AN ABSTRACT OF THE THESIS OF

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Genetic stability of micropropagated and cold-stored *Fragaria x anannasa* cultivar 'Pocahontas' was evaluated by Random Amplified Polymorphic DNA (RAPD) assay. Plantlets grown for eight months on M & S medium with 5 μM and 15 μM N6-benzyladenine (BA) were analyzed. No evidence of mutations was seen in over 200 loci that were PCR amplified with 29 of the 30 Operon primers used. Pocahontas *in vitro* plantlets stored for over 4 years in gas-permeable plastic bags at 4°C also showed no evidence of mutations for the same loci. One primer, OPF-18 (TTCCCGGGTT), generated DNA profiles which were polymorphic for all the BA treated and cold-stored plantlets analyzed. The polymorphism seen was not completely reproducible and no pattern matching any treatment was seen. Southern blots in concert with Hpa II and Msp I restriction enzymes were used to assay for methylation pattern changes. No differences from control plants were seen in most of the samples. One sample from 15 μM BA treatment and one cold-stored sample displayed methylation on the external cytosine in the

CCGG restriction site which was not seen in controls or in other samples. Field-grown plants of BA and cold-stored treatments showed methylation patterns similar to that of controls. This indicates that changes seen were due to tissue culture stress. Field grown plants from 5 μM and 15 μM BA treatments were not significantly different in morphological characteristics, except for plant height. Significant interactions between treatments and explant source were seen for some characters. Cold-stored plants were significantly different from the 5 μM BA treatment plants for most morphological characters. Plants showed less vegetative and reproductive vigor and two-thirds of the plants showed late or no flowering. A variety of factors influenced the RAPD-PCR reaction including the reaction volume, primer, and $MgCl_2$. Optimization for each sample and primer was found to be important.

Genetic Stability of Micropropagated and in vitro Cold-stored Strawberries

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

October 14, 1995

Mohan B. Kumar, Author

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Dr. Barbara Reed was involved in all the stages of experimental design, discussion, support and editing. Dr. Reed Barker let me use his laboratory facilities for molecular biological experiments and assisted with the interpretation of the RAPD results.

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Chapter 1

Introduction

Somaclonal variations in micropropagated strawberries have been reported for a number of cultivars. Variations like white-streak, dwarfness, and chlorosis occur in some strawberry cultivars at high frequency (Sansavini et al., 1990). Thus, micropropagation as a means to produce uniform, true-to-type strawberry nursery stock could be jeopardized.

Some abnormalities in morphology and function can be attributed to epigenetic changes, but others like dwarfism and chlorosis are nuclearly heritable (Sansavini et al., 1990). The role of the tissue culture process and growth regulators in medium on somaclonal variation has been addressed in many studies (Beech et al., 1988; Marcotrigiano et al., 1984; Nehra et al., 1994). So, epigenetic effects and somaclonal variations need to be separated.

This thesis addresses the issue of the effects of N^6-benzyladenine (BA) levels and cold storage on genetic stability and morphological behavior of the strawberry plant upon transplantation. In the studies described, Random Amplified Polymorphic DNA (RAPD) markers were used to determine if BA levels or cold storage lead to genetic changes.

Secondly, studies into methylation pattern changes in the BA-treated and cold-stored plants were undertaken. Methylation patterns have been shown to be altered under

tissue culture conditions, and some growth regulators like IAA and inositol have been implicated in hypo- and hypermethylation of the genome (Arnholdt-Schmitt, 1993). Studies were conducted to assay changes in methylation patterns in BA-treated and cold-stored plantlets. Some field planted material was also analyzed. Finally, morphological and agronomic characteristics of field grown BA and cold-store treatment plants were examined to determine if there were any variation in morphological characteristics.

Chapter 2

Literature Review

Somaclonal variations are any variations from the original, in plants derived from any form of cell, tissue, and organ culture (Larkin and Scowcroft, 1981). Somaclonal variations have been described for many plant species (Garcia et al., 1994) including some strawberry cultivars (Sansavini et al., 1990; Nehra et al., 1994).

Strawberries *(Fragaria sp.)* are propagated by tissue culture to produce true-to-type, (Beech, 1988) virus free clones at a rapid rate (Beech et al.,1988; Moore, 1991; Swartz et al., 1981). However, occasionally micropropagation in strawberries results in variants which are markedly different from mother plants and other tissue culture plantlets (Anderson et al., 1982; Swartz et al., 1981). Variants are much more prevalent in callus cultures (Nehra et al., 1992) although somaclonal variations also occur in meristem cultures too(Sansavini et al., 1990). Common variants seen are dwarf plants, yellow leaves, and white-streaked leaf chlorosis (Swartz et al., 1981). Some of these variations are due to physiological changes and the plants revert back to normal after a period of time, while others persist and remain stable through runner propagation (Nehra et al., 1992) and later sexual generations (Sansavini et al., 1990).

2. 1. Strawberries

2.1.1. Micropropagation

Micropropagation does not generally lead to somaclonal variations, but strawberries are an exception, in that high frequencies of somaclonal variations have been seen in some cultivars. Seven cultivars of micropropagated *Fragaria* evaluated by Sansavini et al. (1990) for somaclonal variations showed differential susceptibility, with cultivar 'Pocahontas' registering the highest incidence of somaclonal variation. Chlorophyll mutants, white stripe, chlorosis and dwarfism were the predominant variations. Symptoms were heritable, affecting selfed (S1) and F1 progenies. *Fragaria annanassa* cultivars 'Redcoat' and 'Veestar' propagated through meristem culture, callus culture, and direct shoot regeneration (from leaf discs) showed genotype and culture dependent phenotypic changes (Nehra et al., 1994). *In vitro* propagated plants had fewer stolons per plant, and more leaves and runners the following year, than standard runner propagated plants. 'Redcoat' flowered earlier and produced more fruits. Micropropagated plants produced more fruits in general. Yellow leaf variants occurred in micropropagated and callus culture regenerated plants (Nehra et al., 1994).

The frequency of multi-apex abnormality in micropropagated strawberries was found to be genotype dependent and the presence of indole butryic acid (IBA), or higher levels of N6-benzyladenine (BA) in the culture medium increased multi-apexing, while it decreased with the addition of gibberellic acid (GA_3) (Anderson et al., 1982). White-streaking, chlorophyll, pronounced yellow mottling, and mosaic mutants occurred at low frequency in strawberries (Boxus, 1989; Swartz et al., 1981). Swartz et al. (1981) also noted the presence of appreciable variation in yield and agronomic characteristics between

micropropagated and runner-propagated strawberries. Plants showed increased vigor and hence small fruit size. Marcotrigiano et al. (1987) observed that 90% of the micropropagated plants of *F. vesca albo marginata* were not true to type. Beech et al., (1988) reported that with cytokinin concentrations between 0.5 and 5.0 μM in the medium, in subsequent cropping and vegetative performance of runner progeny, few significant interactions occurred between the varieties studied and the *in vitro* culture conditions imposed in strawberry micropropagation. These interactions were minimal and restricted to the first year.

Merkle (1993) found that micropropagated strawberry plants had more inflorescence/plant, inflorescence/crown, and flowers/plant, but smaller fruits than standard runner propagated plants. Yield and runner production were growth regulator dependent. Micropropagated plants produced more runners than traditionally propagated plants of strawberry cultivars 'Douglas' and 'Chandler'. The BA concentration (1.48-4.44 μM), salt formulations, or the number of subcultures (1-8), did not influence field behavior (Lopez-Aranda et al., 1994). Moore et al. (1991) discovered that yields of micropropagated 'Olympus' strawberries were no greater than runner-propagated ones, but variability among subclones was high for newly planted clones. These differences were lost upon runner propagation for four years (Moore et al., 1991)

2.1.2. Callus culture regeneration

Callus cultures have been known to produce a wide variety of variants and at a higher frequency than micropropagation. Frequency (10%) of dwarf strawberry plants, when regenerated from callus culture, was found to increase with higher concentrations (20 μM each) of BA and 2,4-Dichlorophenoxyacetic Acid (2,4-D). Frequency of deformed leaf and yellow leaf variants, which were stable through runner propagation was high in 16- and 24-week old callus regenerants. Variants were not polymorphic for isozyme profiles, but plants with variant leaf shapes were aneuploid (Nehra et al., 1992). Ploidy changes were callus age dependent in strawberry callus cultures. Calli initiated from *in-vitro*-leaf explants were more susceptible to ploidy changes than those of green-house-leaf explants. Morphogenetic ability of calli were influenced by ploidy levels, with increased ploidy levels leading to decreased morphogenetic ability (Nehra et al., 1991). Esterase isozyme banding pattern polymorphism was seen in callus culture regenerated strawberry plants, but profiles for four other isozymes were monomorphic (Nehra et al., 1991).

2.2. Other genera

2.2.1. Micropropagation

Micropropagation by axillary bud multiplication does not lead to genetic variation in general, but some prominent examples of somaclonal variants exist.

Walden et al. (1989) found no evidence of somaclonal variations in the case of maize meristem cultures. No somaclonal variations were seen in greenhouse-planted wild beet (*Beta maritima*) after micropropagation from inflorescence pieces when an optimized medium and proper explants (inflorescence tips collected before flowering) were adopted (Zhong et al., 1993). Coleus micropropagation induced epigenetic and heritable changes in leaf variegation. The frequency of variation was genotype dependent (Marcotrigiano et al., 1990).

2.2.2. Callus and suspension-culture regenerated plants

Mutations (sterility, isoenzyme variants, abnormal leaf morphology, chlorophyll deficiency) were isolated in soybeans regenerated from callus (Amberger et al., 1992 and b). Long-term callus cultures of *Allium* exhibited cytological abnormalities including direct elimination of chromatin from the nuclei, multiple chromosome fusions, and formation of polycentric and megachromosomes. The rate of abnormalities increased with culture age (Joachimiak et al., 1993).

Mikhailova-Krumova et al. (1991) found that seeds of pea-callus-culture regenerants showed variation in a legumin polypeptide. Increase in culture duration reduced the amounts of the protein. Many regenerants displayed an altered amino acid balance with drastic changes in the levels of methionine and phenylalanine. Restriction Fragment Length Polymorphism (RFLP) analysis revealed random DNA polymorphism in rice callus cultures, their suspension cultures and regenerants with a similar degree of polymorphism in callus and suspension cultures (Brown et al., 1990).

When generated from immature-embryo cultures, triticale lines showed that lines with telomeric heterochromatin gave rise to more variants than ones without heterochromatin (Bebeli et al., 1993). Prolonged culture in 2,4-D, along with maintenance and regeneration phases, induced changes in the callus culture regenerants of poplar. Stem height and gas exchange had an inverse relationship with BA concentration and a complex relationship with 2,4-D. Gas exchange parameters and morphological characteristics were correlated. Leaf shape variation was found to be an early indicator of divergence (Saieed et al., 1994). Plants propagated by axillary budding (axillary bud proliferation) based on *in vitro* techniques showed no morphological or fruit composition variation in the field, but somatic embryogenesis-regenerated plants of another cultivar showed drastic changes in the sucrose and amino acid composition compared to conventionally produced plants (Booij et al., 1993).

