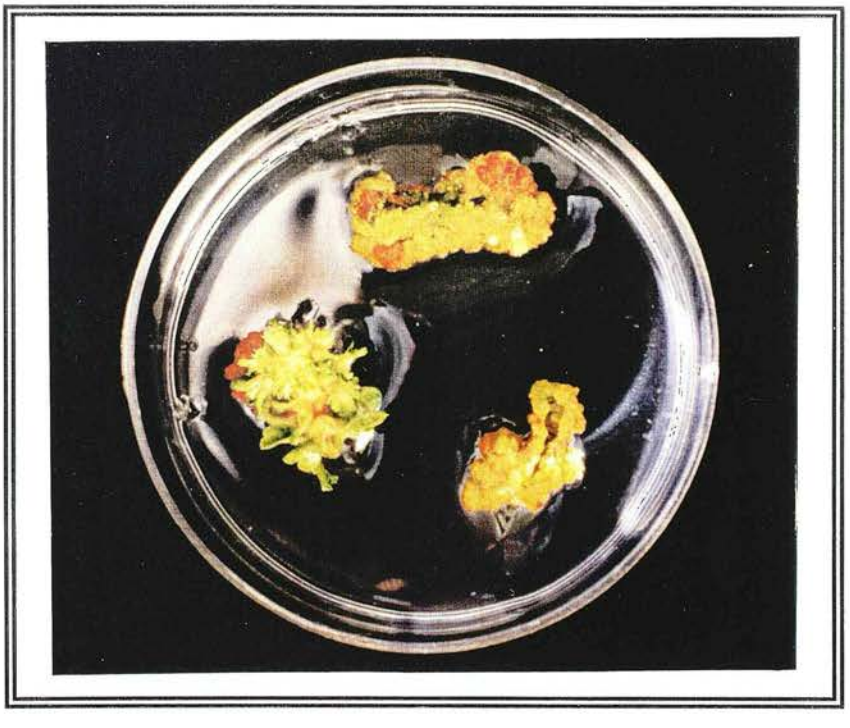


Figure 5.3:3 Plants regenerated from callus of strawberry cultivar Tango

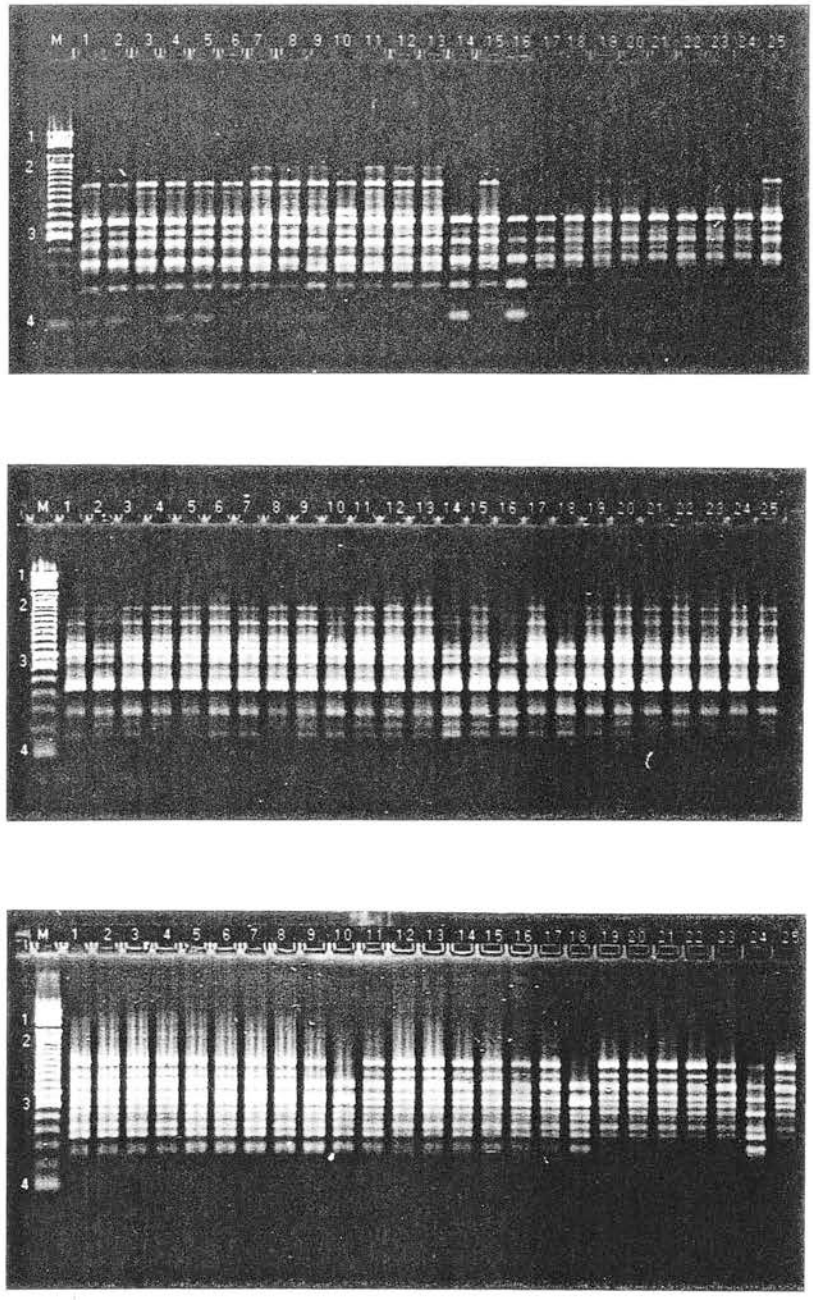


Figure 5.4.1:. Amplification products derived from 24 plants of strawberry cultivar Tango using random 10 base primer A primer No.1. B. primer No.6 and C. primer No. 7. From left to right M represents 100 base pair nucleic acid markers bp represents fragments sizes of the DNA marker in base pairs. From 1-25 are strawberry regenerants except no.5 which is parent explant source

Table 5.2.2. Results of genetic uniformity of plants regenerated via callus culture and regeneration in strawberry cultivar Tango, estimated from RAPD-PCR data generated using 8 primers

Samples No.	Similarity	Variations(%)
1	0.924	7.58
2	0.916	8.33
3	0.993	0.645
4	0.993	0.645
5	1.00	0.00.Parent
6	0.993	0.645
7	0.993	0.645
8	0.993	0.645
9	0.966	3.311
10	0.840	15.942
11	0.987	1.282
12	0.987	1.282
13	0.993	0.645
14	0.909	9.090
15	0.917	8.219
16	0.771	22.834
17	0.96	4.00
18	0.861	13.868
19	0.938	6.122
20	0.953	4.697
21	0.953	4.697
22	0.96	4.00
23	0.939	6.040
24	0.879	12.056
25	0.960	3.947

5.2.5. DISCUSSION.

In order to exploit callus as a source of new plant material, it must produce healthy, well developed and true to type planting material. If it does not alternatively it can be used as a source of genetically variable plants for future selection.

In this study all the plants regenerated were genetically different from their parent explant source, however 56% of the total plants analysed were, found to have less than 5% variation as compared with the parent. Many workers have reported the existence of somaclonal variation among plants regenerated from different callus and detected using different techniques. Damiano et al. (1995) regenerated plant from callus culture derived from anther filaments and observed somaclonal variation using isozyme pattern. Swartz et al. (1981), Anderson et al. (1982) and Kondokova et al. (1983) described the existence of somaclonal variation in regenerated plants detected using morphological techniques. Niemirowicz (1990) described that plants regenerated from anther callus gave rather low yields as compared to standard material and no plant even reached the lowest yielding standard variety used. It was also observed that out of 93% plants which bore fruits 16% produced inviable seed after open pollination and about 50% of those fruiting plants showed very low fertility, and can practically be considered as non-fertile. Nehra et al. (1990) also reported somaclonal variants produced by leaf derived callus culture and regeneration. They further described the possible cause of variation which may be the use of sub-optimal media as high concentration of BAP and increased number of sub-culture before regenerating plants initiates somaclonal variation. The level of variability observed in this study was much higher than that observed by other workers using morphological techniques. This was not surprising and was expected because RAPD-PCR sampled the genome under study most probably randomly without any variation due to ontogenic expression. In the total nuclear DNA a large part of it is simple repetitive thus any mutation occurring in this region cannot be detected using morphological techniques. It has been established by a number of workers that RAPD analysis has potential in the

establishment and estimation of somaclonal variation (Valles et al., 1993; Guzukirmizi et al., 1993). This study supports the view of existing variation at molecular level in the plants regenerated from callus culture and regeneration

In commercial tissue culture off-types individuals are undesirable and are thrown away. It seems, however, that the selection of the varities showing the greatest somaclonal variation would be of interest, but it is necessary to choose the best varities from the point of view of traits to be maintained in future breeding. The focus of most tissue culture studies has been on standardisation of optimal conditions for *in vitro* morphogenesis. However, the conditions that are optimal for plant regeneration may not be optimal for maintaining the genetic integrity of a genotype. Most of the workers concentrate on optimum ratio between cytokinin and auxins. In practice it might work better if concentrate on a narrow range of absolute values instead of the ratio between these hormones.

On the basis of results obtained in this study and in Section 4.3 it is worthwhile mentioning that plants regenerated both from meristem and from callus can undergo genetic changes. Indeed such changes are more likely to occur in a sexually propagated polyploid crop species as they are buffered against otherwise lethal mutation. This study suggests that using callus tissue culture as a means of mass propagation should be carried out with caution and further work on optimisation of absolute concentration of cytokinin and auxin should be carried out in relation to the uniformity of regenerants. Because only micro-amounts of material are necessary, RAPD-PCR can be used to assess each stage of *in vitro* culture. Large sample sizes can be treated rapidly and the technique lends itself to automation (William's et al., 1990; Welsh et al., 1991; Hedrick, 1992).

Indeed results obtained in the study of plants regenerated via micropropagation showed a higher level of variation as compared to the level of variation detected in plants regenerated via callus culture and regeneration. This sort of result was unexpected because micropropagation is considered relatively safe in comparison

with callus culture and regeneration. The actual cause of this result is unknown and cannot be explained by the present study but it indicates the need for further study in this field with more improved methods of detecting variation.

5.3. CALLUS CULTURE AND REGENERATION IN BLACKCURRANT

5.3.1. INTRODUCTION

Success or failure of any programme involving *in vitro* culture depends on some basic tissue culture techniques. These techniques may be specific to genotype or species. Therefore establishment of basic culture technique for each genotype is a pre-requisite for any tissue culture programme. There are many factors which may affect any tissue culture programme, positively or negatively. Decisions are mainly based on the choice of the workers, facilities available, need of the special requirement of the genotype under study. Some of the factors which can affect a tissue culture system are explant type, source, age, size, and sterilisation techniques used, nutrient media, macro and micro nutrients, plant growth regulators their types and concentration, culture conditions and culture time etc. For individual species and genotypes, all the above mentioned factors need to be investigated thoroughly by experimentation. However, this may require a long time, and with the limitation of time available it was not possible to investigate all the possible variables in this study. Most decisions were made on the basis of facilities available and previous work done on *Ribes* species and some other related soft fruit species. Only a few factors, considered essential were examined. The result of these experiments will be presented in this chapter.

The main aim of this study was to develop an appropriate callus culture and regeneration system for the *Ribes* species in order to investigate the potential of this approach for rapid multiplication and then to evaluate the genetic integrity of regenerated plants by using suitable molecular markers. Emphasis focused on the selection of optimum concentrations of auxin and cytokinin for callus initiation and subsequent root and shoot formation.

In the literature, only three groups of researchers have used different auxins and cytokinins. The combinations of auxins and cytokinin used successfully in different

studies involving *Ribes* species were 2, 4-D, BAP, NAA:Kinetin (Joersbo et al., 1992). IAA and kinetin (Harvey and Grasham, 1977). IBA and 2, 4-D with BAP (Graham and McNicol, 1991). From this study it was clear that most of the commercially available auxins and cytokinin can be used successfully but there was no report published about the regeneration of plants from leaf disc callus culture and regeneration. A leaf disc regenerating system is required in order to achieve mass multiplication of the plants. A number of different plant growth regulators combinations were investigated using two cultivars of blackcurrant Ben Sark and Ben Lomond, but both cultivars tested gave generally similar results. Therefore in this chapter only the results obtained using Ben Sark cultivars were presented.