Maize regenerants from immature embryos and their testcross lines generally had lower yield and moisture. Grain yield and plant height decreased with culture age. A great deal of variation for agronomic characteristics was seen among the regenerants (Lee et al., 1988). Embryogenic-callus culture of finger millet was directly implicated in inducing somaclonal variations similar to mutagen treatments and produced a wide range of variations for almost all traits (Pius et al., 1994).

An albino mutant of sunflower was isolated from the selfed progeny of an

in vitro regenerated plantlet. The mutant was defective in anthocyanin biosynthesis, had non-dormant seeds, and low levels of endogenous ABA in cotyledons and leaves which did not increase after drought stress (Fambrini et al., 1993). Genotype dependent variations in levels of chlorophyll a and b and carotenoid content were seen in embryogenic-callus-regenerated king grass plants (Lauzan et al., 1991). Albino plants were detected in immature-embryo regenerants in rye. The frequency of albino plants was similar in the case of embryogenic and organogenic culture regenerants, but the frequency was genotype dependent (Linacero and Vazquez, 1992).

2.3. Types of variants

2.3.1. Isozyme variants

Isozymes are enzymes of which have same function (catalyze the same reaction). Variations in isozyme patterns have been seen in cultured plants. Regenerated tall fescue plantlets derived from shoot-tip callus and suspension culture showed instability in flowering response (with some plants flowering without vernalization during the first year), chromosome organization, pollen fertility, and phosphoglucoisomerase isoenzyme locus (Garcia et al., 1994). No variations in isoenzyme patterns of peroxidases, acid phospatases, 6-phosphogluconate dehydrogenases, and glutamate-oxaloacetate transaminases were seen in potato plantlets regenerated from cell suspensions, but esterase isoenzyme polymorphism was evident (Henn et al., 1993). Karyotype and esterase isoenzyme variation was seen in wheat somaclones, but no polymorphism was seen in three other isoenzyme patterns (Gaponenko et al., 1993).

Co-segregating phenotypes of chlorophyll deficiency and mitochondria malate-dehydrogenase null mutation were seen in one cultivar of soybean along with a null *aconitase-b* mutant of another cultivar upon regeneration via somatic embryogenesis (Amberger et al., 1992). Eight of 18 Persimmon 'Meotagaki' plantlets regenerated from anther-derived callus showed different malate dehydrogenase isozyme profiles than that of field grown trees, but plantlets regenerated from leaf primordia derived calli had normal profiles (Tao et al., 1992).

2.3.2. DNA abnormalities

A variety of DNA abnormalities have been seen in cultured plants. These include chromosome number changes, chromosomal rearrangements, mutations, etc. More instances of DNA abnormalities have been elucidated in callus and cell suspension cultures than in the case of micropropagation.

2.3.2.1. Chromosome number changes

In the case of *Chrysanthemum* , embryogenesis was induced from sectors of diploid calli and non-morphogenic sectors were found to have chromosome numbers ranging from 2n=12 to 72. While diploid regenerated plants were morphologically stable, the chromosomes from non-embryogenic calli showed abnormal behavior during mitosis (Pal, 1992).

On the other hand, Shepard et al., (1980) reported that protoclones of potato possessed a normal karyotype. Petunia plants derived from leaf discs and mesophyll protoplasts, exhibited a high frequency of genetic and chromosomal variation with only

25% of the plants having the normal diploid complement. Aneuploidy in two plants was associated with abnormal development. Heritable morphological differences were documented in other callus derived *Petunia* plants (Lewis-Smith et al., 1990). Meiotic chromosomal changes were seen in primary regenerants from a single embryo culture of winter wheat with translocations being the most common. A third of the wheat plants were chimeras (Whelan, 1990). Frequency of off-types in leaf and head morphology increased with number of subcultures in the case of micropropagated globe artichokes. Off-types were found to have changes in ploidy levels or were chimerical (Pecaut and Martin, 1992). Six to thirty-six week old calli of ornamental *Phlox drummondii* Hook, derived from diploid internodal-segment explants, showed a wide ranging variation in chromosome numbers (from 2n=14 to 100) and 2C nuclear DNA amounts (8.2 to 63.2 pg). The autotetraploid cultures had the same chromosome number and DNA content as that of the autotetraploid mother plants (Raja et al., 1992).

Early prophase nuclei of callus regenerated *Aloe* plants showed 22.5% basic DNA content variation among the diploid plants. Increases and decreases in DNA content related to euchromatin and heterochromatin and rDNA content was also seen. Nuclear DNA content was found to be positively correlated to cell dimensions (Cavallini, 1993).

When chromosome stability of cell suspensions of diploid, tetraploid and hexaploid wheat was compared, differences were seen between ploidy levels and between species within ploidy levels; with diploids the most stable and hexaploids the least stable. Instability was found to be greatest during the first months of culture in all wheat lines. Very few structural changes were found in most lines although morphogenetic capacity was lost in all lines after many months of culture (Winfield et al., 1993).

Deletions of 10-20% of the arms was seen in maize regenerants (Lee and Phillips, 1987).

2.3.2.2. Single gene and gene family mutations

Callus culture regenerants of *Petunia hybrida* showed copy number changes of the 18S-25S rRNA genes (by a factor of 10). A heritable change in the length of the major 5S rDNA repeat was also observed in one petunia somaclone (Anderson et al., 1991). One somaclonal variant regenerated from immature embryos was found to possess an electrophoretic variant of the maize *Adh1* gene (Brettel et al., 1986) which had a single base pair mutation (Dennis et al., 1984). A null Adh1 allele which arose as a somaclonal variant had a point mutation leading to a stop codon (Dennis et al., 1987). All but one family of rRNA genes were found to be stable in plants derived from immature embryo culture of Triticale. The reduction in rRNA copy number was heritable and correlated to reduced C-banding at the *Nor R1* position on chromosome 1R (Brettell et al., 1986).

2.3.2.3. mtDNA somaclonal variations

Multi-recombination events involving mtDNA repeats and deletions, resulting in the formation of new "master chromosomes", were implicated in mtDNA rearrangements in maize cytoplasmically male-sterile type T callus-culture regenerants (Fauron et al., 1992). Restriction analysis of mtDNA revealed structural alterations in callus, suspension cultures from the callus, and regenerated plants of sugar beet (Dikalova et al., 1993). Amplification and deamplification of rearranged forms of mtDNA in varying proportions was found in turnip callus cultures (Shirzadegan et al., 1991).

Structural rearrangements, as a result of reciprocal recombination events, were detected in progeny of immature embryo regenerants of wheat cv. 'Chinese Spring' (Hartmann et al., 1994). Organization of the mitochondrial genome in wheat callus culture was found to be associated with its regeneration capacity. Novel subgenomic

configurations were detected, some of which were organ/tissue specific. Age of culture determined the degree of mtDNA rearrangement. Not all of these novel organizations were systematically detected in all regenerated wheat plants, but were detected in some after passage out of *in vitro* culture (Morere-Le-Paven et al., 1992).

A 14.7 kb EcorRI region with *atpA*, *atp6*, and *cob* genes, was found to be highly variable when sorghum cells were subjected to protoplast culture, but was stable *in planta* (Kane et al., 1992). Point mutations and gross rearrangements were seen in mtDNA in one tobacco cell culture variant resulting in resistance to inhibition of respiration by auxin 2,4-D (Hakui and Nakamura, 1991).

2.3.2.4. Repeat DNA mutations

Significant copy number differences were seen in two anonymous DNA sequences and pea rDNA homologues in alfalfa callus (from immature ovaries) culture regenerants (Kidwell and Osborn, 1993). Methylation patterns and copy numbers of tandemly repeated sequences were identical in short-term callus cultures, regenerated plants, and the explant (cotyledons), but were found to be different in the case of two dispersed repeated sequence families. Copy number of the latter, present in both the nuclear and mitochondrial genomes, was drastically reduced in long term callus cultures (Grisvard et al., 1990).

Chromosomal rearrangements, including a deletion and an isochromosome, were detected using C-banding in the case of an alfalfa regenerant (Masoud et al., 1991). Alteration in gene copy number and RFLPs were seen in the process of callus formation, but were reduced once differentiation and organogenesis began leading to similarity between regenerated plants and controls (Brown et al., 1990).

Vyskot et al. (1991) found contents of moderately repetitive sequences were more stable than highly repetitive sequences (which showed a reduction in number) in protoplast derived regenerants. DNA variability in source material and *in vitro* stress during early stages of regeneration of protoclones greatly influenced formation of modified genomes. Karyological studies and southern blots showed ploidy level changes and RFLPs respectively. Vosman et al., (1992) found that $(GATA)_4$ DNA sequences in callus culture regenerated tomato plants showed no polymorphism. The approximate percentage of variant alleles occurring in any one somaclonal regenerant of sugar, fodder and garden beet was 0.05% isozyme and 0.1% for restriction fragments (Sabir et al., 1992).

2.3.2.5. Random Amplified Polymorphic DNA (RAPD)

RAPD analysis of callus culture regenerated barley plants revealed significant differences in profiles for different regenerants pointing to somaclonal variations (Gozukirmizi et al., 1993). RAPD markers used to determine the genetic stability of somatic embryogenesis derived populations of black spruce detected no variation within clones (Isabel et al., 1993). RAPD analysis detected pre-existing variation, but no *de novo* variation in the protoplast derived plants when compared to their suspension culture regenerants (Valles et al., 1993).

RAPDs used for studying taxonomic relationships of *Brassica*, *Sinapis*, and *Raphanus* demonstrated the usefulness of RAPD markers in distinguishing related plants. A minimum of 10 primers with approximately 100 bands were needed to decipher relationships (Demeke et al., 1992). Over 60% of the 43 primers yielded polymorphic amplification profiles in verifying the identity of strawberry cultivars and in developing a genetic map of the octaploid cultivated strawberry (Hancock et al., 1994). RAPDs were used in determining genetic variation in outcrossing buffalograss (Huff et al., 1993).

2.3.2.6. Methylation changes

Methylation changes (increase and decrease) occurred in callus culture regenerated maize plants and were stably inherited over two generations (Kaeppler and Phillips, 1993). DNA polymorphism was seen among rice plants regenerated from different callus cultures and sibling plants from a single callus. Basal medium did not influence genetic stability, but the age of the callus was a major factor. In this case, methylation changes and DNA polymorphism were closely correlated (Muller et al., 1990). Changes in genomic methylation and copy number of DNA sequences (deamplification) occurred to a great extent in carrot callus cultures. Growth regulators indirectly influenced the amplification process by regulating cell division (Arnholdt-Schmitt, 1993). DNA content and methylation changes were genotype dependent in callus and regenerated shoots of pea (Cecchini et al., 1992). Repeat induced point mutations were also suspected to be active in undifferentiated callus cultures of plants (Phillips et al., 1994). Changes in methylation patterns were seen in suspension cultures of soybean; the patterns showed gradual alteration after prolonged culture (Quemada et al., 1987a). Cytokinin was found to have no effect on the variation in 5-methyl cytosine in carrot embryogenic-callus cultures, but exogenously added auxin positively correlated with methylation of cytosine. Induced changes were fast and reversible but when accompanied with hypermethylation or hypomethylation embryogenesis was irreversibly impaired (LoSchiavo et al., 1989). Rice protoplast culture regenerants displayed increased RFLPs compared to control plants. The polymorphisms were wide spread and were non-specific. Methylation changes were not found to be a major factor (Brown et al., 1990).