5.3.2 EFFECT OF NAA AND BAP ON CALLUS INITIATION FROM LEAF DISC EXPLANT OF BEN SARK.

The aim of this experiment was to establish a suitable range of NAA and BAP for callus initiation using leaf disc as explant. Sixteen different media tested in this experiment differed only in their growth regulator concentrations (Table 5.3.1). Explant preparation and sterilisation methods were exactly similar to those described in Sections 2.2.3, 2.24 and 2.25. The range and type of growth regulators was decided on the basis of a previous paper (Joersbo et al., 1992).

Table 5.3.1. Combinations of NAA/BAP added to culture media. All figures are in mg\l

NAA / BAP	0	2.5	3.5	4.5
0	0/0	0/2.5	0/3.5	0/4.5
1	0/1	2.5/1	3.5/1	4.5/1
2	0/2	2.5/2	3.5/2	4.5/2
3	0/3	2.5/3	3.5/3	4.5/3

The experimental design used was 4x4 factorial (five replications). After six weeks of initiation, the callus was scored as described in Section 2.3.3 and subcultured on fresh media for another four week The data was transformed and analysed using standard Analysis of variance. ANOVA revealed highly significant differences (s.e.d = $\pm$ 0.128) $p<0.01$ among the level of NAA and $p<0.001$ among the levels of BAP and interactions Appendix 5.3.1.

Figure 5.3.1 shows that NAA alone is necessary for good callus growth. There was no callus initiation at all when the concentration of both NAA and BAP was zero or when the NAA is absent. Maximum callus initiation in this experiment was on the medium with 3.5 mg\l of NAA with zero BAP.

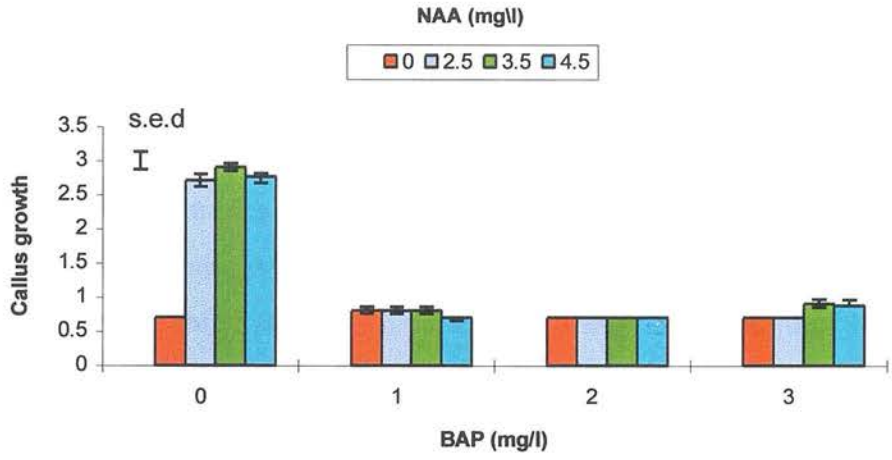
5.3.3. EFFECT OF NAA AND BAP ON CALLUS INITIATION FROM LEAF PETIOLE EXPLANT OF BEN SARK.

The aim of this experiment was to find out the optimum range of NAA and BAP for callus initiation of Ben Sark cultivar using leaf petiole as an explant source. On the same media described in Table 5.3.1 All other conditions were exactly similar to the previous experiment 5.3.1 except the explant source where leaf discs were

replaced with leaf petiole. After six weeks of incubation the calli were scored and data was analysed using standard statistical techniques. Analysis of variance revealed highly significant differences among the level of hormones, individually as well as in interaction Appendix 5.3.2 (s.e.d. ± 0.194, $p < 0.001$).

Figure 5.3.2 again shows that NAA alone is necessary for callus initiation and growth, from blackcurrant cultivar Ben Sark leaf petiole explant. Again no callus initiation occurred at all when NAA was absent. The maximum callus initiation in this experiment was again on medium with 3.5 mg\l of NAA and zero BAP. From the result of experiments it was clear that cytokinin (BAP) is not necessary for callus initiation in both types of explant sources. In addition plant growth regulators requirements of leaf disc explant source and leaf petiole explant source is similar. Both give excellent callus growth at 3.5 mg\l of NAA without any cytokinin (BAP). When BAP was added to the medium it severely reduced the callus growth. Therefore it was decided that in further experiments examining other plant growth regulators would be performed on leaf discs only. The results would be applied to both explant sources.

(a).



(b).

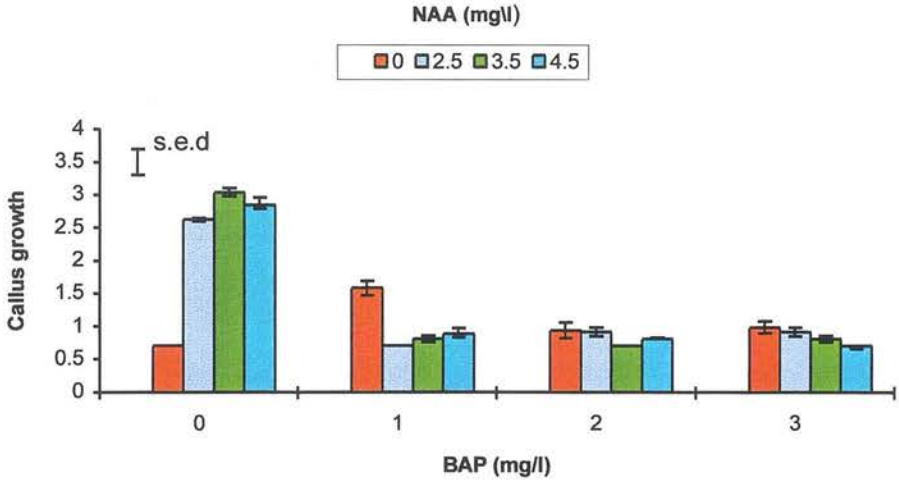


Figure 5.3.1. Influence of different levels of NAA and BAP on callus initiation of blackcurrant cultivar of Ben Sark. Data collected after six weeks of incubation. Mean ±SEM. N=5.

(a) Effect of NAA and BAP using leaf disc explant source for callus initiation

(b) Effect of NAA and BAP using leaf petiole explant source for callus initiation

5.3.4. EFFECT OF DIFFERENT TYPES AND LEVEL OF AUXINS AND THE LEVEL OF BAP AS CYTOKININ ON CALLUS INITIATION FROM LEAF DISC EXPLANT OF BEN SARK.

Callus initiation on leaf disc of cultivar Ben Sark was investigated using two auxins, IBA and BAP, with different levels of BAP as cytokinin. The new range of BAP was selected on the basis of results of the previous experiments in this section. New concentrations studied ranged from 0.0-0.6 mg\l of BAP with 0.0-4.5 mg\l of IBA (Table 5.3.2), BAP 0.0-0.3 mg\l and 0.0-6.0 mg\l of IAA (Table 5.3.3).

Table 5.3.2. Combinations of IBA/BAP added to culture media. All figures are in mg\l

IBA / BAP	0	2.5	3.5	4.5
0	0/0	0/2.5	0/3.5	0/4.5
0.2	0/0.2	2.5/0.2	3.5/0.2	4.5/0.2
0.4	0/0.4	2.5/0.4	3.5/0.4	4.5/0.4
0.6	0/0.6	2.5/0.6	3.5/0.6	4.5/0.6

Leaf disc explants were excised, placed on the surface of media and pressed gently to the surface of media. Three leaf discs were cultured in each Petri dish, and each Petri dish was considered as a single treatment. A total of 5 replications were used for each treatment and all the Petri dishes were randomised in the culture room. Cultures were observed weekly and callus growth scores following transformation were subjected to statistical analysis.

Table 5.3.3. Combinations of IAA/BAP added to culture media. All figures are in mg\l

IAA / BAP	0	2	4	6
0	0/0	0/2	0/4	0/6
0.1	0/0.1	2/0.1	4/0.1	6/0.1
0.2	0/0.2	2/0.2	4/0.2	6/0.2
0.3	0/0.3	2/0.3	4/0.3	6/0.3

5.3.4.a. EFFECT OF IBA AND BAP

Analysis of variance revealed highly significant differences among the levels of hormones individually as well as for interaction (s.e.d. 0.151, $p<0.001$) Appendix 5.3.3. Maximum callus growth was observed on the medium with 3.5mg\l of IBA in the absence of BAP Figure 5.3.2.a. There was good callus growth on 3.5 mg\l of IBA with the combinations of 0.2 and 0.4mg\l BAP but on the 0.6 mg\l of BAP growth was decreased and the colour of callus turned in brownish, while on 0.2 and 0.4 mg\l BAP with the combination of 3.5 mg\l of IBA green and compact callus growth was observed. The presence of BAP at 0.2 and 0.4 mg\l levels slightly improved the greenish colour and compactness of the callus as compared to the callus growth at IBA alone callus growth was slightly slower.

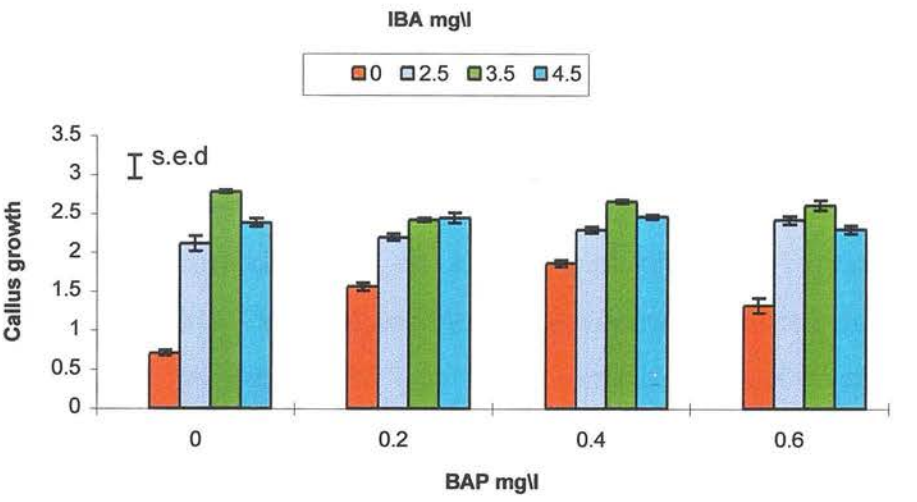
5.3.4.b. EFFECT OF IAA AND BAP

In this experiment IBA was replaced by IAA at slightly higher concentration and the BAP concentration used ranged from 0.0-0.3 (Table 5.3.3). Callus growth was scored after six weeks of initiation and data subjected to statistical analysis. Analysis of variance revealed highly significant differences among the levels of hormones individually and in interaction (s.e.d. $= \pm 0.195$ $p<0.001$) Appendix 5.3.4. Maximum callus growth was observed on 4mg\l of IAA. On 2mg\l of IAA

there was reasonable growth but the callus was of brownish colour. Presence of BAP at 0.1 mg\l slightly increased the callus growth (Figure 5.1.2 b).

From an overall review of experiments performed up to this stage it was clear that the blackcurrant cultivar Ben Sark does not require any cytokinin for callus initiation, only auxins. The requirement of plant growth regulators for both leaf discs and leaf petioles explant was the same. BAP in very low concentrations such as 0.1, 0.2, 0.3 and 0.4mg\l with the combinations of auxins at 2.5 and 3.5 mg\l gave slightly less but more greenish and compact callus. Callus arising through NAA alone and combinations with BAP was strongly green and compact as compared to callus arising through IBA and IAA alone and in combinations with BAP. It was also observed in all the subsequent experiments that callus grew only from leaf veins present in leaf discs and not from the cut surface.

(a)



(b)

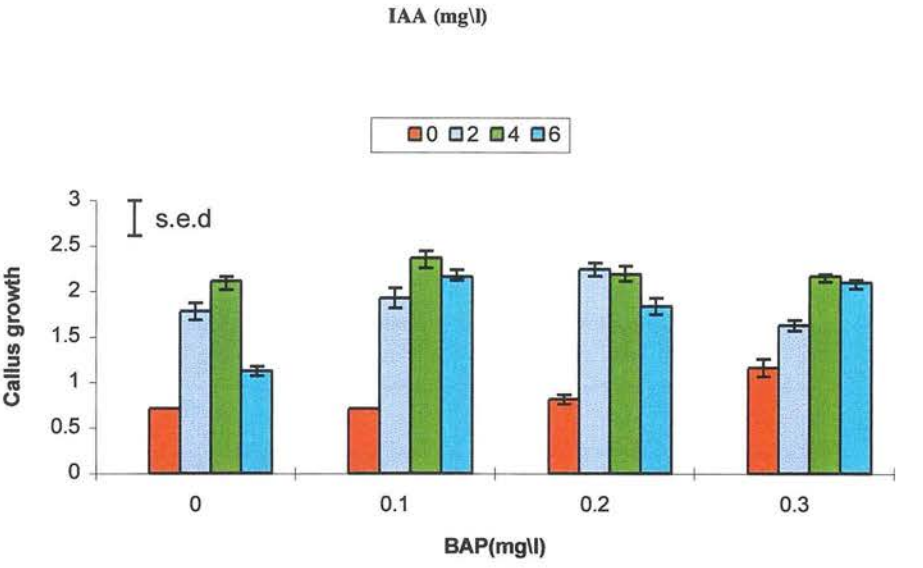


Figure 5.3.2. Influence of different levels of IBA, IAA and BAP on callus initiation and subsequent callus culture of blackcurrant cultivar of Ben Sark. Data collected after six weeks of incubation. Mean ±SEM. N=5.

(a) Effect of IBA and BAP using leaf disc explant source for callus initiation

(b) Effect of IAA and BAP using leaf disc explant source for callus initiation

5.3.5. INVESTIGATING THE REGENERATION OF PLANTS FROM CALLUS

Following the optimisation of the level of growth regulators for callus initiation, the next requirement of the project was the optimisation of the level of auxins and cytokinin for shoot regeneration in the blackcurrant cultivar Ben Sark under study. Out of the three reported works on *Ribes* regeneration, two indicated shoot formation on BAP alone, both root and shoot formation on media containing auxins and cytokinin on 1:1 ratio while very small number of shoot formation was observed on media containing auxin alone (Joersbo et al., 1992; Graham et al. 1991) However, Harvey et al. (1977) suggested, contradictory to others, that root formation at low auxin/kinetin ratio, both root and shoot formation at intermediate ratio, and shoot formation at high ratio. It was decided that 3.5 mg\l of NAA, IBA and IAA with the combinations of 0.0, 0.2, and 0.4mg\l of BAP will be used to multiply the callus for regeneration purpose using two different types of explant sources i.e. leaf disc and leaf petiole in Ben Sark cultivar of blackcurrant. (Table 5.3.4).

Table 5.3.4. Combinations of plant growth regulators used to initiate callus for regeneration

BAP / Auxins	0	0.2	0.4
NAA	C1	C2	C3
IBA	C4	C5	C6
IAA	C7	C8	C9

On the basis of literature available on *Ribes* and other related literature, a programme of experiments was carried out to investigate the appropriate level of auxins and cytokinin for shoot regeneration. For this experiment 0.1, 1, 5 and 10 mg\l of IAA with combinations of 0.1 1.0 and 10mg\l of kinetin were used (Table

5.3.5) to initiate shoot regeneration from the callus produced on nine different media as described in Table 5.3.4. While studying the regeneration in tissue culture it was not possible to measure the regeneration quantitatively. Therefore the regeneration was measured as described. -= no growth at all and + = regeneration started, ++ = fair +++ = good and ++++ = ample growth.

Table 5.3.5. ***Effects of growth regulators on callus growth and redifferention of leaf disc explant of blackcurrant cultivar Ben Sark.***

Callus initiation	Regeneration media mg\l				Callus	Callus initiation	Regeneration media mg\l				Callus
Media	IAA	Kinetin	Shoots	Roots	growth	Media	IAA	Kinetin	Shoots	Roots	growth
C1	0	0	-	++++	++++		5.0	0.1	-	++	++
	0.1	0.1	-	-	+			1.0	-	++	++
		1.0	-	-	-			10.0	-	-	--
		10.0	-	-	-		10.0	0.1	-	+++	++++
	1.0	0.1	-	+	+			1.0	-	++	+++
		1.0	-	-	+			10.0	-	-	+
		10.0	-	-	+	C6	0	0	-	++	++++
	5.0	0.1	-	++	+++		0.1	0.1	-	-	+
		1.0	-	++	++			1.0	-	-	+
		10.0	-	-	--			10.0	-	-	-
	10.0	0.1	-	+++	++++		1.0	0.1	-	-	+
		1.0	-	++	++++			1.0	-	-	+
		10.0	-	-	++			10.0	-	-	-
C2	0	0	-	+++	++++		5.0	0.1	-	+	++
	0.1	0.1	-	-	+			1.0	-	+	++
		1.0	-	-	+			10.0	-	-	-
		10.0	-	-	-		10.0	0.1	-	++	++++
	1.0	0.1	-	-	+			1.0	-	+	+++
		1.0	-	-	+			10.0	-	-	-
		10.0	-	-	-	C7	0	0	-	++	++++
	5.0	0.1	-	+	++		0.1	0.1	-	-	++
		1.0	-	+	++			1.0	-	-	-
		10.0	-	-	-			10.0	-	-	-
	10.0	0.1	-	++	++++		1.0	0.1	-	-	++
		1.0	-	+	+++			1.0	-	-	+
		10.0	-	-	-			10.0	-	-	-
C3	0	0	-	++	++++		5.0	0.1	-	++	++++
	0.1	0.1	-	-	++			1.0	-	+	++++
		1.0	-	-	-			10.0	-	-	-
		10.0	-	-	-		10.0	0.1	-	+++	++++
	1.0	0.1	-	-	++			1.0	-	++	+++
		1.0	-	-	+			10.0	-	-	++
		10.0	-	-	-	C8	0	0	-	++	++++
	5.0	0.1	-	++	++++		0.1	0.1	-	-	++
		1.0	-	+	++++			1.0	-	-	-
		10.0	-	-	-			10.0	-	-	-
	10.0	0.1	-	+++	++++		1.0	0.1	-	-	++
		1.0	-	++	+++			1.0	-	-	++
		10.0	-	-	++			10.0	-	-	-
C4	0	0	-	++	+++		5.0	0.1	-	+	+++
	0.1	0.1	-	-	++			1.0	-	-	+++
		1.0	-	-	-			10.0	-	-	+
		10.0	-	-	-		10.0	0.1	-	+++	+++
	1.0	0.1	-	-	++			1.0	-	+	+++
		1.0	-	-	++			10.0	-	-	++
		10.0	-	-	-	C9	0	0	-	++	++++
	5.0	0.1	-	+	+++		0.1	0.1	-	-	+
		1.0	-	-	+++			1.0	-	-	-
		10.0	-	-	+			10.0	-	-	-
	10.0	0.1	-	+++	+++		1.0	0.1	-	-	++
		1.0	-	+	+++			1.0	-	-	++
		10.0	-	-	++			10.0	-	+	+++
C5	0	0	-	++	+++		5.0	0.1	-	+	+++
	0.1	0.1	-	-	+			1.0	-	-	+++
		1.0	-	-	-			10.0	-	-	+
		10.0	-	-	-		10.0	0.1	-	+++	+++
	1.0	0.1	-	-	++			1.0	-	+	+++
		1.0	-	-	++			10.0	-	-	++
		10.0	-	+	+++						

- = No growth at all + = growth started. ++ fair amount of growth +++ good growth ++++ Excellent growth

The results indicated (Table 5.3.5) that no regeneration media initiated shoots, only roots were observed. It was also observed that at higher concentrations of auxins more strong, healthy and long roots were formed as well as further vigorous callus growth. If cytokinin was added, callus growth and root formation was reduced and at yet higher concentrations (5 and 10 mg\l of kinetin) growth ceased altogether and the media changed to dark brown colour. This pattern was followed by all types calli initiated on different callus initiation media.

5.3.6. REGENERATION MEDIA

From the experiments described above it was clearly observed that callus initiated from all the combinations of hormones generally gave the same results. For the next set of experiments it was decided to use only NAA at 3.5mg\l for callus initiation because this hormone gave maximum growth of good compact and green callus. Regeneration media tried under this study were R1 with no plant growth regulators, R2 contained BAP at 1mg\l based on observation of the requirement for micropropagation, R3 contained BAP 1mg\l with 40 mg\l of adenine. Adenine was added since it has been known for some time to promote bud formation in other species (Skoog & Miller, 1957). Nitsch et al. 1967 found that cytokinin such as kinetin had little or no effect in promoting bud formations in *Plumbago indica* and pith tissue of *Nicotiana tabacum* even when the auxin level in the medium was lowered to zero. They observed that, if adenine was incorporated into the media in addition to cytokinin, abundant bud formation occurred. They reported that adenine seems to be essential, together with a cytokinin, for promoting the formation of adventitious buds on the material used. R4 contained 5mg/l gibberellic acid alone because gibberellic acid was reported successfully to regenerate plants from embryo culture in blackcurrant and redcurrant (Zatyko et al., 1979). All the above four media were used on full and half strength MS basal salt mixture. Half strength contained half dilution of recommended concentration of basal media with normal concentration of other constituents such as sugars and plant growth regulators. The results obtained were presented in the Table 5.3.6.

Table 5.3.6. ***Effects of growth regulators on callus growth and redifferention of leaf disc explant of blackcurrant cultivar Ben Sark***

Full Strength Media				Half Strength Media			
Regeneration Media	Shoots	Roots	Callus Growth	Regeneration Media	Shoots	Roots	Callus Growth
R1	-	++	++	R1	-	++	+
R2	-	-	-	R2	-	-	-
R3	-	-	-	R3	-	-	-
R4	-	-	++	R4	-	-	++

As described in the Table 5.3.6 none of the media gave shoot regeneration. The media without hormones gave roots on both full and half strength media but after six weeks, callus growth stopped. The media containing BAP turned brown along with callus after four weeks and then dark brown. After six weeks the solid media containing BAP with and without adenine subsequently turned into a brownish liquid. Death of the callus was confirmed by transferring sub sets to initiation media. All the above media were attempted on callus derived from leaf petiole and leaf disc explant sources and on B5 as well as MS media with full and half strength media. All attempts to regenerate shoots from both types of calli were unsuccessful.

5.3.7. DISCUSSION.

The aim of the different experiments described in this chapter was to select an explant source suitable for callus induction, subsequent callus culture and plant regeneration, and to optimise levels of auxins and cytokinin for callus induction, subsequent callus cultures, shoot regeneration and root formation on regenerated shoots. Results achieved from different experiments will be discussed under this section.

The first observation from these studies was that both the explant, studied, were able to induce callus. Leaf petiole gave callus from cut ends while in leaf disc callus mostly arises from the veins present in the leaf discs. Plant growth regulators requirements of both types of explants were same. Joersbo et al. (1992) obtained the similar results i.e. only auxin was required to initiate callus in *Ribes* species. It was also observed that the level of exogenous auxin required did not depended on the explant source. Cytokinin such as BAP although not necessary for callus induction, did at low concentration (such as 0.2-0.6 mg\l improve callus colour and compactness as well as retarding roots development, At higher level such as 1mg\l, BAP became harmful for callus growth.

While studying shoot regeneration from callus induced by both types of explants it was observed that high concentrations of auxins initiated root formations only while cytokinin at low concentration inhibits root formations. At higher concentration cytokinin becomes harmful for callus growth. These results are contradictory to the results obtained by Harvey et al. (1977) who observed root formations at low IAA/kinetin (0.0001/0.001) (g\l), both root and shoot formation at intermediate ratio (0.001/0.001), and shoot formation at high ratio (0.01/0.001) using explant of stem cortex. One possible explanation of this phenomenon could be that different explants may require different concentration to initiate shoot formations.

Joersbo et al. (1992) also reported shoot regeneration from callus on 1mg\l of 2,4-D, 22% of calli gave shoots and on 1mg\l of NAA and kinetin in combination, 76% calli formed shoots while on 1mg\l BAP, 97% calli gave shoots using bud explant source. This showed that it is the cytokinin which is important in the initiation of shoot. But in this study none of the regeneration media investigated, was able to initiate shoot regeneration. It might be possible that regenerative ability of different explant material is different more generally that the effects could be genotype specific.

CHAPTER 6

GENERAL DISCUSSION

6.1. INTRODUCTION

Demands for high quality virus-free material have necessitated a move away from traditional propagation systems. New cultivars with selected advantages must be propagated rapidly, in order to introduce them into commercial plantations as soon as possible. The only answer to this problem is to use highly efficient vegetative methods of propagation such as tissue culture techniques. During the last two decades tremendous progress has been made in the area of *in vitro* culture of soft fruits (Bajaj, 1986).