2.4. Absence of somaclonal variations

A number of examples of somaclonal variations have been reported among different forms of culture, but in a majority of cases tissue culture leads to uniform, true-to-type plants. Regenerated *Haworthia* plants from adventitious buds showed no somaclonal variations once transferred to the greenhouse (Rogers, 1993). Wofford et al., (1992) found that callus induction and regeneration in *Desmodium* was as much genotype dependent as it was medium dependent and regenerated plants showed no somaclonal variations. Limited negative variations were found in barley somaclones (Baillie et al., 1992).

2.5. Explant effect on somaclonal variations

Proper explant use is one of the most important aspects of tissue culture in order to control the frequency of variants. Explant choice was the most critical factor for maintenance of original foliar variegation in micropropagated *Rhododendron* with shoot tips being better than leaf or floret explant (Pogany and Lineberger, 1990). Strawberry regenerants were less genetically stable when leaf explants were employed. Genetic stability was also dependent on the degree of differentiation of somatic explants and age of donor mother plants. Regenerants from roots were the most unstable (Stanys, 1992). The source and location of explant was found to play an important role in stability of ploidy levels in melon regenerants. Immature cotyledons, proximal sections from immature cotyledons, and 18 to 22 days-after-planting-harvested embryos were found to produce high frequencies of tetraploids (Adelberg et al., 1994).

2.6. Genotype dependency of somaclonal variations

The adaptability of plant to tissue culture conditions mainly depends on its genotype. Each genotype responds to tissue culture and growth regulator conditions in a different way. Variant types increased with the number of subcultures in micropropagated globe artichokes. Leaf shape, offshoot and harvest date variations were genotype dependent (Pecaut and Martin, 1992). Regenerated plants of rye exhibited higher chiasma frequencies and more distally located chiasmata than source plants. The nature of cytological variation was genotype dependent (Puolimatka and Karp, 1993). Regenerants from immature-embryo cultures of rye had chromosomal mosaicism, abnormal meiosis, supernumery spikelets and albinism. Among variants, mutations were dominant or homozygous, more than one mutation was present in some cases and some loci were more susceptible than others. Frequency of somaclonal variations was genotype dependent (Linacero and Vazquez, 1992).

2.7. Mechanisms for somaclonal variations

Ever since somaclonal variations were observed in cultured plants, the possible mechanisms have been speculated and studied. Each variation may be due to different mechanism or a single mechanism could lead to different types of variations. Late or delayed replication of heterochromatin was implicated in a variety of chromosomal rearrangements in callus cultures of *Crepis* where breakpoints corresponded to late DNA synthesis regions (Sacristan, 1971). Simultaneous breaks in homologous chromosomes was implicated in duplications and deficiencies and simultaneous breaks in non-homologous chromosomes was suggested to lead to reciprocal interchanges in maize callus cultures (Lee and Phillips, 1987). Genotype dependency of frequency was suggested

to be related to the species specific distribution of heterochromatin blocks (Lee et al., 1988). Nucleotide pool imbalances was suggested to contribute to a variety of mutations-nuclear, chloroplast, and mtDNA- rearrangements, point mutations, mitotic recombination, and aneuploidy (Lee et al., 1988).

Significant changes in the oxygen regime of tissues and cells during culture in an *in vitro* environment result in an increase in level of free radical oxidation of membrane lipids (lipid peroxidation). This leads to oxidation products (oxygen radicals, conjugated diene, malondialdehyde and Schiff's base) that cause structural rearrangements of DNA such as single- and double-stranded breaks, malfunctioning of enzyme systems involved in realization and reproduction of genetic information, and methylation. The Fe-EDTA complex used in growth media was a strong pro-oxidant leading to lipid peroxidation (Konstantinov and Mashenenkov, 1990).

Regenerated maize plants from two independent embryo cell lines contain an active Ac transposable element, but original explants contained no active *Ac* element (Peschke et al., 1987). Subsequently, *Spm* activity was detected in two R1 progeny of a single regenerant plant derived from a friable embryogenic callus (Peschke and Phillips, 1991).

2.8. *In vitro* cold storage

In vitro cold storage is being used as an alternative form of germplasm conservation (Reed, 1991; 1992). *In vitro* cold storage combines the advantage of micropropagation with the advantages of smaller space and low-maintenance requirements, but its effect on the genetic stability of plants needs further investigation. Strawberries and other temperate species have been successfully cold-stored in gas-permeable plastic bags (Reed, 1991; 1993).

2.8.1. Survival during and after cold storage

Strawberry accessions cold-stored in gas-permeable polyethylene bags in hormone-free MS medium could be stored for a period of 15 to 18 months (Reed, 1991). Survival rate of *Rubus* plantlets stored in gas-permeable bags (at 4°C) on hormone-free MS medium was high and 12 h photoperiod was better than darkness (Reed, 1993). *In vitro* cold storage of micropropagated Rubus species at 4°C on a diluted MS medium devoid of growth regulators increased survival of plantlets and reduced proliferation (Hughes, 1991). Cold storage of *Rubus* in plastic bags delayed decline and reduced contamination as compared to cold storage in tubes (Reed, 1993). Cold storage of suspension cultures at 4°C extended the period of regeneration-competence and increased regeneration frequency (Creemers-Molenaar et al., 1992).

2.8.2. Morphological changes in cold-stored plants

Red raspberry plants which were *in vitro*-cold-stored had longer canes, and internodes, and fewer roots than greenhouse controls after field planting (Prive and Sullivan, 1991). Increasing post-*in vitro* storage duration of *Lilium* bulblets decreased time for first leaf emergence and percentage of field plants producing shoots within 36 weeks, but increased the number of days to shoot emergence, anthesis, leaf number and flower bud number (Higgins and Stimart, 1990). Albino, red pigmented, and rosette type growth pattern variants of hybrid poplar were seen following storage of shoot cultures at 4°C for 5 years. Local disruptions of cambium connections and accumulation of chemicals in some cells were evident upon examination after cold storage (Son et al., 1991). Cold-

stored somatic-embryo regenerated celery plantlets were morphologically identical to plants regenerated from non-stored embryos (Nadel et al., 1990).

Chapter 3

Molecular and Morphological Analysis of Micropropagated *Fragaria x annanasa* Cultivar 'Pocahontas'

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3.1. Abstract

Random Amplified Polymorphic DNA (RAPD) markers were used to determine if cold storage or supraoptimal levels of N6-benzyladenine (BA) in the culture medium caused genetic changes in micropropagated strawberries. Micropropagated *Fragaria x annanasa* 'Pocahonatas' plants stored at 4°C for over four years were compared with pot grown mother plants. Five ''Pocahontas'' explants were initiated into tissue culture, multiplied and later grown on medium with two different BA concentrations for seven months. Possible changes in methylation patterns of rRNA genes of strawberries were examined. No indication of mutation was detected in over 200 loci amplified by 29 of 30 Operon primers tested. One primer, OPF-18 (TTCCCGGGTT), generated inconsistent polymorphism which was random and did not correlate with any treatments. Changes in methylation patterns were observed in one sample from plants grown on 15 μM BA medium and in one of the cold-stored plants. Length polymorphism was observed in two samples from plantlets derived from one explant. Most of the morphological characteristics did not differ for field grown plants micropropagated on 5 μM and 15 μM BA medium, but cold-stored plants differed from BA-treated plants.

3.2 Introduction

Micropropagation of strawberries (*Fragaria sp.*) can lead to production of a relatively high proportion of somaclonal variations (up to 0.46% in 'Pocahontas') (Sansavini et al, 1990). Changes include leaf color variants and dwarf plants among others (Sansavini et al, 1990 ; Cameron and Hancock, 1986 ; Swartz et al., 1981). These variants pose a problem to production of uniform, true-to-type plants. Some symptoms are delayed and are masked by other variations (Sansavini et al, 1990). Some symptoms are epigenetic in nature and disappear over time (Koruza and Jeleska, 1993). Numerous authors report that genetic changes including insertions, deletions, point mutations, and other rearrangements occur in tissue culture conditions (Anderson et al., 1991; Kane et al., 1992) and few of the phenotypic symptoms are heritable (Sansavini et al., 1990)

Methylation pattern changes were detected in suspension cultures of soybean (*Glycine max.*) (Quemada et al., 1987) and maize callus culture regenerants (Kaeppler and Phillips, 1993). The role of growth regulators as agents of mutation was studied by several investigators. Arnholdt-Schmitt (1993) discovered that IAA and inositol in the growth medium caused DNA rearrangements and methylation changes in callus cultures. On the other hand, cytokinin levels did not influence cropping and vegetative performance of runner progeny of micropropagated strawberry (*Fragaria sp.*) plants (Beech et al, 1988).

The objective of this study was to determine if cold storage or BA concentration in the culture medium during multiplication, influenced the genetic stability of 'Pocahontas'

plantlets as detected by RAPD markers and planted and analyzed for differences in morphological characteristics.

3.3. Materials and methods

3.3.1. Effect of N^6-benzyladenine (BA) concentration

3.3.1.1. Plant material

Strawberry *Fragaria x annanasa* cv. 'Pocahontas' screenhouse mother plants were used for explant collection for initiation into micropropagation. Five runner tips of 'Pocahontas' were collected from two screenhouse mother plants. Explants were washed in running water for 10 min and then disinfected for 10 min with 10% (v/v) bleach (Clorox, Oakland, CA) (5.25% sodium hypochlorite) and a drop of Tween 20 (Sigma, St. Louis, MO) (50% lauric acid). The base of the explant was recut and placed in 16 mm glass tubes with 10 ml of NCGR-FRA medium: (Reed, 1991) M & S salts with NaH_2PO_4 (1.42 mM), adenine sulfate (0.434 mM), indole-3-acetic acid (IAA) (5.71 μM), giberrelllic Acid (GA_3) (0.26 μM) (Sigma, St. Louis, MO) with 4.4 μM of BA. Agar (Bitec, Difco,

Detroit, MI) and gelrite (Kelco, San Diego, CA) were added at the rate of 3 g l^{-1} and 1.25 g l^{-1} respectively.

3.3.1.2. Initial experiments

Initial experiments were conducted to determine threshold level of BA tolerance of strawberry plantlets. Plantlets were grown in Magenta boxes on NCGR-FRA medium with 5, 10, 15 or 20 mM BA. Concentrations of 5 and 15 μM BA were finally adopted for the experiment.

3.3.2. Culture conditions

Growth room conditions were 16 hr days (25 μEm^{-2} s^{-1} at the level of the plants) and 8 hr nights at 25°C. After six weeks plantlets were transferred to boxes with 40 ml NCGR-FRA medium with 4.4 μM BA for multiplication. After six weeks, ten uniform plantlets were randomly selected and placed in Magenta boxes (Magenta, Chicago, IL) on 40 ml of NCGR-FRA medium with 5 or 15μM BA.

Plantlets were subcultured onto the same medium every 3 weeks four times to obtain a total population of 400 plantlets for each treatment. From the fifth transfer onwards, one plantlet from each clump was chosen randomly to be transferred, and 20 plants were placed in each Magenta box. Plantlets were subcultured for a total of eight months before sample collection for DNA extraction. Twelve randomly chosen samples, (representing the five original explants) were initially used for RAPD analysis. Eighty

other samples were used for further RAPD analysis. Whole plantlets were used for DNA isolation. DNA from samples was immediately isolated or samples were stored at -70°C until extraction. Young leaf samples were collected from the two screenhouse mother plants for use as controls.

A completely randomized design was employed for the Magenta boxes in the growth room. Boxes were labeled with the treatment, explant number and date of transfer. Plantlets from each explant were distinctly maintained with four boxes per explant.

3.3.3. Cold storage

3.3.3.1. Plant material

Five 'Pocahontas' plants (A-E) which were *in vitro* cold-stored at 4°C for four years were used. Plantlets were stored in gas-permeable plastic bags ("Star*pacs", Agristar, Conroe, TX) with 10 ml of NCGR-FRA medium free of growth regulators with agar (3.5 g l^{-1}) and gelrite (1.45 g l^{-1}) in the medium. During storage period plantlets were removed from cold storage yearly and repropagated on NCGR-FRA medium for 3 to 4 subcultures of three weeks each and returned to NCGR-FRA medium without growth regulators in Star*pacs for further storage. After 4 yr. of storage, plantlets transferred to Magenta boxes with 40 ml of NCGR-FRA medium in the growth room. Plantlets were subcultured four times prior to sampling for DNA extraction.

3.3.3.2. Culture conditions

Culture conditions were identical to those in the BA experiments with 16 hr days (25 $\mu Em^{-2} s^{-1}$ at the level of the plants) and 8 hr nights at $25^{o}C$.

3.3.4. DNA isolation

Total cellular DNA from samples was extracted using a modified Weeden and Garvin minipreparation protocol (Torres et al., 1993) with 2% CTAB extraction buffer (with 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0). The DNA was quantified using a TKO-100 Minifluorometer (Hoefer Scientific Instruments, San Francisco) and diluted to 5 ng μl^{-1}. These samples were used for RAPD-PCR reactions. For follow-up experiments, leaf samples were collected from five screenhouse-grown (SH) cultivars ; 'Conrad', 'Earliglow', 'Guardian', 'Gorella' and 'Pocahontas'. Each cultivar was represented by two plants in the screenhouse and each was collected separately. Samples were collected on four separate dates from one week to three months apart. Freshly emerged leaves were also collected from two month-old pot grown cold-stored plants and DNA extracted for PCR amplification.

For the methylation experiments, total cellular DNA was isolated using a modified Doyle and Doyle DNA preparation with 4% CTAB (0.5% PVP, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0). DNA was quantified using a spectrophotometer. Samples were RNased an additional time (100 mg ml^{-1}) when absorbance values were higher than 1.8. These samples were used for digestions with restriction enzymes. Young whole daughter plants produced by field plants from the BA-treatments, cold-stored, and control plants were also used for DNA extraction along with *in vitro* samples.

3.3.5. Random Amplified Polymorphic DNA (RAPD) analysis

PCR amplification was carried out with 10-mer Operon oligonucleotides (Operon Technologies, Inc., Alameda, CA) using a cocktail adopted from Huff et al. (1993) with minor variations. The PCR reaction was initially optimized for DNA amplification. A range of DNA template, DNA polymerase and $MgCl_2$ concentrations were used in the process of optimization. The PCR amplifications were performed in 12 µl volumes. Conditions included 0.5 ng μl^{-1} strawberry DNA , Stoffel buffer (10 mM KCl and 10 mM Tris-HCl), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM Operon 10-mer primer and 0.042 U μl^{-1} of Amplitaq DNA polymerase, Stoffel fragment (Perkin Elmer, Norwalk, CT).

Reactions were carried out in 96-well flexible assay plates (Falcon, Oxnard, CA) with reaction mixtures overlaid with 3 drops of mineral oil. The thermocycle profile was adopted from Huff et al. (1993). Each thermocycle consisted of DNA melting at 94 °C for 30 sec, primer annealing at 36°C for 1 min, a 1 sec-1 ramp leading to 72°C for 2 min for primer extension. This cycle was repeated 43 times followed by an final extension at 72°C for 5 min. The thermocycle profile was preceded by an initial denaturation for 7 min at 94°C and an 1 min annealing at 36. DNA amplifications were performed in a PTC100 MJ thermocycler (MJ research, Inc., Watertown, MA).

3.3.5.1. Primer screening

Approximately one hundred 10-mer arbitrary primers were initially screened for suitability in terms of producing deep, low background bands. The PCR conditions used

for this purpose included 0.5 ng μl^{-1} of DNA and 1.0 U μl^{-1} of Amplitaq Polymerase, Stoffel fragment, of reaction volume with other components identical to conditions indicated earlier. A bulk sample of cold-stored plantlets (leaf samples) was used to screen primers. Young leaves from screenhouse (SH) mother plants were used as controls.

Almost all of the 100 primers screened yielded monomorphic amplification profiles. Band fragments ranged from 100 bp to more than 2 kb in length. Only five primers produced polymorphic amplification profiles during the screening process. Two primers OPM-11 and OPF-18 (with sequences GTCCACTGTG & TTCCCGGGTT) continued to produce polymorphic amplification profiles upon replication. OPM-11 profiles were later discarded as artifacts after experiments varying DNA, $MgCl_2$ concentrations and replications. A total of 30 arbitrary primers were chosen for final DNA amplification to generate a minimum of 200 bands for RAPD analysis.

3.3.5.2. Visualization and analysis

Amplification products were separated in 7.5% acrylamide/bis gel (37:1) in 0.375 M Tris. pH 8.8 buffer, and visualized using silver staining (Caetano-Anolles and Gresshoff, 1992) with minor variations. After electrophoresis, gels were fixed in a 7.5% acetic acid fixer solution for 10 minutes followed by 3 washes of 4 to 5 minutes in double-distilled water. Silver impregnation was performed with neutral silver solution (0.15% silver nitrate and 0.056% formaldehyde) for 10 minutes followed by a brief double-distilled water rinse. Image development was carried out with developer solution (3% sodium carbonate, 0.056% formaldehyde and 2 mg/l sodium thiosulfate) and the were dried between membranes. The gels were scored for polymorphism by visual scoring. All PCR reactions were replicated using the second subsample.

3.3.6. Methylation analysis of rDNA genes

Methylation changes in cold-stored and BA-treated samples as compared to control screenhouse-mother plants was analyzed using Southern blots. About 15 mg of DNA samples were digested with *Hpa II* and *Msp I* restriction enzymes (New England Biolabs, Beverly, MA) independently overnight. The samples were further digested with a fresh aliquot of enzymes for an additional 4 hr and run on 0.8% agarose (Gibco BRL, Gaithersburg, MD) gels using standard procedures. The DNA was Southern transferred overnight onto nylon membranes. All protocols were standard and performed according to procedures outlined in Current Protocols in Molecular Biology (1995). Random primed rDNA probes were hybridized to the nylon membranes overnight at 37 °C. Membranes were placed in film cassettes with Kodak film (Eastman Kodak Co., Rochester, N. Y.) and autoradiographed for one week at -70°C.

3.3.7. Morphological analysis of field-grown plants

One hundred plantlets from each treatment of the BA experiments and fifty cold-stored plantlets were planted in the field. Plants were potted in seedling mixture (containing peat moss, perlite and vermiculilte 1:1:1) in trays, placed in mist bed for a week, then moved to greenhouse benches. After 7 to 10 days, when sufficient roots had been produced, they were moved to 600 c.c. pots with potting mixture (fir bark, peat and vermiculite 2:1:1) and placed in greenhouses to be hardened for 3 to 4 weeks. Plants were planted in the field in matted rows 30" apart with 12" between plants. A completely randomized design was employed. Control plants (crown divisions), were planted, but were not used for comparison because of their extremely low vigor.

3.3.9. Data collection and statistical analysis

A total of 13 morphological characteristics were measured for each plant. The parameters included leaf length, angle (leaf angle was defined as the angle between the lines connecting the first serration and the base of the leaf), width, petiole length, runnering, plant height, spread, vigor (rated 1-5 with 5 being most vigorous), number of trusses, average number of fruits per truss, primary fruit weight, color (rated 1-5 with 1 being white and 5 being dark red) and shape(1-globose, 2-conical, 3 malformed), and total fruit weight. All the leaf characteristics were measured on three different central leaflets.

Data was evaluated with one-way analysis of variance (ANOVA) along with multifactorial analysis of variance using Statgraphics Statistical Graphics System (Statgraphics Graphics Corp., Rockville, Madison, MD) and SAS Analytical System (SAS Institute Inc., Cary, NC).

3.4. Results and discussion

3.4.1. Tissue culture morphology

'Pocahontas' plantlets grown on 15 μM BA medium were compact and pale green in color while those on 5 μM BA were taller and dark green (Fig. 3.1). Height variation persisted in the potted plants. Cold-stored plants were not morphologically different from 5 μM BA plants.

Fig. 3.1. 'Pocahontas' plants grown on 5 μM or 15 μM BA medium for 8 months were transplanted to pots for hardening. 5 μM BA plant (right) and 15 μM BA plant (left) showing differences in height.

3.4.2 Random Amplified Polymorphic DNA (RAPD)

3.4.2.1. BA treatments

The BA treatments did not yield polymorphism in any samples when 29 of 30 primers were used for DNA amplification. Number of bands in a profile varied between 3 to 18 depending on primer (Fig. 3.2.; primer OPA-F profile is shown here). Primer OPF-18 produced wide-spread polymorphism among all samples. Amplification profiles for the two SH mother plants were also polymorphic for OPF-18 (lanes 1 and 12). SH controls showed variations between PCR reactions run using the DNA sample from the same extraction. No pattern correlating to any treatment or explant could be deciphered for

OPF-18 primer amplification (Fig.3.3.). Negative controls devoid of DNA in reaction mixtures, yielded no amplification products for primers used, including OPF-18.

3.4.2.2. Cold-stored plants

Five cold-stored samples were analyzed using 30 primers. Twenty eight of the primers yielded monomorphic profiles. OPM-11 and OPF-18 produced polymorphism, but as earlier, OPM-11 was discarded. Profiles for OPF-18 primer were highly polymorphic for all samples analyzed. Profiles were not entirely reproducible across different PCR reactions.

Follow-up experiments with the OPF-18 primer were conducted to determine if the PCR reaction conditions were optimal. OPF-18 PCR amplification profiles of DNA samples from 'Pocahontas' and four other cultivars were polymorphic for samples collected from the same plant on three different dates (Fig. 3.6). DNA amplification profiles were monomorphic and reproducible when a primer which has 60% sequence identity- OPF-17 (AACCCGGGTT) was used.

The PCR conditions were further optimized for the OPF-18 primer by varying $MgCl_2$ concentration (from 2.5 to 3.5 mM) and reaction volume (from 12 µl to 36 µl). In both cases number of bands was reduced and the profiles were slightly more reproducible when compared to original conditions. In general, there was a reduction in number of amplification products in cold-stored sample profiles when compared to screenhouse samples.