The problem of lack of genetic stability of soft fruit cultivars propagated by *in vitro* culture is very important. For the release of a variety, conditions related to trueness-to-type are very strict. Using tissue culture systems, with enormous potential for multiplication, the risk of multiplying an undesirable mutation is increased. The aim of this study was to investigate the effect of tissue culture regimes on the genetic uniformity of regenerated plants using appropriate markers, and to investigate the potential of callus culture and regeneration as an alternative multiplication system. Special references were given to 1) the selection of markers and their ability for cultivar identification. 2) The investigation of the effect of generation cycle/sub-culture on the genetic uniformity of regenerated plants, 3) The study of the potential of callus culture and regeneration system as an alternative propagation system and the effect of different *in vitro* conditions on the production of genetically uniform plants

6.2. SELECTION OF MARKERS

SDS-PAGE electrophoresis and RAPD-PCR were evaluated for cultivar identification. Electrophoresis of proteins is well recognised for potential cultivar identification and has many advantages over morphological techniques. In this study this technique failed to produced enough polymorphism to identify the ten blackcurrant cultivars. Therefore it was considered unlikely to be sufficiently sensitive to reveal polymorphism in the plants regenerated from micropropagated plants of a single cultivar and which are supposed to be a clone. Extensive polymorphism was obtained using RAPD-PCR among the cultivars tested. All the cultivars used could be separated using as few as two primers. The cultivar identification based on morphological assessment is subjective while RAPD-PCR assay could be a simple fast and reliable diagnostic technique for cultivar identification. Certainly it is a technique that merits further consideration for the identification of cultivars. In addition the RAPD-PCR products have value for examining the systematic relationships. This analysis gave important information regarding the blackcurrant cultivars under study. It is likely that many founding clones, although given different names, were in fact very similar in terms of their genetic contribution. It would be expected that the genetic similarity values obtained from the banding pattern would be higher in value than those obtained from parental contribution which was confirmed in this study. The latter was considered the lowest level of similarity since it ignores the fact that many early cultivars were nothing more than a supposedly superior selection from wild species. The values obtained from banding patterns are likely to be a more accurate reflection of the genetic relationship than that based on pedigree.

Indeed the use of pedigree information to indicate relationships has certain weaknesses because the parentage provided for some the genotypes are not as descriptive as desired. For example Baldwin is 150 years old and of unknown derivation, and other older types are described as open pollinated seedlings from a particular genotype, so the male lineage is effectively lost. The information

obtained using RAPD-PCR can serve to fill the gaps and to develop a clear picture of the relationships that can be of value for future breeding strategies.

All propagation systems aim to produce progeny which are identical to the mother plant clone. However, even with traditional systems of propagation, variants can arise. The oldest growing blackcurrant cultivar Baldwin, was evaluated and sub-samples were collected from different nurseries. Indeed no two samples were found identical in their banding patterns. This shows the ability of RAPD-PCR markers to differentiate the individuals within a clone, supposedly maintained as a single cultivar. This variation may be the result of mutations that occur but do not result in any change to those characters used for cultivar identification. Chimera especially bud sports, are quite well known to occur, especially in raspberry. Mutation may occur in a single bud and lead to different genotypes not recognisable as different using morphological means.

A comparative study was conducted between different gel separating and staining methods after the DNA amplification to increase the efficiency of the RAPD-PCR reaction. The results obtained indicate that the agarose gel electrophoresis followed by ethidium bromide staining gave more accurate results in keeping with the known history of the source material. The possible explanation of this could be the high sensitivity of polyacrylamide gel and silver staining. Weak and unreliable bands produced by RAPDs can be easily distinguished in agarose gel whilst following silver staining these bands are difficult to recognise and hence this affects the overall results when included in the analysis. These weak and un-reliable bands are produced in RAPD-PCR when primers bind (with reduced efficiency) to targets containing one or two mismatches. Although by discarding the weak bands, incorrect results can be avoided and reliability of results increased, this may be at the expense of additional information which may be lost as it might be not due to mismatches.

6.3. MICROPROPAGATION

Most workers use meristem culture as the favoured approach to rapid multiplication partly because of the true-to-type nature of the products. However, regenerated plants were usually evaluated using morphological methods only and very few off-types have been reported by different workers. In this study the effect of sub-culture/generation cycles on the genetic uniformity of regenerated plants was evaluated using RAPD-PCR analysis. In *Ribes* up to 14 subcultures were evaluated and all the plants were found to be 100% similar to the explant parent. On the other hand, in *Rubus,* plants regenerated up to the 4th sub-culture seemed genetically uniform and then haphazard variation was observed (Table 4.3.2). In strawberry all the plants produced at sub-culture 3 were analysed and only 5.8 % plants showed 100% similarity with parent. The nature of this variation and its appearance and disappearance in subsequent generations in *Rubus* was surprising. There could be several possible explanations. The most important factors ensuring the genetic stability of the shoot apex are probably the number of cells and their organisation into discrete tissue layers. Because mutation is a single cell event, only one tissue layer at a time is usually affected, inevitably leading to the formation of chimerical shoots, most of which are unstable and the mutant tissue is confined to a limited area in developing organs. Occasionally stable periclinal chimeras are formed when a complete layer of mutant tissue is established. A wholly mutant shoot can only result from the establishment of a sectorial chimera and the subsequent spread of the mutation to all tissue layers in an auxiliary meristem. An additional possibility is that of disruptions occurring in the layering of established periclinal chimeras, either spontaneously or as a result of the immersion of the shoot apex in liquid or agar media. The possibility of developing chimeras artificially by mixed callus cultures has been demonstrated in tobacco, adventitious shoots being produced in a chimeral arrangment from two separate species (Marcotrigiano, 1985).

Thus chimeras, either pre existing or tissue culture induced may give rise to a genetically variable population. The sampling methodology in combination with

chimera population may account for presence/absence of individual PCR bands. At any one sub-culture regenerants may have been a mutation and for which the leaf samples were collected. These may not survive in the next sub-culture, in which case leaf samples would have been collected from their normal 'sibs'. This may lead to haphazard variation detected by RAPD-PCR analysis. Another possible explanation of the unstable expression of variation could be the involvement of transposable elements. Environmental stress can result in a burst of transposable activity (Peterson, 1985, Doring and Starlinger, 1986). *In vitro* culture is indeed an environmental stress and may stimulate activity of a previously silent transposon. In other species, workers have suggested that tissue culture may act as a possible trigger of a previously silent transposon such as in alfalfa (Reisch and Bingham, 1981).

In adventitious regeneration, the whole shoot apex or a significant part of it ,often including one or more axillary meristems, is derived from a single totipotent cell. The formation of wholly mutant plants rather than chimera is therefore much more likely. Thus, provided it remains totipotent, any mutant cell, or one in which the genome has been permanently altered during differentiation, will be the potential source of variation. Although there is no shortage of possible mechanisms, the occurrence and onset of somaclonal variation within the plant body is as yet poorly understood. There is evidently considerable diversity between species and between tissue of the same species in the degree of variation arising from the tissue culture regenerated plants. Blackcurrant and raspberry are diploid species and showed uniformity or less variability than polyploid strawberry in micropropagation. It may be that some sort of complication occurs more frequently during rapid cell division in *in vitro* culture in larger and more complicated genomes. For the future, careful attention should be given to the nature of the starting material, the hormone levels and the morphological behaviour of the cultures. Finally the results obtained in the study of genetic uniformity of the micropropagated plants support the idea that genetic uniformity of micropropagated plants is genotypically dependant. The variation detected using RAPD-PCR may be of academic value

and may not be reflected in changes of morphologically or other economically important traits. This technique provides a useful opportunity for fine tuning the multiplication systems for the production of genetically uniform plants using *in vitro* culture techniques.

6.3. CALLUS CULTURE. AND REGENERATION

An additional aim of this study was to evaluate the potential of alternative propagation systems such as callus culture and regeneration system and to evaluate the effect of different environmental variables on the genetic uniformity of the regenerated plants. The results clearly indicate that callus culture and subsequent regeneration could be achieved successfully in *Fragaria* species. Plant regeneration was achieved both on the initiation media and following the placing of callus on regeneration media. In strawberry four groups of plants were regenerated from callus on four different variable systems but in this study only one group could be compared with a group of control micropropagated plants using RAPD-PCR. The results obtained from both types of regenerated plants showed that generally plants regenerated via micropropagation were more variable genetically as compared with plants regenerated via callus culture and regeneration. These results were unexpected as micropropagated plants are generally believed to be more uniform as compared with callus culture and regeneration and therefore difficult to explain here. Plants regenerated from *in vitro* explant source produced more plants as compared with the glasshouse-grown explants under the same conditions. This may be the result of greater adaptation to artificial environments and exogenous hormones of *in vitro* explant sources.

Callus culture was achieved successfully in *Ribes* when all types of auxins were added to the media. This may be due to the internal requirements of the explant sources or the presence of sufficient endogenous cytokinin. Very strong root formation was observed on the blackcurrant callus when placed on a higher level of auxins but all the attempts to initiate shoot formation were unsuccessful. According to George (1993) morphogenesis has now been observed *in vitro* in

numerous plants of many genera but it cannot yet be induced universally. Even within a species, varieties can be found which are recalcitrant. Plant regeneration in some species may be difficult, but may or may not be impossible. It is evident from the literature that most plant species have potential for *in vitro* regeneration which needs to be explored thoroughly. In blackcurrant we know that callus obtained from leaf discs had morphogenic potential as indicated by the large number of strong roots produced. The failure to switch on shoot initiation might be due to some limitation in the techniques adapted and/or culture conditions.

Genotypic effects are also influential in the regeneration of adventitious organ formation from callus tissue. Regeneration is readily obtained in plants of certain families or genera, and is obtained with difficulty in others. In most instances, callus has been obtained from one kind of explant of several different plants varieties on a single medium, but if then treated in the same way, the callus from each variety may have a different organogenetic potential as reported in soft fruits (Graham et al., 1990). In addition there is the question of the site specific nature of tissue culture work to be considered. The air pollution such as hydrocarbons, humidity level, extent and quality of day light on the explant all may be investigated. Regeneration from callus has been reported in strawberry by a large number of researchers but in *Rubus* and *Ribes* plant regeneration from leaf disc is very difficult. This might be due to the plant behaviour in nature *Fragaria* as polyploids in nature are usually well adapted to vegetative propagation while *Rubus* and *Ribes* as diploid can produce viable seeds and are not well adapted to the vegetative system and this may also affect behaviour in *in vitro*.

6.4. SUGGESTIONS FOR FUTURE RESEARCH

6.4.1. MOLECULAR MARKERS IN CULTIVAR IDENTIFICATION AND PHYLOGENETIC STUDIES

The appearance of RAPD products is often not consistent. Using the RAPD-PCR approach for cultivar identification, therefore, requires substantial screening of primers and selection of those primers so that under certain PCR conditions can generate reproducible polymorphic products. Only consistent and reproducible polymorphic products should be considered. The reproducibility and reliability of RAPD can be confirmed by using more individual plants of a cultivar and the bands present in all individuals of a cultivar should be used for identification. The reproducibility of RAPD-PCR products can be even more strongly established by cloning, sequencing the two ends and then distinguishing fully matched primers for PCR amplification under high stringency conditions (Paran and Michelmore, 1993). Another alternative is the use of more reliable and reproducible techniques such as AFLP (Lin and Kuo, 1995). The cost, time and use of hazardous chemicals such as radioactive chemicals can be avoided by modification to the standard protocol. In AFLP after PCR amplification, the products can be separated on ready made polyacrylamide gel followed by silver staining. This separation technique is much more environmentally friendly, very quick and much cheaper as compared to the standard protocol involving radioactive isotopes followed by X-rays photography. Although the number of bands detected in silver staining is less as compared to the standard procedure, these can be increased using more primers if needed. To study the phylogenetic relationship one should produce saturated phylogeny using a large numbers of bands by increasing number of primers used. A saturated phylogeny will reflect the true relationship among the cultivars under study. The information obtained from saturated phylogeny studies can be used in future breeding programmes

6.4.2. MICROPROPAGATIONS

In soft fruits there have been several reports about producing off-types from micropropagated regenerants. It would be important to investigate the level of variability among the regenerants by sampling more systematically and by analysing all the regenerated plants at each sub-culture along with their all recurrent parent explant sources. This type of analysis will provide valuable information on the level of variation produced in each sub-culture. For the production of genetically uniform plant absolute values of individuals cytokinin and auxins rather than cytokinin /auxin ratio should be evaluated. Comparison of RAPD-PCR genetic variation with a very close scrutiny of the morphological characters may have important repercussions for the certification schemes operating and the number of cycles in culture.

6.4.3. CALLUS CULTURE AND REGENERATION

The production of genetically variable plants is well documented in literature but the actual cause is still unknown. There are several causes it would be important to identify the major causes of variations by evaluating the effects of different variables using molecular markers. On the other hand this variation could be used for better selection of cultivars as has been used in other crop plants such as rice. Several reports have shown that organogenetic potential is influenced by genotype and explant origin. It is therefore important to investigate more genotypes and different explant sources within genotypes for a callus regeneration system. It would be important to initiate callus on low auxin level rather than more callus which requires higher levels of auxins and produced roots on callus.