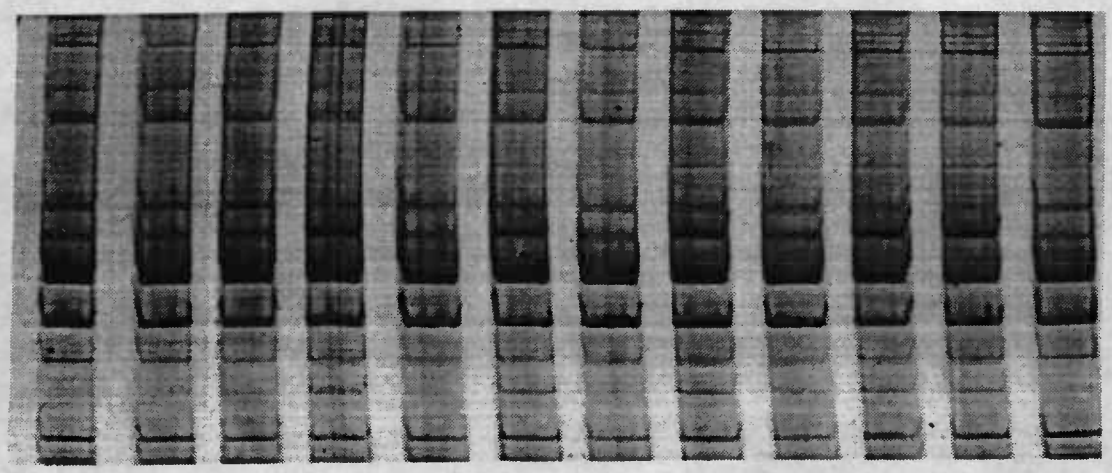

Fig. 3.2. DNA profiles generated by OPA-7 primer (GAAACGGGTT) of 'Pocahontas' plants grown on 5 µM BA or 15 µM BA for 8 months shown with mother plant controls. Lane 1. Screenhouse A. 2. Treatment5/ Runner-explant1. 3. Treatment15/R1. 4. Treatment5/R2. 5. Treatment15/R2. 6. Treatment5/R3. 7. Treatment15/R3. 8. Treatment5/R4. 9. Treatment15/R4. 10. Treatment5/R5. 11. Treatment15/R5. 12. ScreenhouseB.

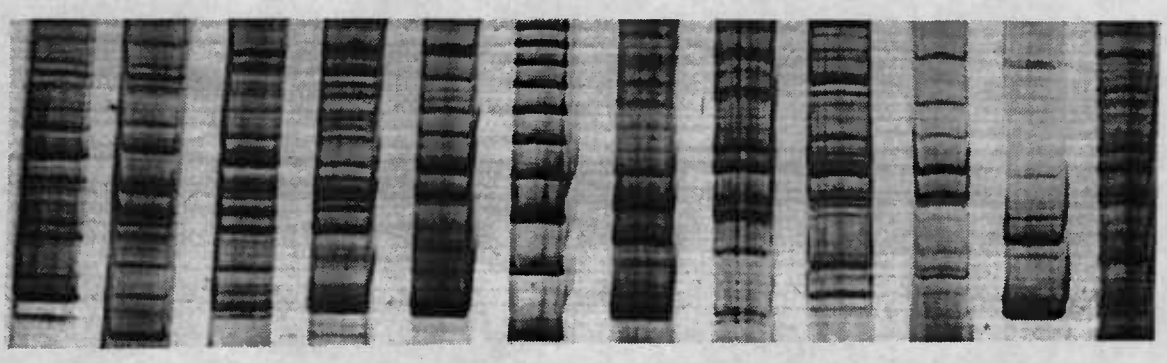

Fig. 3.3. DNA profiles generated by OPF-18 primer (TTCCCGGGTT) of 'Pocahontas' plants grown on 5 µM BA or 15 µM BA for 8 months shown with mother plant controls. Lane 1. Treatment5/ Runner-explant1. 2. Treatment15/R1. 3. Treatment5/R2. 4. Treatment15/R2. 5. Treatment5/R3. 6. DNA marker.7. Treatment15/R3. 8. Treatment5/R4. 9. Treatment15/R4 10. Treatment5/R5.11. Treatment15/R5. 12. ScreenhouseB.

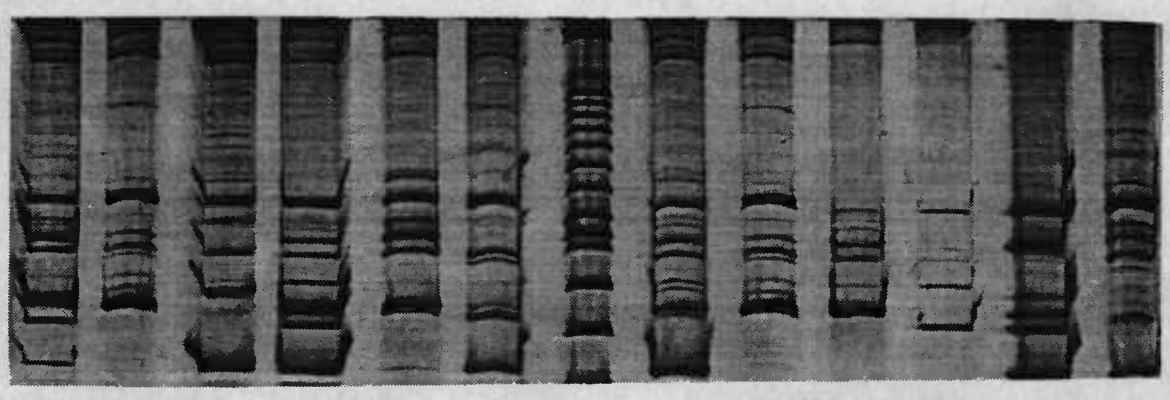

Fig. 3.4. DNA smaples from five different strawberry cultivars were collected at three different times and amplified with OPF-18 primer (TTCCCGGGTT).
Lane 1. 'Pocahontas' SH A (7/13). 2. SH A (7/19). 3. SH A (7/29). 4. 'Pocahontas' SH B (7/13). 5. SH B (7/19). 6. SH B (7/29). 7. DNA marker. 8. 'Guardian' SH A (7/13). 9. SH A (7/19). 10. SH A (7/29). 11. SH B (7/13). 12. SH B (7/19). 13. SH B (7/29). Numbers in parentheses indicate month and date of collection in 1994.

While 29 of the 30 primers revealed no evidence of mutations, OPF-18 primer yielded high polymorphism under certain conditions (the primers, their sequences and number of amplified fragments are presented in Table 3.1). Profiles at high reaction volumes and high $MgCl_2$ concentration were slightly more reproducible in that the main bands were repeatable, although profiles were still polymorphic and showed reduction in number of RAPD alleles of the OPF-18 primer. The OPF-18 loci polymorphism may be the result of non-specific priming or it may be a highly variable repeat. The OPF-18 primer contained a CCGG sequence in the middle. Internal cytosine in CCGG sequences is often methylated which, upon spontaneous deamination, leads to a C-->T transition mutation. A

high proportion of base pair mutations are repaired, but about 6% are not (Phillips et al., 1994). The high level of OPF-18 polymorphism seen with the micropropagated plants could be the result of methylation induced point mutations. Loss in the number of OPF-18 alleles in tissue culture and cold-stored plants may be due to loss of priming sites for amplification following point mutations. Variation in amplification profiles were seen when single base-pair mutations were introduced in various sites of a single primer (Tingey et al., 1992). Methylation induced variation would be totally random which would make profiles highly polymorphic, but presence of polymorphism in samples collected from the same plant on different dates is hard to explain. Optimization using a higher reaction volume reduced level of polymorphism significantly, but the role of methylation in these loci needs to be further investigated. This polymorphism may also be the result of deamplification. Deletions and rearrangements in repeat DNA were demonstrated under pressure in other organisms (Smith, 1976). The fact that over 96% of the primers revealed no sign of mutation, points to the stability of these alleles after micropropagation and four years of cold storage.

Table 3.1. Primers used for DNA amplification and their sequences.

Primer ID	Sequence	Number of amplified fragments
OPA1	CAGGCCCTTC	8
OPA2	TGCCGAGCTG	11
OPA7	GAAACGGGTG	8
OPA11	CAATCGCCGT	8
OPA20	GTTGCGATCC	7
OPB10	CTGCTGGGAC	12
OPC9	CTCACCGTCC	6
OPC17	TTCCCCCCAG	6
OPD1	ACCGCGAAGG	8
OPD2	GGACCCAACC	4
OPD11	AGCGCCATTG	19
OPD18	GAGAGCCAAC	4
OPF2	GAGGATCCCT	3
OPF3	GGGATATCGG	8
OPF7	CCGATATCCC	14
OPF9	CCAAGCTTCC	2
OPF10	GGAAGCTTGG	10
OPF17	AACCCGGGAA	8
OPF18	TTCCCGGGTT	5-17*
OPH4	GGAAGTCGCC	4
OPH5	AGTCGTCCCC	7
OPH8	GAAACACCCC	8
OPH11	CTTCCGCAGT	9
OPH12	ACGCGCATGT	10
OPH14	ACCAGGTTGG	12
OPH15	AATGGCGCAG	10
OPM11	GTCCACTGTG	7
OPM17	TCAGTCCGGG	7
OPM20	AGGTCTTGGG	8
OPZ2	CCTACGGGGA	13
OPZ6	GTGCCGTTCA	12

* Polymorphism seen, but profiles not reproducible

3.4.3. Methylation studies

3.4.3.1. BA treatments

Methylation changes were detected in only one DNA sample (treatment 15/ runner-explant 5 representative; Fig. 3.6.; lanes 12-15) when probed with a 3.11 kb *Bam* HI fragment containing 18S, 5.8S and a part of the 26S rRNA genes. All other samples showed no indications of methylation changes (Fig. 3.5.). Both *Msp I* and *Hpa II* restriction enzymes were unable to digest the DNA. This would indicate methylation on the external cytosine of the CCGG restriction site (although partial digestion cannot be ruled out). External cytosine methylation was not found in other samples or the control plants. In all other samples *Hpa II*, as expected, was unable to digest DNA when methylated. Differences in hybridization signals were also seen in some samples and may indicate differences in copy number of rRNA genes. Length polymorphism was seen one sample from explant 5. Field plants from BA-treatments were also analyzed. Differences in methylation patterns were seen between field planted and *in vitro* samples from one treatment (15 μM BA/Runner-explant 5). Methylation studies were carried on a limited number of samples and these results need to be further substantiated.

3.4.3.2. Cold storage

Changes in methylation patterns were seen in one sample from cold-stored plants. External cytosine in the CCGG sequence was methylated. There were also changes in hybridization signals with other samples. Differences in methylation patterns were seen between field grown and *in vitro* samples in one case, sample E (Fig. 3.6; lanes 8-11).

rRNA genes are tandemly arranged multi-copy genes. Brettel et al. (1986) reported that rDNA spacer sequences were stable in plants derived from immature embryo

culture, but in one family of rRNA (IR rDNA spacer sequence) genes, a marked decrease in copy number was seen. The overall stability of strawberry rRNA genes under tissue culture with high BA concentrations was also evident in most of our samples. There were changes in methylation patterns between field grown and *in vitro* samples in which case the methylation pattern of the field grown plants resembled controls.

Many sequences of the plant genome are highly methylated. Estimates indicate about 32% methylation of various sequences in plant genomes (Brown, 1989). Methylation patterns may be altered as a result of tissue culture in regenerated plants (Brown, 1989). A considerable proportion of 5-methylated cytosine (m^5C) in plants is in CpG and CpNpG sequences. Very little is known about the role of methylation and how disruption of methylation patterns affects overall functioning of the plant. There are some indications that methylation may serve as a secondary somatic imprint which plays a role in gene regulation (Ohlsson et al., 1994).

Fig. 3.5. BA-treated 'Pocahontas' DNA samples were digested with *Hpa* II and *Msp* I and southern hybridized with a 3.11 kb *Bam* HI fragment containing 18S, 5.8S and part of 26S rRNA genes. H- *Hpa* II M-*Msp* I. Lane 1(H) and 2 (M). SH sample. 3(H) and 4 (M). Treatment5/R1. 5(H) and 6 (M). Field-planted Treatment15/ Runner-explant 2. 7(H) and 8 (M). Field-planted Treatment15/Runner-explant 3. 9(H) and 10. (M).Treatment15/Runner-explant 4. 11(H) and 12 (M). Field-planted Treatment15/R4.

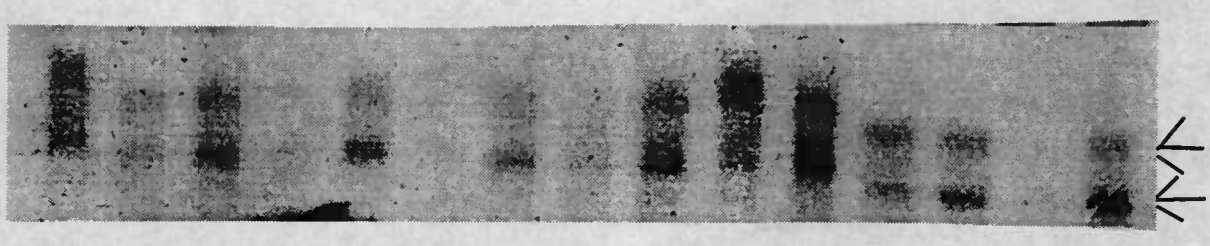

Fig. 3.6. BA-treated 'Pocahontas' and cold-stored DNA samples were digested with *Hpa* II and *Msp* I and southern hybridized with a 3.11 kb *Bam* HI fragment containing 18S, 5.8S and part of 26S rRNA genes. H- *Hpa* II M-*Msp* I. Lane 1(H). Treatment 15/ Runner-explant 4. 2 (M) and 3(H). Field-planted treatment 15/Runner-explant 1. 4 (M) and 5(H). Treatment 5/ Runner-explant 1. 6 (M) and 7(H). Cold-stored sample D. 8 (M) and 9(H). Cold-stored sample E. 10 (M) and 11(H). Field-planted cold-stored sample E. 12 (M) and 13(H). Treatment15/Runner-explant 5. 14 (M) and 15(H). Field-planted treatment 15/Runner-explant 5.

One factor that is crucial in methylation is that m^5C in a CCGG sequence could undergo spontaneous deamination which would lead to C(cytosine)-->T(thymidine) transition mutations. It is speculated that in about 90% of the cases, DNA repair enzymes correct T back to C, but in half the cases methylation is not maintained (Phillips et al., 1994). Changes in methylation patterns brought about by tissue culture or growth regulators in the medium could either favor or impair the deamination processes. On the other hand, methylation changes and phenotypic abnormalities may not go hand in hand (Brown, 1989). Methylation needs to be further studied to decipher its role and the consequences of disruption. Tissue culture provides a means of changing methylation levels using compounds like 5-azacytidine. Our results suggested that although methylation changes are seen in some plants *in vitro* plants seem to revert to normal once

taken to the field. This could possibly mean that selection pressure may be active in tissue culture conditions which may lead to deviation in methylation pattern. Alteration in DNA amplification profiles were also observed in pot grown samples from *in vitro* cultured plantlets.

3.4.4. Morphological studies

One field grown plant from the 15 μM BA treatment bore distorted, crinkled and rough leaves, with poor vegetative vigor and no flower production (Fig 3.7.). The runner-produced daughter plants also bore distorted leaves. No cyclamine mites were seen on the plants and symptoms did not match those of cyclamine mite attack. Distortion was restricted to this plant and did not spread to other nearby plants, and persisted for the season. At the end of the season the plant started to bear normal leaves which indicated that these variations were epigenetic changes. No other gross variants were observed. A few semi-dwarf plants were seen in both treatments.

3.4.4.1. BA treatments

Overall, there were no significant differences between 5 μm and 15 μm BA treatments on most of the parameters measured (Table 3.1). The only character which showed significant differences between the two treatments was plant height (7.13 ± 0.11 (15 μM) and 6.76 ± 0.13 (5 μM) ; p-value = 0.034 (all at 95% confidence intervals).

Fig. 3.7. 'Pocahontas' plants were either grown on 5 μM or 15 μM BA medium and representative samples were field planted. One plantlet grown on 15 μM BA medium upon transplanting bore distorted leaves.

For the following morphological characteristics there were significant interactions between treatments and runner-explants (Appendix A): plant height, number of trusses per plant, vigor, and total number of fruits. There were no significant interactions for the remaining parameters.

Increase in BA concentrations in the medium did not seem to affect morphological characteristics except plant height, where it was confounded with significant interactions between the treatments and the runner-explants. Significant interactions existed in three other parameters (petiole length, total truss number and total fruit number), but there were no significant differences between the two treatments. Presence of significant interactions indicates that the choice of runner explant plays a

Table 3.2. Morphological analysis of field grown plants from 5μM and 15 μM BA treatment

Character	5μM plants	15μM plants	P-value (0.05)
Leaf Angle (mm)	107.76±0.99	109.26±1.16	0.337
Leaf length (mm)	74.13±1.01	76.09±1.11	0.198
Leaf width (mm)	66.06±0.82	67.08±1.05	0.454
Petiole length (mm)	123.04±2.84	126.41±2.58	0.3929
Plant height (cm)	6.76±0.13	7.13±0.11	0.034*
Plant spread (cm)	6.76±0.20	7.23±0.202	0.100
Plant vigor [a]	4.4±0.11	4.52±0.11	0.440
Runnering	11.4±0.48	11.6±0.46	0.766
Number of trusses	1.56±0.15	1.62±0.13	0.766
Total fruit number	13.16±1.16	14.3±1.10	0.485
Primary fruit wt. (gm)	12.24±0.88	11.44±0.96	0.546
Primary fruit color [b]	4.26±0.26	3.9±0.26	0.347
Total fruit weight(gm)	33.5±2.6	30.18±3.01	0.415

[a] plant vigor rating: 1 (poor) - 5 (highly vigorous)
[b] fruit color rating: 1(white)-5(dark red)

* Significant at P≤ 0.05 (One-way ANOVA/LSD)

Table 3.3. Morphological analysis of field grown plants from 5 mM BA and cold-stored treatments.

Character	5μM plants	Cold-store plants	P-value (0.05)
Leaf Angle (mm)	107.76±0.99	104.76±1.18	0.054
Leaf length (mm)	74.13±1.01	70.57±1.42	0.442
Leaf width (mm)	66.06±0.82	62.65±1.33	0.032*
Petiole length (mm)	123.04±2.84	118.98±2.79	0.310
Plant height (cm)	6.76±0.13	6.35±0.113	0.020*
Plant spread (cm)	6.76±0.20	6.2±0.17	0.04*
Plant vigor [a]	4.4±0.11	3.82±0.11	0.004**
Runnering	11.4±0.48	13.64±0.51	0.002**
Number of trusses	1.56±0.15	0.5±0.11	0.000***
Total fruit number	13.16±1.16	5.02±1.10	0.000***
Primary fruit wt. (gm)	12.24±0.88	3.46±0.80	0.0000***
Primary fruit color [b]	4.26±0.26	1.3±0.30	0.0000***
Total fruit weight(gm)	33.5±2.6	7.02±1.98	0.0000***

a plant vigor rating: 1 (poor)-5 (very vigorous)
b fruit color rating: 1(white)-5(dark red)

* Significant (P-value ≤ 0.05) to *** highly significant (P-value ≤ 0.001)

major role in controlling variations. Numerous reports have pointed to the importance of proper explant choice (Adelberg et al., 1994). Characterization of runner-explants would be necessary to further determine the role of runner-explants.

3.4.4.2. Cold storage

There were significant differences between cold-stored plants and 5 μM BA-treatments for most characteristics measured. Some of the measurements were significantly lower for cold-stored plants. Most leaf measurements showed no significant differences from the 5 μM BA plants (Table 3.3.).

Cold storage affected most of the characteristics measured. Cold-stored plants were generally less vigorous, vegitatively and reproductively, and a high proportion (about 66%) of the plants did not flower. The cold-stored plants showed delayed ripening of fruits, and had more runners. The lack of sufficient vegetative vigor may have lead to a dramatic decrease in reproductive vigor of the cold-stored plants. Higgens and Stimart (1990) reported a positive correlation between number of leaves and flower bud number in onion bulblets. Completely chilling strawberry plants has been known to inhibit flower induction. Freezing injury of initiated floral buds could also lead to loss of flower induction. Vernalization has often been found to decrease the days required for flower initiation, but long term cold storage cannot be equated to vernalization (Higgens and Stimart, 1990). Decrease in vegetative, and thus, reproductive vigor may be the direct result of cold storage. Increased number of runners, which was the only vegetative characteristic to be larger for the cold-stored plants, may be due to the decrease in reproductive vigor which would channel photoassimilates to runnering. The high density planting used in this study, could also lead to higher runnering (Marcotrigiano et al., 1984).

Absence of polymorphism in RAPDs and lack of major changes in DNA methylation, combined with field data indicates that many of the changes in cold-stored plants are epigenetic in nature. The one variant plant in the BA treatments could be due to changes in gene expression. However, Sansavini et al., (1990) reported that some variations are masked by other characteristics and tend to show in later seasons. RAPD-PCR amplifies non-coding DNA inverted repeats which may have no phenotypic consequences by themselves, but may serve as markers which indicate changes. The fact that no polymorphisms were seen when DNA was amplified with a particular set of primers, does not exclude the possibility of mutations in other repetitive DNA segments or coding regions (genes) of the genome. Together the primers amplify about 200 loci, which would account for only a fraction of the genome. Additional characterization of micropropagated plants by transcript, protein analysis, and other factors is needed.

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Chapter 4

Summary

Strawberry micropropagation provides a way of producing large amounts of true-to-type nursery stock. However somaclonal variations lead to production of undesirable off-types. Information about the influence of the tissue culture medium and growth regulators on the genetic stability of micropropagated strawberries would be valuable in determining the optimal conditions for micropropagation and to minimize off-types. Epigenetic changes in field plants can be confused for somaclonal variations. Molecular tools provide a way of distinguishing the two. The role of methylation in determing the overall functioning of the plant is yet to be fully understood although methylation may be involved as a secondary somatic imprint in gene regulation. Tissue culture provides a way of deciphering the consequences of changes in methylation patterns.