6.5. CONCLUSIONS

1. RAPD-PCR combining speed and simplicity of operation and high resolution power, has obvious potential for identification, certification and protection of commercial soft fruit cultivars. Variation could arise from the traditional vegetative propagation systems which could remain undetected using morphological analysis and can be efficiently detected by RAPD-PCR technology. Agarose gel electrophoresis followed by ethidium bromide staining gave more reliable results as compared to polyacrylamide and silver staining in RAPD-PCR.

2. The relationship among the blackcurrant cultivars based on pedigree contained certain gaps. These gaps can be filled using RAPD-PCR analysis of the cultivars. The information obtained from RAPD-PCR in combination with pedigree analysis can be of great importance for the future breeding strategies.

3. Micropropagation could be used for mass multiplication in blackcurrant cultivars. In raspberries and strawberries micropropagation produced off-types and for mass production should be used with caution.

4. Callus culture and regeneration can produce off-types. The high level of variable plants produced by micropropagation in strawberry as compared to callus culture and regeneration is unexpected and needs further examination / investigation. However, variability detected in both micropropagation and callus culture could be used for further improvement and selection of new cultivars and is an important source of variability to be exploited.

5. In blackcurrant callus could be easily induced from leaf discs on media containing only auxins. In morphogenic behaviour callus behaves normally (i.e. root formation was directly related with the increase in auxin level).

7. REFERENCE CITED

AHLOOWALIA, B.S. (1976). Chromosomal changes in parasexually produced rye-grass. In *Current Chromosome Research* (K. Jones; P.E. Brandham eds.). North Holland, Amsterdam, Pp 77-87.

AHLOOWALIA, B.S. and SHERINGTON, J. (1985). Transmission of somaclonal variation in wheat. *Euphytica.* **34**, 525-537.

ANDERSON, H.H., ABBOT, A.J. and WILTSHIRE, S. (1982). Micropropagation of strawberry *Fragaria* x *ananassa* plants, *in vitro* effect of growth regulators on incidance of multi-apex abnormality. *Scientific Horticulture.* **16** 331-342.

ANDERSON, W.C. (1980). Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *R. occidentalis*. *Acta Horticulturae.* **112** 13-20.

ARULSEKAR., S., BRINGHURST, R.S. and VOTH. V. (1981). Inheritance of PG1 phosphoglucoisomerase and leucine aminopeptidase isozymes in octaploid cultivated strawberry. *Journal of the American Society for Horticultural Science.* **106**, 679-683.

BACHMANN, K. (1994). Tansley review No. 63: Molecular markers in plant ecology. *New Phytologist.* **126**, 403-408.

BARBIER, M. and DULIEU, H.L. (1983). Early occurrence of genetic variants in protoplast cultures. *Plant Science Letters.* **29**, 201-206.

BECKMANN, J.S, and SOLLER, M. (1986). Restriction fragment length polymorphism and genetic improvement of agricultural species *Euphytica.* **35,** 111-124.

BERGMANN, L. (1960). Growth and division of single cells of higher plants *in vitro*. Journal of Genetics and Physiology. **43**, 841-851

BERNATZKY, R. and TANKSLEY, S.D. (1989). Restriction fragments as molecular markers for germplasm evaluation and utilization In: *The Use of Plant Genetic Resourecs*. (Brown, A.D.H., Frankel O, Marshall, D.R. and William, J.T. ed) Cambridge University Press, Cambridge UK. Pp 354-362.

BOURGIN, J.P and NITSCH, J.P. (1967). Production of haploid Nicotiana from excised stamens. *Annales de Physiologie Vegetable*. **9**, 377-382.

BOXUS, P. (1974). The production of strawberry plants by *in vitro* micropropagation. *Journal of Horticultural Science.* **49**, 209-210.

BOXUS, P., QUOIRIN, M. and LAINE, J. M. (1977). Large scale propagation of strawberry plants from tissue culture In: *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture* (J.Reinert. and Bajaj Y.P.S. ed). Springer Velag; New York, Pp 130-143.

BRENNAN, R., DAVIDSON, D., WILSHIN, A and MILLAM, S. (1989). An assessment of the *in vitro* multiplication rates of fourteen blackcurrant cultivars. *Journal of Horticultural Science.* **64**, 679-681.

BRENNAN, R.M., MILLAM, S., DAVIDSON, D. and WILSHIN, A. (1990). Establishment of an *in vitro Ribes* germplasm collection and preliminary investigations into long-term low temperature germplasm storage. *Acta Horticulturae.* **280**,109-112.

BRESSAN, R.A., SINGH, N.K., HANDA, A.K., KONONOWICZ, A. and HASEGAWA, P.M. (1985). Stable and unstable tolerance to NaCl in cultured tobacco cells. In: *Plant Genetics* (M. Freeling ed.), Vol 35. Allan R. Liss, Inc., Pp 755-769.

BRINGHURST, R.S., ARULSEKAR, J.F. HANCOCK, and VOTH, V. (1981). Electrophoretic characterization of strawberry cultivars. *Journal of the American Society for Horticultural Science.* **106** 684-687.

BROWN, P.T.H. (1989). DNA methylation in plants and its role in tissue culture. *Genome.* **31**(2), 717-729.

BROWN, P.T.H., LANGE, F.D., KRANZ, E. and LÖRZ, H. (1993). Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. *Molecular and General Genetics.* **237**, 311-317.

BRYNE, D.H. and LITTLETON, T.G. (1988). Electrophoretic characterization of diploid plum of the Southeastern United States. *Journal of the American Society for Horticultural Science.* **106** 684-687.

CALOS, M.P. and MILLER, J.H. (1980). Transposable elements. *Cell.* **16**, 739-752.

CARLSON, J.E., TULSIERAM, L.K. GLAUBITZ, J.C., LUK, V.W.K., KAUFFELDT, C. and RUTLEDGE, R. (1991). Segregation of random amplified DNA markers in F1 progeny of conifers. *Theoretical and Applied Genetics.* **83**, 194-200.

CHANDLER, S.F., MANDAL, B.B. and THORPE, T. A. (1986). Effect of sodium sulphate on tissue culture of *Brassica napus* cv. Wester and *Brassica campestris* cv. Tobin. *Journal of Plant Physiology.* **126**, 105-117.

COOKE, R.J. (1984). The characterisation and identification of crop cultivars by electrophoresis. *Electrophoresis*. **5**, 59-72.

COUSINEAU, J.C. and DONNELLY, D.J. (1989). Identification of raspberry cultivars by starch gel electrophoresis and isoenzyme staining. *Acta Horticulturae*. **262,** 269-268.

COUSINEAU, J.C. and DONNELLY, D.J. (1992). Use of isoenzyme analysis to characterize raspberry cultivars and detect cultivar mislabeling. *HortScience.* **27** 1023-1025.

COUSINEAU, J.C., ANDERSON, A.K. and DAUBENY, H.A. (1993). Characterization of red raspberry cultivars and selections using isozyme analysis. *HortScience.* **12**,1185-1186.

COUSINEAU, J.C.and DONNELLY, D.J. (1991) Advantitious shoot regeneration from leaf explant of tissue cultured and greenhouse grown raspberry. *Plant Cell Tissue and Organ Culture*. **27**(3), 249-255.

D'AMATO, F. (1977). Chromosomal number variation in cultured cells and regenerated plants. In *Frontiers of Plant Tissue Culture* (T.A. Thrope ed.), IAPTC/University of Calgary Press, Pp 287-295.

DAMIANO, C, ASCARCLLI, A. FRATTARCLLI, A and LAURI, P. (1995). Adventitious regeneration and genetic variability in strawberry. *Acta Horticulturae.* **392,** 107-114.

DAMIANO, D. (1980). Strawberry micropropagation. In: *Procedings of the Conference on Nursery Production of Fruit Plants Through Tissue Culture*. Zimmerman R.H.(ed). USDA, Pp 11-21.

DARROW, G.M., (1928). Notes on thornless blackberries. *Journal of Heredity.* **19**, 139-142.

DENTON, I.R., WESTCOTT, R.J. and FORD-LOYD, B.V. (1977). Phenotypic variation of *Solanum tuberosum* L. Cv. Dr.McIntosh regenerated directly from shoot tip culture. *Potato Research.* **20,**131-136.

DERMAN, H. (1947). Histogenesis of some bud sports and variegation. *Procedings of the American Society for Horticultural Science*. **99**, 352-358.

DEVRIES, H. (1912). Species and varieties: their origin by mutation. *Open court publishing Co., Chicago.*

DIETERT, M.F., BARRON, S.A. and YODER, O.C. (1982). Effect of genotype on *in vitro* culture in the genus *Brassica. Plant Science Letters.* **26**, 233-240.

DIJKSTRA, J. (1987). Results of experiments with runner plants of tissue culture strawberry plants (cv. Gorella). In: *In Vitro Culture of Strawberry Plants.* (Boxus, P and Larvor, P ed.) C.E.C., Biological Science, EUR, **10871**, 11-14

DIXON, G.R. (1987). The practicalities and economics of micropropagation for the amenity plant trade. In: *Micropropagation in Horticulture: Practice and Commercial Problems.* Proceedings of the Institute of Horticulture Symposium, University of Nottingham, School of Agriculture.

DIXON, R.A. (1985). Isolation and maintenance of callus and cell suspension cultures: In *Plant Cell Culture: A practical Approach* (R.A. DIXON ed.). IRL Press, Pp 1-20.

DONNELLY, D.J. and DAUBENY, H.A.. (1986). Tissue culture of *Rubus* species. *Acta Horticulturae.* **183**, 305-314.

DORING, H.P. and STARLINGER, P. (1986). Molecular genetics of transposable elements in plants. *Annual Review of Genetics.* **20**, 175-200

DOUCHES, D.S. and LUDLAM, K. (1991). Electrophoretic characterisation of North American potato cultivars. *American Potato Journal.* **68**, 767-780.

DOYLE, J.J. and DOYLE, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin.* **19**(1), 11-15.

DUNCAN, D.R. and WIDHOLM, J.M. (1986). Cell selection for crop improvement. In: *Plant Breeding Review*, vol. 4 (J. Janick ed.). AVI Publishing Company. Inc., Pp 153-169.

DYER, A.F. (1979). Investigating Chromosomes. Wiley, New York

EDALLO, S., UCCHINALI, C., PERENZIN, M. and SALAMINI, F. (1981). Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and plant regeneration in maize. *Maydica.* **26,** 39-56.

ELLSWORTH, D.L., RITTENHOUSE, K.D. and HONEYCUTT, R.L. (1993). Artifactual variation in Random Amplified Polymorphic DNA banding patterns. *Biotechniques.* **14**, 214-217.

EVANS, D.A. and BRAVO, J.E. (1986). Phenotypic and genotypic stability of tissue cultured plants. In: *Tissue Culture as a Plant Production System for Horticultural Crops*. (Zimmerman, R.H., Griesbach, R.J., Hammerschlag, F.A. and Lawson R.H. ed). Martinus Nijhoff Publishers.

EVANS, D.A. and SHARP, W.R. (1986). Somaclonal and gametoclonal variation. In *Hand Book of Plant Cell Culture*: Techniques and Application (D.A. Evans, W.R. Sharp and P.V. Ammirato, eds.), vol. 4. Macmillan Publishing Company, New York. Pp 97-132.

FEUCHT, W., SCHMID, P.P.S., SCHIMMELPTENG, H., THEILER-HEDTRICH, C. and SCHMITT, A. (1985). Large-scale trial of raspberries propagated *in vitro*: 0.68% of off-types and enzyme pattern of atypical fruits. *Erwerbsobstbau.* **27**, 167-169.

FLEGMANN, A.W. and WAINWRIGHT, H.(1981). Shoot doubling time: a quantitative parameter for characterizing shoot cultures *in vitro*. *Plant Cell Tissue and Organ Culture.* **1**, 85-92.

GAMBORG, O.L., MILLER, R.A. and OJIMA K. (1968). Nutrient requirments of the suspension culture of soyabean roots cells. *Experimental Cell Research.* **50**, 151-158.

GEORGE, E.F. (1993). Plants tissue cultures techniques. In: *Plant Propagation by Tissue Culture*, 2nd edition. Exegetics Limited, Pp 3-36.

GEORGE, E.F. and SHERINGTON, P.D. (ed.) (1984). In *Plant Propagation by Tissue Culture*. Exegetics Ltd: Basingstoke, U.K.