In this study, BA concentrations did not affect the genetic stability of *in vitro* plantlets grown at either concentration. Changes in patterns, polymorphism or otherwise, were not seen. Monomorphic profiles were recorded at both BA concentrations with 29 of the 30 primers. Micropropagation at 5μM or 15 μM BA had no effect on the genetic stability of the loci that were amplified compared with SH controls. None of the micropropagated plants assayed showed any evidence of mutation in about 95% of the loci examined, and polymorphism profiles were not entirely reproducible.

No changes in methylation patterns were evident in a majority of our samples. One BA-treatment and one cold-stored plant showed methylation on the external cytosine which was absent in the controls and other samples. BA or micropropagation did not seem to significantly alter the pattern of methylation. Two samples from explant 5 exhibited length polymorphism which may indicate deletions.

There were few differences between the plants grown on 5 μM and 15 μM BA. Cold-stored plants, on the other hand, were significantly different from the 5 μM and 15 μM plants. They generally were vegetatively and reproductively less vigorous in the field.

The absence of RAPD-detectable genetic changes along with the field data suggest that the changes in cold-stored plants may be epigenetic in nature. This study indicates that some loci in the strawberry genome are not affected by micropropagation and cold storage. Questions about the fate of other parts of the genome and role of methylation have been raised and need to be resolved.

RAPD-PCR was influenced by a variety of factors including primers, genome size, and $MgCl_2$ concentration in the reaction mixture. DNA amplification with 10-mer primers will need to be optimized more for some primers. The effect of $MgCl_2$ and genome size needs to be elucidated further to improve the reliability of some primers. Some primers may be clouded by pseudopolymorphism and hence, may need additional work.

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APPENDICES

Appendix A

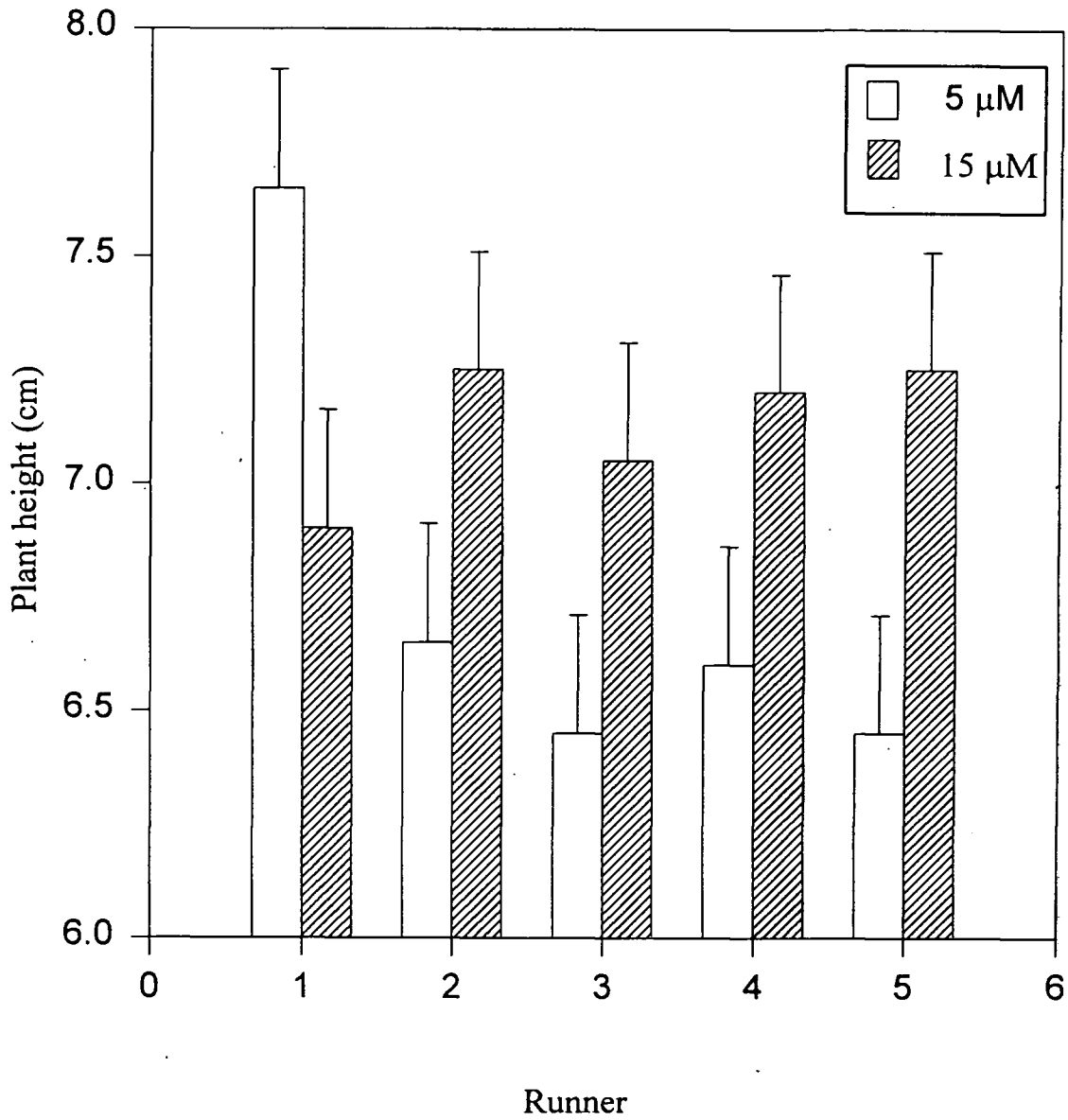

Fig. A.1. Interaction between runner-explant and BA concentrations influencing plant height. 'Pocahontas' plantlets micropropagated on NCGR-FRA medium with 5 µM or 15 µM BA were field planted and compared morphologically. Multivariate analysis was performed for interactions between explant sources and BA concentrations influencing plant height.

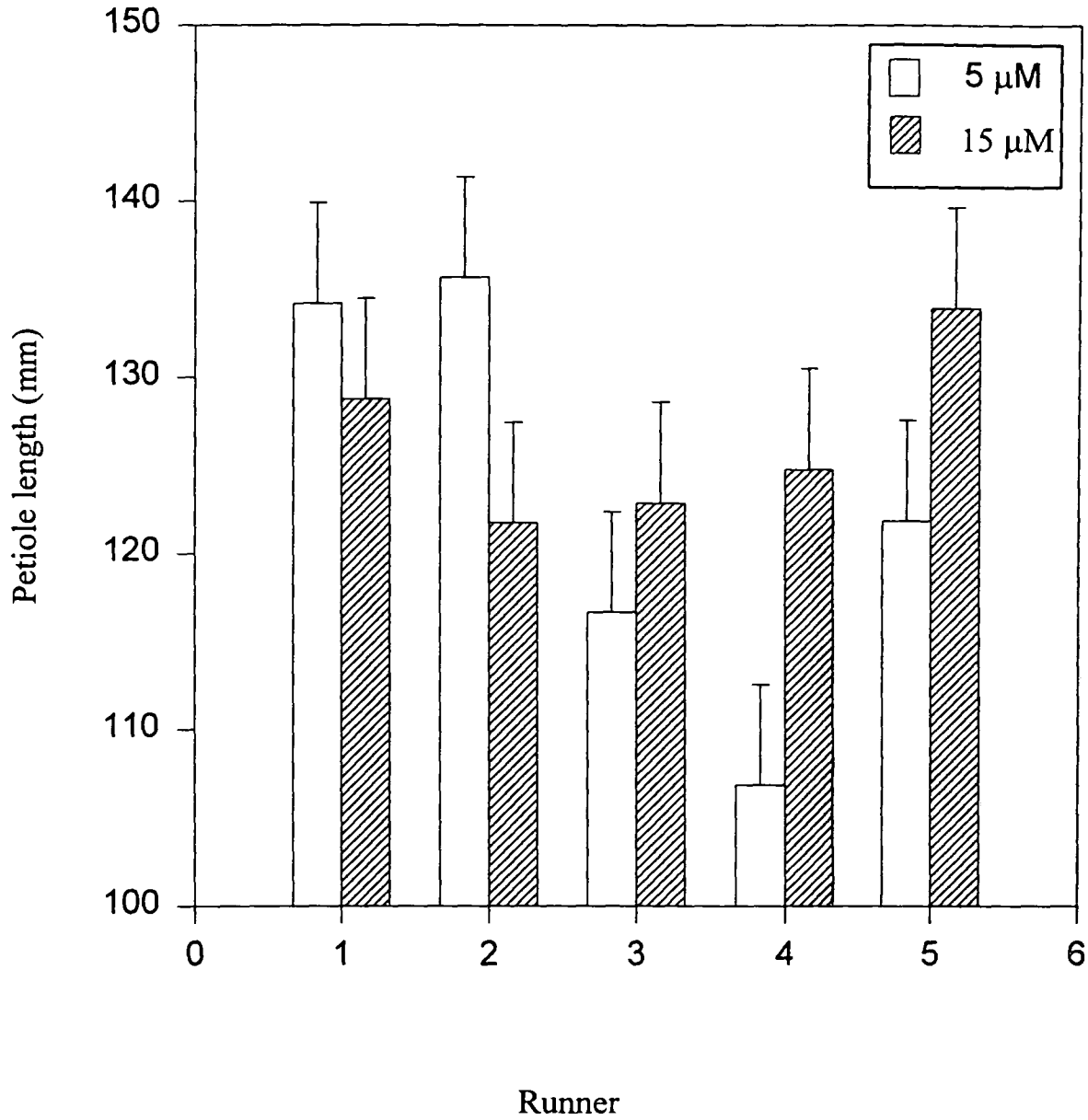

Fig. A.2. Interaction between runner-explant and BA concentrations influencing petiole length. 'Pocahontas' plantlets micropropagated on NCGR-FRA medium with 5 µM or 15 µM BA were field planted and compared morphologically. Multivariate analysis was performed for interactions between explant sources and BA concentrations influencing petiole length.