GIDONI, D., ROM, M., KUNIK, T., ZUR, M., IZHAR, S. and FIRON, N. (1994). Strawberry-cultivar identification using Randomly Amplified Polymorphic DNA (RAPD) markers. *Plant Breeding.* **113**, 339-342.

GÖBEL, E., BROWN, P.T.H., and LÖRZ, H. (1985). *In vitro* culture of *Zea mays* and analysis of regenerated plants. In *Nuclear Techniques and In Vitro Culture for Plant Improvements.* IAEA/FAO, Vienna, Pp 21-27.

GRAHAM, J. and McNICOL, R. J. (1990). Plant regeneration and genetic transformation in soft fruit species. *Acta Horticulturae.* **280**, 517-522.

GRAHAM, J. and McNICOL, R. J. (1991). Regeneration and transformation of *Ribes*. *Plant Cell Tissue and Organ Culture.* **24**, 91-95.

GRAHAM, J. McNICOL, R. J. and McNICOL, .J.W. (1996). A comparasion of methods for the estimation of genetic diversity in strawberry cultivars. *Theoretical and Applied Genetics.* **93**, 402-406

GRAHAM, J. McNICOL, R. J., GREIG, K. and VAN DE VEN, W.T.G. (1994). Identification of red raspberry cultivars and an assessment of their relatedness using fingerprints produced by random primers. *Journal of Horticultural Science*. **69** (1) 123-130.

GRATTAPAGLIA, D., CHAPARRO, J., WILCOX, P., McCORD, S., WERNER, D., AMERSON,H., McKEAND, S., BRIDGEWATER,F. WHETTEN,R., O'MALLY,D. and SEDEROFF. R. (1992). Mapping in woody plants with RAPD markers: Application to breeding in forestry and horticulture, In: Application of RAPD Technology to Plant Breeding. *Joint Plant Breeding Symposium Series Minneapolis*. Pp 37-40.

GROOSE, R.W. and BINGHAM, E.T. (1984). Variation in plants regenerated from tissue cultures of tetraploid alfalfa hetrozygous for several traits. *Crop Science.* **24**, 655-658.

GROOSE, R.W. and BINGHAM, E.T. (1985). An unstable anthocyanin mutation recoverd from tissue culture of alfalfa (*Medicago sativa*) high frequency of reversion upon reculture.Plant Cell Report. **5**, 104-107.

GROUT, B. and ASTON, M. (1977). Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and xylem regeneration. *Horticulture Research.* **17**, 1-7.

GRUNEWALDT, J. and DUNEMANN, F. (1991). Variation and selection *in vitro*. *Gartenbauwissenschaft.* **56** (1), 1-5.

GUZUKIRMIZI, N., ARI, S., GUREL, F., FUMUSEL, F. and CIRAKOGLU, B. (1993). Fingerprinting barley genome using PCR with arbitrary primer in barley plants regenerated from tissue culture. *Biotechnology in Agriculture. Proceedings of the First Asia-Pacific Conference on Agricultural Biotechnology,* Beijing, China (C.B. You; Z.L. Chen; Y. Ding eds.). Kluwer Academic Press, Pp 185-188.

HABERLANDT, G. (1902). 'Kulturversuche mit isolierten Pflanzenzellen'. Sitzungsber. *Akad. Wiss. Wien, Math.' Naturwiss*. K1., Abt **111**, 69-92.

HADRYS, H., BALRICK, M. and SCHIERWATER, B. (1992). Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology.* **1**, 55-63.

HAMES, B. D. (1981). An introduction to polyacrylamide gel electrophoresis. In: *Gel Electrophoresis of Proteins a Practical Approach.(*B.D. Hames and D. Rickwood eds). IRL press Ltd., London and Washington DC. Pp 1-91.

HANCOCK, J.F. and CALLOW, P.A. (1994). Random Amplified Polymorphic DNAs in the cultivated strawberry, *Fragaria* x *ananassa*. *Journal of American Society for Horticultural Science*. **119**(4), 862-862

HARTMANN, H.T., KESTER, D.E. and DAVIES, F.T. (ed) (1990). Source selection and managment in vegetative propagation In: *Plant Propagation: Principles and Practices*, 5th edition. Prentice-Hall, Englewood Cliffs, New Jersey. Pp 165-192

HARVEY, A.E. and GRASHAM, J.L. (1977). Callus proliferation and differentiation of *Ribes* spp. in vitro. *USDA Forest Service Research Note International*. 222.

HASKEL, G. and GARRIE, J.B. (1966). Fingerprinting raspberry cultivars by empirical paper chromatography. *Journal of the Science of Food and Agriculture*. **17**,189-192.

HATTON, R.G. (1920). Blackcurrant varieties. A method of classification. *Journal of Pomology* **1**, 65-80, 145-154.

HEDRICK, P. (1992). Shooting the RAPDs. *Nature*. **335**, 679-680.

HEDRICK, U. P.(1938). The Cyclopedia of Hardy Fruits, 297-300.

HELENTJARIS, T and BURR, B. (ed) (1989). Development and application of molecular markers to problems in plant genetics. In: *Current Communications in Molecular Biology* Cold Spring Harbor Laboratory., Cold Spring Harbor. New York.

HENNERTY, M. J., HUNTER, S.A and FOXE, M.J. (1987). Field performance of tissue cultured strawberry plants. In: (Boxus, P and Larvor, P ed). In Vitro Culture of Strawberry Plants. C.E.C., *Biological Science*, EUR, 10871, 41-46.

HOASKA, K., MATSUBAYASHI, M. and KAMIJIMA, O. (1985). Peroxidase isozymes in various tissues for discrimination of two tuberous *Solanum* species. *Japan Journal of Breeding*. **35**, 375-382.

HU. J. and QUIROS, CF. (1991). Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Reports*. **10,** 505-511.

HUANG, D.H., WU, J.Y. and ZHOU E. (1985). Shoot tip culture of blackcurrant (*Ribes nigrum* L.) *in vitro* and its cytological and histological observation. *Genetic Manipulation in Crop Newsletter.***1**(1) 51-53.

HUDSON, J.P. (1955). The regeneration of plants from roots. *Procedings 14th International Horticulture Congress*. **2**, 1165-1172.

HUSSEY, G. (1985). Vegetative propagation of plants by tissue culture. In: *Plant Cell Culture Technology* (M.M. Yeoman ed.). Blackwell Scientific Publications, Pp 29-66.

HUSSEY, G. (1986). Problems and prospects in the *in vitro* propagation herbaceous plants. In: *Plant Tissue Culture and its Agricultural Applications* (L.A. Withers; P.G. Alderson eds.). Butterworth, Pp. 69-85.

HUSSEY, G. and STACEY, N.J. (1981). *In vitro* propagation of potato *(Solanum tuberosum L.). Annals of Botany*. **48** 787-796.

HUSSEY, G. (1983). *In vitro* propagation of horticultural and agricultural crops. In: *Plant Biotechnology* (Mantell, S.H. and SMITH, H. ed). Cambridge Univerisity Press, Cambridge. Pp 111-138.

HUTH, W. (1979). Culture of raspberry plants from apical meristem. *Gartenbauwissenschaft.* **44**, 53-55.

INNIS, M.A. and GELFAND, D.H. (1990). Optimisation of PCRs. In: *PCR Protocol. A Guide to Methods and Application* (M.A. Innis; D.H. Gelfand; J.J. Sninksky; T.J. White eds.). Academic Press, Inc., Pp 3-12.

JAIN, R.K., JAIN, S., NAINWATEE, H.S. and CHOWDHURY, J.B. (1990). Salt tolerance in *Brassica juncea* L. 1. *In vitro* selection, agronomic evaluation and genetic stability. *Euphytica.* **48**(2), 141-152.

JINKS, J.L. (1964). Extrachromosomal inheritance. Englewood Cliffs, N. J Prentice-Hall. Inc.

JOERSBO, M., ENEVOLDSEN, K. and ANDERSEN, J.M. (1992). Oil glands on blackcurrant callus. *Journal of Plant Physiology.* **140,** 96-100.

JONES, O.P., WALLER, J.B. and BEECH, M.G. (1988). The production of strawberry plants from callus cultures. *Plant Cell Tissue and Organ Culture.* **12**, 235-241.

KARP, A. (1991). On the current understanding of somaclonal variation. *Oxford Survey of Plant Molecular and Cell Biology.* **7**, 1-58.

KARP, A. and BRIGHT, S.W.J. (1985). On the causes and origin of somaclonal variation. *Oxford Survey of Plant Molecular and Cell Biology.* **2**, 199-234.

KEEP, E. (1995). Currants *Ribes spp. (Grossulariaceae)* In: *Evolution of crop plants* (N.W.Simmonds, ed), Longman Group Limited, London. Pp 235-239

KONDAKOVA, V., KACHARMAZOV, V., and KHRISTOV, R. (1983). Effect of different nutrient media on organogenesis in strawberry tissue culture. *Gradin Loz Nauka.* **206,** 61-64.

LANDSMANN, J. and UHRIG, H. (1985). Somaclonal variation in *Solanum tuberosum* detected at the molecular level. *Theoretical and Applied Genetics.* **71**, 500-505.

LANHAM, P.G., BERNNAN. R.M., HACKETT. C. and McNICOL. R.J. (1995). RAPD fingerprinting of blackcurrant (*Ribes nigrum* L.) cultivars. *Theoretical and Applied Genetics.* **90**, 166-172.

LARKIN, P.J. and SCOWCROFT, W.R. (1981). Somaclonal variation a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics.* **60**, 197-214.

LIN, J.J. and KUO, J. (1995). AFLP. A novel PCR based assay for plant and bacterial DNA fingerprinting. *Focus.* **17**, 52-66.

LIU, Z.R. and SANFORD, J.C. (1988). Plant regeneration by organogenesis from strawberry leaf and runner tissue. *HortScience.* **23**, 1057-1059.

LÖRZ, H. and SCOWCROFT, W.R. (1983). Variability among plants and their progeny regenerated from protoplasts of Su/su heterozygotes of *Nicotiana tabacum. Theoretical and Applied Genetics.* **66**, 67-75.

M.A.F.F. (Ministry of Agriculture, Fisheries and Food) (1981). *Bush Fruits. Reference Book 4*, H.M.S.O., London.

MARCOTRIGIANO, M. And GOUIN. F.R. (1984). Experimentally synthesised plant chimeras. *In vitro* recovery of *Nicotiana tabacum* L. Chimera from mixed callus culture. *Annals of Botany.* **54**, 503-511.

MAZHOROV, E.V. (1977). Dietetic and medicinal properties of strawberry. *Byull VIR Imeni N I Vavilova.* **77**, 41-46.

MEINS, F. (1983). Heritable variation in plant cell culture. *Annual Review of Plant Physiology.* **34**, 327-346.

MOREL. G. (1960). Producing virus-free *Cymbidium. American Orchid Society Bullitin.* **29**, 495-497.

MORI, M., HOSAKA, K., UMEMURA, Y. and KANEDA, C. (1993). Rapid identification of Japanese potato cultivars by RAPDs. *Japan Journal of Genetics*. **68**, 167-174

MULLER, E., BROWN, P.T.H., HARTKE, S. and LORZ, H.T.I (1990). DNA variation in tissue-culture-derived rice plants. *Theoretical and Applied Genetics* **80**(5), 673-679.

MULLIN, R.H. (1976). Cold storage maintenance of strawberry meristem plantlets. *Horticulturae Science*. **11**, 100-101.

MURALIDHARAN, K. and WAKELAND, E.K. (1993). Concentration of primers and template qualitatively affects products in random amplified polymorphic DNA PCR. *Biotechniques.* **14**, 362-364.

MURASHIGE, T. (1974). Plant propagation through tissue cultures. *Annual Review of Plant Physiology.* **25**, 135-166.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum*. **15**, 473-497.

MURPHY, R.W.,SITES, J.W., BUTH, and HAUFLER, C.H. (1990). Proteins I: Isozymes electrophoresis In: *Molecular systematics* (ed Hills D.M. and Moritz C.). Sinauer Association sunderland, Massachusetts.

MURRAY, M.G., MA, Y., ROMERO-SEVERSON, J., WEST, D.P. and CRAMER, J.H. (1988). Restriction fragment length polymorphisms: What are they and how can breeders use them? *Annual Corn and Sorghum Research Conference*. **43**, 72-87.

NAGL,W. (1979). Differential DNA replication in plants: A critical review. Z. Pflanzenphysiol. **95**, 283-314.

NEAL, D.B. and WILLIAM, G.C. (1991). Restriction fragment length polymorphism mapping in conifers and applications to forest genetics and tree improvement. *Canadian Journal of Forest Research.* **21**, 545-554

NEHRA, N.S., KARTHA, K.K. and STUSHNOFF, C. (1990). Nuclear DNA content and isozyme variation in relation to morphogenic potential of strawberry (*Fragaria* x *ananassa*) callus cultures. *Canadian Journal of Botany.* **69**, 239-244.

NEHRA, N.S., STUSHNOFF, C. and KARTHA, K.K. (1990). Regeneration of plants from immature leaf-derived callus of strawberry *(Fragaria×ananassa). Plant Science.* **66**, 119-126.

NEI, M. and LI, W.H. (1979). Mathematical model for studying variation in terms of restriction endonuclease. *Proceedings of National Academy of Science* USA. **76**, 569-5273.

NIEMIROWICZ-SZCZYTT, K. (1990). Somaclonal variation in strawberry. In: Biotechnology in Agriculture and Forestry II Somaclonal Variation in Crop improvement (Y.P.S. Bajaj, ed.). Spring-Verlog, Pp 511-528.

NISHI, S. OOSAWA, K. (1973). Mass production method of virus-free strawberry plants through meristem callus. *Journal of Agriculture Research.* **7**, 189-194.

NITSCH, J.P. and NITSCH, C. (1969). Haploid plants from pollen grains. *Science.* **163**, 85-87.

NITSCH, J.P., NITSCH, C., ROSSINI, L.M.E. and BUI DANG HA, D. (1967). The role of adenine in bud differentiation. *Phytomorphology.* 446-453.

NYANGE, N. (1994). The development and potential utilisation of *in vitro* techniques in coffee breeding for resistance to *Colletotrichum kahawae* sp. nov. Ph.D. thesis, University of Dundee. Pp 34-35.

O'BRIEN, S.J. (ed.). (1990). Genetic maps. *Cold Spring Harbor Press, Cold Spring Harbor, New York.*

OGIHARA, Y. TSUNEWAKI, K. (1979). Tissue culture in *Haworthia.* 3. Occurrence of callus variants during subcultures and its mechanism. *Japan Journal of Genetics.* **54**, 271-293.

ORLIKOWSKA, T. (1984). Micropropagation of rood knop cv. blackcurrant. *Fruit Science Report.* **11**, 5-17.

ORLIKOWSKA, T., LISEK, A. and SERWIK, M. (1991). Micropropagation of 9 genotypes of blackcurrant. *Fruit Science Reports.* 18(2)51-62.

ORTON, T.J. (1983). Spontaneous electrophoretic and chromosomal variability in callus cultures and regenerated plants of celery. *Theoretical and Applied Genetics.* **67**, 17-24.

PARAN, I. and MICHELMORE, R. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics.* **85**, 985-993

PARAN, I., KESSELI, R., and MICHELMORE, R., (1992). Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance in lettuce using near-isogenic lines. *Genome.* 1021-1027

PARENT, J.-G., FORTIN, M.G. and PAGE, D. (1993). Identification of raspberry cultivars by random amplified polymorphic DNA (RAPD) analysis. *Canadian Journal of Plant Science*. **73**,1115-1122.

PENNER, G.A., BUSH, A.WISE, R., KIM, W., DOMIER, L., KASHA, K., LAROCHE, A., SCOLES, G., MOLNER,S.J. and FEDAK, G. (1993). Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR Methods and Applications*. **2**, 341-345

PETERSON, P.A. (1985). Virus-induced mutations in maize: on the nature of stress-induction of unstable loci. *Genetical Research* **46**, 207-217.

POWELL, W.BONAR, N. BAIRD, E. RUSSALL, J. and WAUGH, R. (1994). Molecular marker techniques for barley genome analysis and breeding. *Annual Report Scottish Crop Research Institute, Dundee*. Pp 48-58.

RAFALSKI, J.A., TINGEY, S.V. (1993). Genetic diagnostics in plant breeding: RAPDs, microsatellites and mechanics. *Trends in Genetics*. **9**, 275-280.

RAFALSKI, J.A., VOGEL, J.M., MORGANTE, M., POWELL, W., ANDRE, C. and TINGEY, S.V. (1996). Generating and using DNA markers in plants. In: *Non-mammalian Genomic Analysis* (Birren B. and Lai. E. ed.). Pp 75-134.

REISCH, B. and BINGHAM, E.T. (1981). Plants from ethionine resistant alfalfa tissue cultures: variation in growth and morphological characteristics. *Crop Science*. 21, 783.

SAIKI, R.K. (1989). The design and the optimisation of the PCR. In: *PCR Technology.* (Erlich H.A. ed.). M Stockton Press, Pp 7-16.

SAITOU, N. and NEI, M. (1987). The neighbour-joining method. A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* **4**(4), 406-425.

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. (eds.) (1989). In: *Molecular Cloning, A laboratory Manual* (2nd ed.), book 1,2 and 3.

SANSAVINI, S., ROSATI, P., GAGGIOLI, D. and TOSCHI, M.F. (1990). Inheritance and stability of somaclonal variation in micropropagated strawberry. *Acta Horticulturae*. **280**, 375-384.

SCOWCRAFT, W.R. and RYAN, S.A. (1986). Tissue culture and plant breeding. In: *Plant Cell Culture*, vol. 23. Blackwell Scientific Publications. Pp. 67-95.

SHAEFFER, G.W., DAMIANO, C., SCOTT, D.H., McGREW, J.R., KRUL, W.R., ZIMMERMAN, R.H. (1980). Transcription of a panel discussion on the genetic stability of the tissue culture propagated plants. *Procedings Conference of Nursary Production of Fruit Plant Through Tissue Culture-Application and Feasibility*. USDA- Arkansas. Pp 64-79.

SHEPARD, J.F. (1982). The regeneration of potato plants from leaf cell protoplasts. *Scientific American.* **246**(5), 112-121.

SIBI, M. (1986). Non-mendelian heredity. Genetic analysis of variant plants regenerated from *in vitro* culture: epigenetics and epigeneics In: *CEC Symposium somaclonal variation and crop improvement,* (Semal, J. ed.) Gembloux, Belgium. Pp 53-83.

SIMMONDS, N.W. (1976). Principals of crop improvement. *Longman, London.*

SIMPSON, M.J.A. and Withers, L.A. (1986). Characterisation of plant genetic resources using isozyme electrophoresis: a guide to the literature. *International Board for Plant Genetic Resources*, Rome.

SKOOG, F. and MILLER, C.O. (1957). Chemical regulation of growth and formulation in plant tissues cultured *in vitro*. In: *The Biological Action of Growth Substances* (H. K. Porter ed.) Cambridge University Press, Pp 118-131.

SKOOG, F. and TSUI, C. (1948). Chemical control of growth and bud formation in tobacco stem segments and callus culture *in vitro*. *American Journal of Botany.* **35**, 782-787.

SMITH, .J.S.C. (1992). PLANT BREEDERS RIGHTS IN THE USA: Changing approaches and appropriate technologies in support of germ plasm enhancement. *Plant Varieties and Seeds.* **5**, 183-189.

SMITH, J.S.C. and SMITH O.S. (1992). Fingerprinting crop varieties. *Advances in Agronomy.* **47**, 85-140.

SMITH, M.K. and DREW, R.A. (1990). Current application of tissue culture in plant propagation and improvement. *Australian Journal of Plant Physiology.* **17**, 267-289.

STACE-SMITH, R. (1984). Red raspberry virus diseases in North America. *Plant Disease*. **68**, 274-279.

STEGEMANN, H., FRANCKSEN, H and MACKO, V. (1973). Potato protein: Genetic and physiological changes, evaluated by one- and two-dimensional PAA-gel techniques. *Zeitschrift für Naturfors-Chung*. **28**, 722-732.

STREET, H.E. (ed.) (1977). In: *Botanical monograph:* Plant Tissue and Cell Culture, vol. II (2), Blackwell, Oxford.

SWARTZ, H.J., GALLETTA, G.J. and ZIMMERMAN, R.H. (1981). Field performance and phenotypic stability of tissue culture-propagated strawberries. *Journal of the American Society for Horticultural Science.* **27** (10) 1108-1110.

SWARTZ, H.J., LINDSTROM, J.T. and FIOLA J.A. (1987). The use of tissue culture propagation of strawberry in the United States. In: *In Vitro Culture of Strawberry Plants*. (Boxus, P., and Larvor P. ed.) CEC, Biological Sciences, EUR, 10871, 79-100.

TANKSLEY, S.D., BERNATZKEY, R., LAPITAN, L. and PRINCE, J.P. (1988). Conservation of gene repertoire but not gene order in pepper and tomato. *Procedings Natural Academy of Sciences*. U.S.A. **85**, 6419-6423

TANKSLEY, S.D., YOUNG, N.D., PATERSON, A.H. and BONIERBALE, M.W. (1989). RFLP mapping in plant breeding: New tools for an old science. *Biotechnology* **7**, 257-264

THOMAS, E and DAVEY M.R. (1975). The history and development of plant tissue culture In; *From Single Cells to Plant.* (MOTT, N., NOAKES, G.R., YAPP, W.P. ed.). Wykeham Publications London. The Wykeham Science Series 38.

THROPE, T.A. (1979). Regulation of organogensis *in vitro* In: Propagation of Higher Plants Through Tissue Culture (Hughes, K.W., Henke, R. And Constantin, R. Ed.). Springfield,Va:, National Technical Information Service U.S.department of Commerce, Pp 87-101.

TORRE L.C, and BARRITT, B.H. (1979). Red raspberry establishment from root cutting. *Journal of the American Society for Horticultural Science.* **104**, 28-31

TORREY, J.C. (1977). Cytodifferentiation in cultured cells and tissues. *HortScience.* **12**(2), 14-15.

TOYODA, H., HORIKOSHI, K., INABA, K. and OUCHI, S. (1990). Plant regeneration of callus tissues induced from leaf explant of strawberry. *Plant Tissue Culture Letter.* **7**(1),38.

TURNER, A.P., and DICKINSON, N.M. (1993). Copper tolerance of *Acer pseudoplatanus* L. (sycamore) in tissue culture. *New Phytologist.* **123**, 523-530.

VALLÉS, M.P., WANG, Z.Y., MONTAVON, P., POTRYKUS, I. and SPANGENBERG, G. (1993). Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds). *Plant Cell Report.* **12** (2), 101-106.

WAINWRIGHT, H. and FLEGMANN, A.W. (1984). The influence of light on the micropropagation of blackcurrant. *Journal of Horticultural Science.* **59** (3)387-393.

WAINWRIGHT, H. and FLEGMANN, A.W. (1985). The micropropagation of gooseberry (*Ribes uva-crispa* L.): II. *In vitro* proliferation and *in vivo* establishment. *Journal of Horticultural Science.* **60**, (4) 485-491.

WAINWRIGHT, H. and FLEGMANN, A.W. (1986). Studies on the micropropagation of *Ribes* species. *Acta Horticulturea.* **183,** 315-321.

WAINWRIGHT, H. and HAWKES, H.Y. (1984). The influence of the length of hardwood cuttings on the propagation of blackcurrant. (*Ribes nigrum*). *Acta Horticulturae.* **227**, 266-268.

WEEDEN, N.F. and LAMB,R.C. (1985). Identification of apple cultivars by isozyme phenotypes. *Journal of the American Society for Horticultural Science*. **110**, 509-515.

WEEDEN, N.F. and WENDEL, J.F. (1989). Genetics of plant isozymes. In: *Isozymes in plant biology* (Soltis,D, and Soltis P.eds.). Dioscorides Press, Portland, Oregon. Pp 46-72.

WEEDEN, N.F.,TIMMERMAN, G.M., HEMMAT, M., KNEEN, B.E. and LODHI M.A. (1992). Inheritance and reliability of RAPD markers, In: *Application of RAPD technology to plant breeding*. Joint Plant Breeding Symposia Series, Minneapolis. Pp 12-17.

WEILL, J.C. and REYNAUD, C.A. (1980). Somatic Darwinism *in vivo*. *Biosystems*. **12**, 23-25.

WELANDER, M. (1985). *In vitro* culture of raspberry (*Rubus ideaus*) for mass propagation. *Journal of Horticultural Science.* **60**(4) 493 499.

WELSH, J. and McCLELLAND, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research.* **18,** (24) 7213-7218.

WHITE, P.R. (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. *Plant Physiology*. **9**, 585-600

WILLIAMS, J.G.K., KUBELIK, A.R. HAUK, K.J., RAFALKSKI, K.J. and TINGEY, S.V. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research.* **18**, 6531-6535.

WILLIAMS, M.W. and NORTON R.A. (1959). Propagation of red raspberries from soft wood cuttings. *Procedings of the American Society for Horticultural Science.* **74**, 401-406.

WOLFE, K.H., LI. W.H. and SHARP, P.M. (1987). Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Procedings Natural Acadamy of Sciences*.USA. **84**, 9054-9058.

WOLFE, W.H. (1976). Identification of apple cultivars by isozyme phenotypes. *American Journal of Enology and Viticulture.* **27**, 68-73.

ZATYKO, J.M. (1979). Complete red currant (*Ribes rubrum* L.) plants from adventive embryos induced *in vitro*. *Current Science.* **55**, 355-357.

APPENDICES

Appendix 4.1.

THE SCOTTISH OFFICE

Agriculture, Environment and Fisheries Department

INSPECTION AND CERTIFICATION OF SOFT FRUIT PLANTS 1996

MICROPROPAGATION

The Department is prepared to accept applications for the inspection and certification of stocks of *Fragaria, (Strawberries),* Rubus, (Raspberries including Blackberry and Hybrid Berries) and *Ribes,* (blackcurrants, including Redcurrants and Gooseberries), propagated by micropropagation at Standard, Elite and Super Elite grades. A separate leaflet and application formcovers stocks at Foundation grade.

APPROVAL

Premises to be used for micropropagation and growing on of stocks for certification must be approved by the Department. This is to establish the suitability of the business, facilities, equipment and the standard of hygiene maintained for the production of soft fruit stocks. See also under Plant Health and the Single Market below.

ELIGIBLE MATERIAL

Parent material must have a valid certificate at Standard, Elite or Super Elite level. Exceptionally other material may be accepted but full details of origin and health status must be submitted to the Department for approval prior to propagation.

GRADING

Stocks derived by micropropagation will be inspected for health and vigour only as no guarantee can be given on the trueness to type of such stocks. These stocks will be identified by having the suffix "M" included in the certificate number. See section on Certificates below. Grading will be at the same level as the parent material but stocks failing to achieve the necessary standards will be downgraded or rejected as appropriate.

PLANT HEALTH AND THE SINGLE MARKET

The plant health Single Market legislation requires all growers of raspberry and strawberry plants, who trade with other professional growers or wholesalers, to be registered with the Department. In addition all raspberry, strawberry and blackcurrant growers including those who retail direct or supply the retail trade must be accredited under the Marketing of Fruit Plant Material Regulations 1995.

Details on registration and accreditation can be obtained from the address below or by telephone on 0 1 3 1 244 6303.

APPLICATIONS

Application must be made on the appropriate form for micropropagation prior to the commencement of any propagation prog.me. A separate form must also be completed for each species, giving full details of the origin, grade or health status of parent material.

Forms can be obtained from the Department at the address shown below or by telephone on 0131 244 6303.

FEES

Fees will be charged at the following rates and must accompany your application:

Up to the first 10,000 plants	£70 + VAT
each additional 5,000 plants up to 50,000 plants	£30 + VAT
each additional 20,000 plants thereafter	£10 + VAT

For the purposes of the fee calculation applications for strawberries, raspberries or blackcurrants, although entered on separate forms will be treated as one application eg. applications for 3,000 plants each of strawberries, raspberries and blackcurrants will be charged as 9,000 plants at £70 +,VAT.

INSPECTIONS

Material entered for certification will normally be inspected once prior to despatch.
An annual inspection will be made to check records on propagation and sales.

ISOLATION

Material under propagation, weaning and growing on must at all times be held under protected environmental conditions. Only stocks entered for certification below Foundation grade should occupy the growing unit and a physical barrier must be maintained between stocks of different grades. Any other plant material or stocks of a different health status must be contained in separate growing area.

GENERAL CONDITIONS

a) Laboratory propagation areas must be equipped with suitable equipment for aseptic excision and transfer of propagules.

b) The culture method is usually non-adventitious shoot micropropagation.

c) The maximum time any material may be maintained in culture is one year or not more than I 0 sub-cultures whichever is the sooner.

d) All material for certification must be held in a suitable protected environment system with insect **proofing,** double doors and disinfectant foot baths at the entrance.

e) All production must take place out of contact with the base soil. Glasshouse and tunnel structure floors must be covered with a suitable barrier, such as 500 gauge polythene, to prevent contamination from the soil. In all cases surplus water must drain away rapidly.

f) Sterile growing medium or soil-less compost must be used while the plants are under protected environment conditions.

g) An approved pesticide treatment regime must be implemented during the propagation period. Pesticides known to mask symptoms of Phytophora and Verticillium must not be used.

h) Any unwanted plant material and growing medium should be routinely disposed of, off site **or** at least at a reasonable distance from the growing on units.

i) The growing structures must be sterilised annually with an approved sterilant. Pots and containers should be new for each batch of plants or of a material suitable for sterilisation and sterilised between batches. Damaged floor coverings must be replaced

j) Weeds must not be allowed to grow in propagation or growing on areas.

k) Access to propagation and growing on areas must be restricted to working personnel only. Footbaths with disinfectant must be used at all access points

1) Pallets, boxes, trays etc. which leave the area and are retumed after delivery should be sterilised or steam cleaned before being retumed to the growing area for re-use. Non-retumable packagin. should be used whenever possible.

LABELLING

All stocks of parent material must be labelled clearly.

All stocks under propagation must be labelled with variety, clone and batch numbers, and
arranged in order to minimise the risk of mixing taking place.

PEST AND DISEASE CONTROL

A high standard of efficiency and hygiene must be maintained throughout the unit.

An effective pest and disease control regime must be carried out on both parent and propagated stocks.

Particular attention should be paid to the control of red spider mite and mildew. The use
of predators for the control of red spider mite is acceptable.

RECORD KEEPING

Propagation prog.mes:- Where appropriate each prog.me shall be given an identifying code and quoted on the application for certification.

Mother plants:- Where appropriate each mother plant must be allotted an identifying code
which shall be used in the identification of all material in the clone.

Subcultures:- Each sub-culture of a clone must be recorded to enable the number of
subcultures to be monitored.

Log sheets:- Details of the parent plant, progeny, dates of propagation of each sub-culture and storage of batches of plants shall be recorded on a log sheet. These log sheets must be made available to the Departrnent for inspection at any reasonable time and must be kept for two years after the certified plants have been sold.

Pest and disease control:- Details of all pesticide treatments must be recorded and made
available for inspection.

Sale of stocks:- The requirements of the Plant Health (Great Britain) Order 1993 and the Marketing of Fruit Plant Material 1995 must be observed when marketing stocks. This includes the quoting of the certificate number on sales documents.

STANDMWS OF INSPECTION

Inspection standards will be as for field grown stocks (See appropriate Appendix). Stocks
failing to meet the prescribed standards will be downgraded or rejected as appropriate.

CERTIFICATES

Certificates will be issued as appropriate following satisfactory inspections. They will be
valid until the stocks for which they were issued are either:-

a) entered for a further cycle of propagation or:

b) planted out for commercial production.

The certificate number issued to micropropagated stocks will include the suffix 'M' denoting certification for health and vigour only and a statement will be added to the certificate with the wording:-

"No guarantee as to the trueness to type is given, but the plants have been propagated
from virus tested nuclear stock under approved conditions".

Where appropriate, the 'M' suffix can be removed once the material has been fruited and the trueness to type has been confirmed. Growers will be responsible for ensuring that trueness to type checks are carried out by suitably qualified personnel.

GENERAL CONDITIONS OF THE SCHEME

The Department reserves the right to withdraw any certificate issued, if it is found that:-

a) the requirements of the scheme have not been met in relation to the stock in respect of which the certificate was issued; or

b) the conditions under which the certificate has been issued have not been strictly observed.

Before withdrawing a certificate the Department will give written notification of the reasons for the action proposed and give the certificate holder the opportunity to make representations.

Growers must, if so required, furnish the Department with full information concerning the distribution of plants from stocks for which a certificate has been issued.

Applications are accepted on the strict understanding that the Departments decision on all matters shall be final. No liability will be attached to the Department or any of its officers in connection with any inspection carried out, or in respect of certificates and reports which are based on the result,of the examination of stocks or in any other way in connection with the operation of these arrangements for inspection and certification.

The act of application for a certificate will be deemed to imply acceptance by the applicant of these conditions.

PLANT BREEDERs RIGHTS

In those cases where a variety entered for certification under the scheme is subject to plant breeders rights, and as such is distributed under the tertns of a licence from the owner of the rights, it is the responsibility of the grower to ascertain the terms of any such licence and to conform to such propagation or other conditions as may apply.

Horticulture and Marketing Unit

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1996

Appendix. 5.1.1. Analysis of variance for total number of shoots produced from *in vitro*-grown leaf explant of strawberry cultivar Tango on different callus initiation media Table (5.1.1) n=15

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	5	899.87	179.97	3.55**
Residual	84	4258.53	50.70	
Total	89	5158.40		

Appendix 5.1.2. Analysis of variance for total number of plants produced that survived after transferring to glasshouse from *in vitro*-grown leaf explant of strawberry cultivar Tango on different callus initiation media Table (5.1.1) n=15

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	5	120.356	24.071	5.48***
Residual	84	368.933	4.392	
Total	89	489.289		

Appendix. 5.1.3. Analysis of variance for total number of shoots produced from glasshouse-grown leaf explant of strawberry cultivar Tango on different callus initiation media Table (5.1.1) n=15

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	5	422.19	84.44	3.78**
Residual	84	1876.53	22.34	
Total	89	2298.72		

Appendix. 5.1.4 Analysis of variance for total number of plants produced that survived after transferring to glasshouse from glasshouse-grown leaf explant of strawberry cultivar Tango on different callus initiation media Table (5.1.1) n=15

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	5	72.189	14.438	3.71**
Residual	84	326.933	3.892	
Total	89	399.122		

Appendix. 5.1.5. Analysis of variance for total number of shootsproduced from glasshouse-grown leaf explant. callus initiated on media 3 and transferred on different regeneration media described in Table (5.1.3) n=20

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	3	4154.7	1384.9	7.00***
Residual	76	15028.9	197.7	
Total	79	19183.6		

Appendix. 5.1.6. Analysis of variance for total plant survived from glass house-grown leaf explant. callus initiated on media 3 and transferred on different regeneration media described in Table (5.1.3) n=20

Source of variation	d.f	Sum of squares	Mean square	F-ratio
Media	3	179.44	59.81	4.81**
Residual	76	944.95	12.43	
Total	79	1124.39		

Appendix. 5.3.1. Analysis of variance of callus growth from leaf disc explant of blackcurrant cultivar Ben Sark on different levels of NAA and BAP. Levels of plant growth regulators tested ranged from 0-4.5 mg\l for NAA and 0-3 mg\l for BAP n = 5

Source of variation	d.f	Sum of squares	Mean square	F-ratio
NAA	3	1.0513	0.3504	3.72**
BAP	3	29.9696	9.9899	106.09***
Interaction	9	19.1348	2.1261	22.58***
Residual	64	6.0297	0.0942	
Total	79	56.1824		

Appendix. 5.3.2. Analysis of variance of callus growth from leaf petiole explant of blackcurrant cultivar Ben Sark on different levels of NAA and BAP.Levels of plant growth regulators tested ranged from 0-4.5 mg\l for NAA and 0-3 mg\l for BAP n = 5

Source of variation	d.f	Sum of squares	Mean square	F-ratio
NAA	3	4.5714	1.5238	37.46***
BAP	3	34.3026	11.4342	281.11***
Interaction	9	12.1348	1.3483	33.15***
Residual	64	2.6032	0.0407	
Total	79	53.6121		

Appendix 5.3.3. Analysis of variance of callus growth from leaf disc explant of blackcurrant cultivar Ben Sark on different levels of IBA and BAP. Levels of plant growth regulators tested ranged from 0-4.5 mg\l for IBA and 0-0.6 mg\l for BAP n = 5

Source of variation	d.f	Sum of squares	Mean square	F-ratio
IBA	3	7.838	2.613	46.54***
BAP	3	3.188	1.063	18.93***
Interaction	9	5.477	0.6085	10.84***
Residual	64	3.593	0.0561	
Total	79	20.095		

Appendix. 5.3.4. Analysis of variance of callus growth from leaf disc explant of blackcurrant cultivar Ben Sark on different levels of IAA and BAP. Levels of plant growth regulators tested ranged from 0-4.5 mg\l for IAA and 0-0.3 mg\l for BAP n = 5

Source of variation	d.f	Sum of squares	Mean square	F-ratio
IAA	3	20.6801	6.8934	72.38***
BAP	3	1.7890	0.5963	6.26***
Interaction	9	3.5404	0.3934	4.13***
Residual	64	6.0945	0.0952	
Total	79	32.1049		