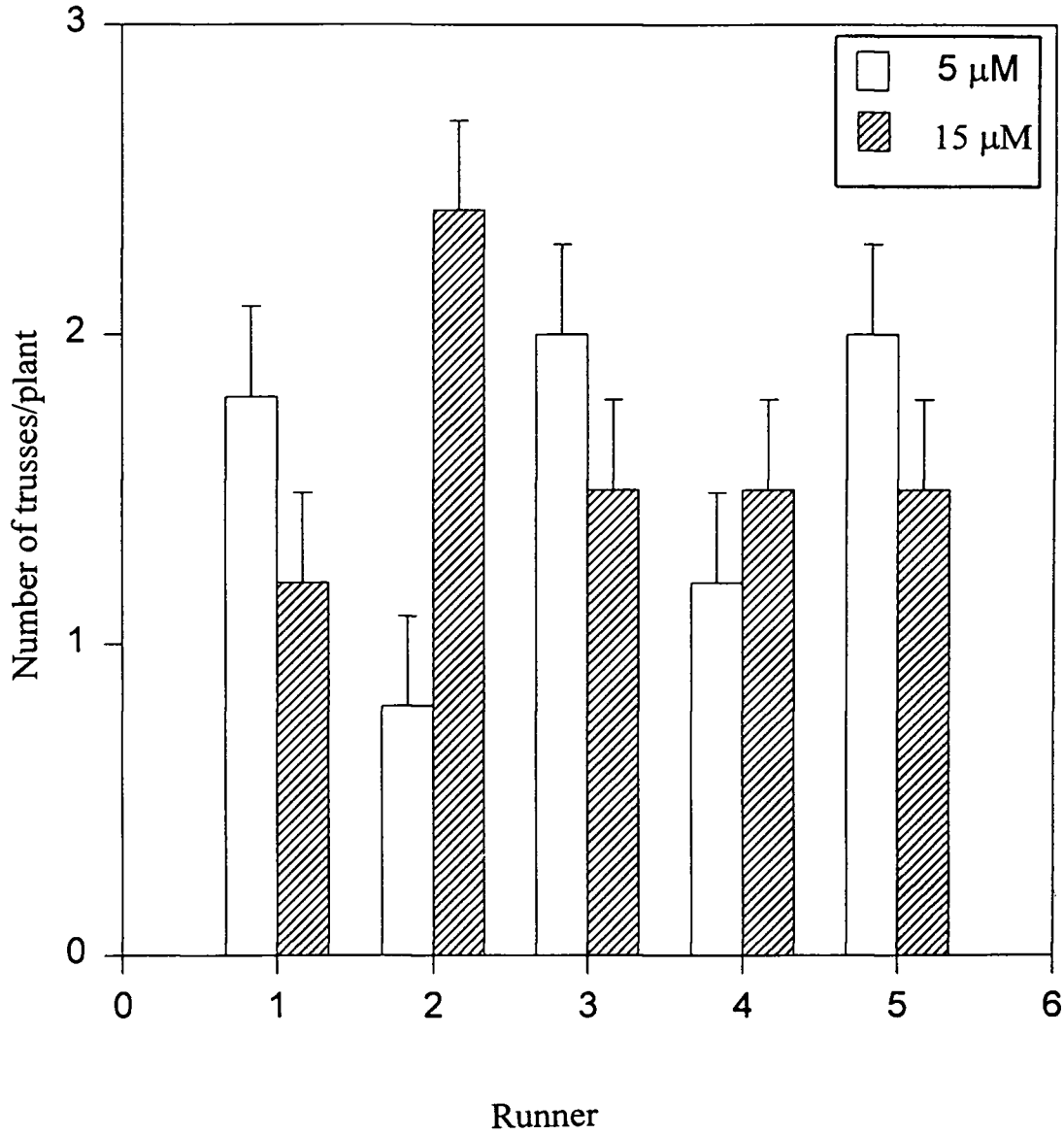

Fig. A.3. Interaction between runner-explant and BA concentrations influencing trusses per plant. 'Pocahontas' plantlets micropropagated on NCGR-FRA medium with 5 µM or 15 µM BA were field planted and compared morphologically. Multivariate analysis was performed for interactions between explant sources and BA concentrations influencing number of trusses per plant.

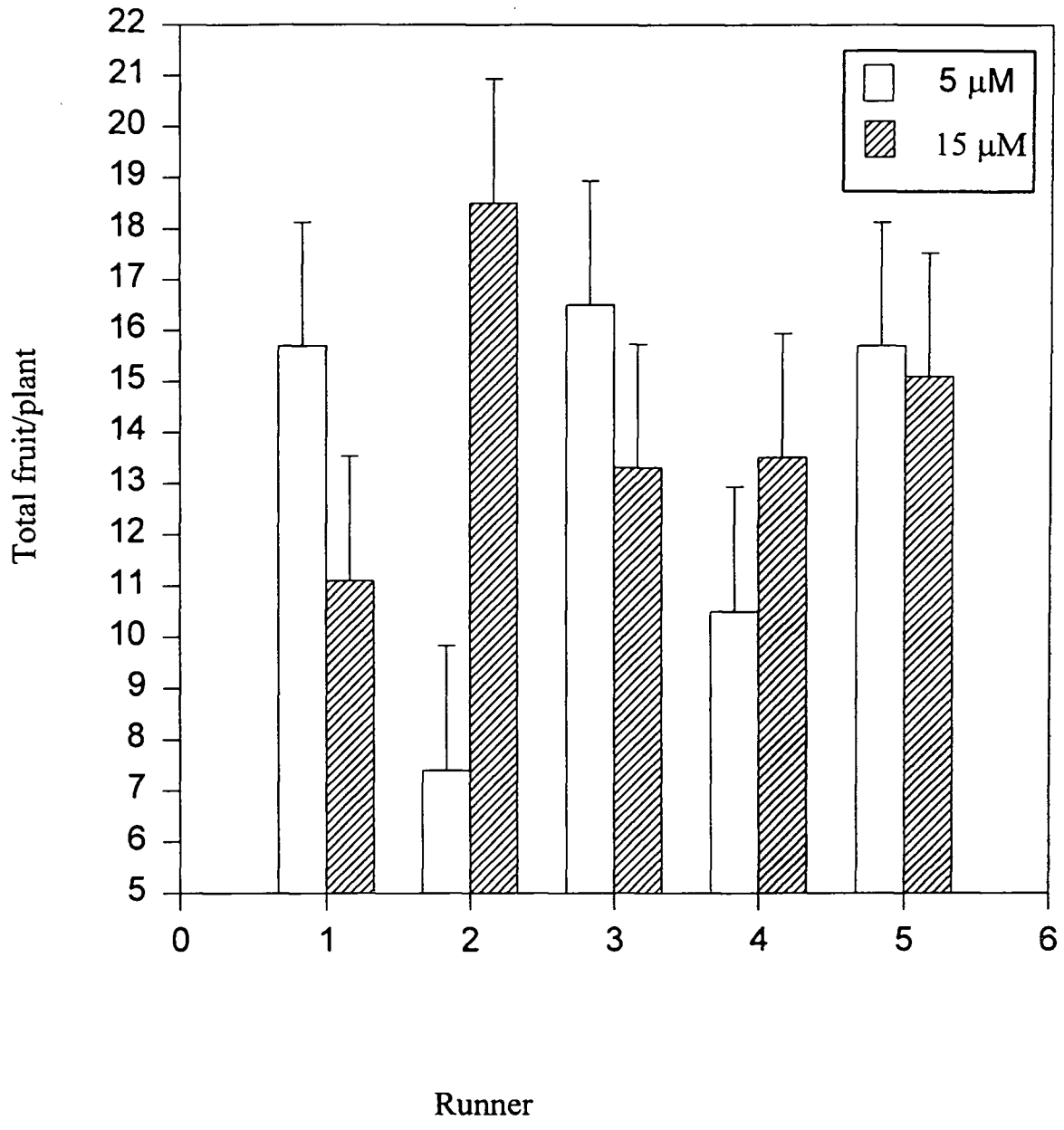

Fig. A.4. Interaction between runner-explant and BA concentrations influencing total fruit per plant. 'Pocahontas' plantlets micropropagated on NCGR-FRA medium with 5 µM or 15 µM BA were field planted and compared morphologically. Multivariate analysis was performed for interactions between explant sources and BA concentrations influencing total fruit per plant.

Appendix B

Factors affecting RAPD-PCR in a Polyploid, *Fragaria x annanassa* cultivar, 'Pocahontas'

Factors affecting RAPD-PCR in a Polyploid, *Fragaria x annanassa* cultivar, 'Pocahontas'

B.1. Abstract

A variety of factors influence the outcome of RAPD-PCR reactions. The $MgCl_2$ concentration, annealing temperature, primer, and genome size are among a few of the factors. Here, we describe a few of the factors which were found to influence DNA amplification in the case of a strawberry (*Fragaria x. annanasa*) cultivar, 'Pocahontas'.

B.2 Introduction

Random amplified polymorphic DNA (RAPD) analysis has been used for genome mapping, gene tagging, and population studies (Williams et al., 1990 ; Huff et al., 1993). RAPD-PCR amplification is usually performed using arbitrary 10-mer primers and low stringency conditions in terms of $MgCl_2$ and primer annealing temperature (Yu and Pauls, 1992; Liu and Berry, 1994). These conditions particularly affect the reliability of RAPD analysis. $MgCl_2$ concentration has been found be extremely important in RAPD-PCR (Park and Kohel, 1994). Here, we report factors which affect RAPD-PCR in the case of a polyploid species, *Fragaria x annanassa* cultivar 'Pocahontas'.

B.3 Materials and methods

Strawberry DNA amplification was carried out in 12 μl reaction volumes. PCR amplification was carried out using 10-mer Operon oligonucleotides (Operon Technologies, Inc., Alameda, CA) using a cocktail adopted from Huff et al. (1993) with minor variations. The conditions included 0.5 ng μL^{-1} strawberry DNA , Stoffel buffer (10 mM KCl and 10 mM Tris-HCl), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM Operon 10-mer primer and 0.042 U μL^{-1} of Amplitaq DNA polymerase, Stoffel fragment (Perkin Elmer, Norwalk, CT).

Reactions were carried out in 96 well flexible assay plates (Falcon, Oxnard, CA) with reaction mixtures overlaid with 3 drops of mineral oil. The thermocycle profile was adopted from Huff et al.(1993), with slight modifications. The thermocycle profile was preceded by an initial denaturation for 7 min at 94°C. DNA amplification was performed in a PTC100 MJ thermocycler (MJ research, Inc., Watertown, MA). $MgCl_2$ concentration of the reaction mixture was altered by either increasing the $MgCl_2$ concentration from 2.5 mM to 3.5 mM or decreasing to 1.5 mM from 2.5 mM . The reaction volume was not altered. Next, reaction volume was increased three-fold (3X) to 36 μl without altering DNA concentration. For altering annealing temperature a "touchdown" PCR thermoprofile was used (Don et al, 1991). A high-end temperature of $39^{O}C$ was adopted. The profile was programmed to decrease annealing temperature by $1^{O}C$ $cycle^{-1}$ to reach $36^{O}C$. The rest of the profile was similar to that used for other amplifications. Amplification products were separated in 7.5% acrylamide/bis (37:1) gel in 0.375 M Tris pH 8.8 buffer, and visualized using silver staining (Caetano-Anolles and Gresshoff, 1992).

B.4. Results and discussion

B.4.1. Primers

Most of the primers tested produced reproducible profiles but one primer OPF-18 (TTCCCGGGTT) produced erratic profiles. OPF-18 amplification profiles were different for samples collected across three time periods and separate reactions. An alternative primer with 60% identity with OPF-18 primer (AACCCGGGAA) yielded monomorphic amplification profiles which were entirely reproducible. Each primer thus needs to be dealt with independently for optimization of reactions to be achieved. The primer sequence- 3' end nucleotides and the GC/AT ratios of sequences have been reported to be extremely important (Tingey et al., 1992).

B.4.2. $MgCl_2$ concentration

We found that the OPF-18 primer produced fewer amplification products and was slightly more reproducible with 3.5 mM $MgCl_2$ than with lower concentrations.(Fig. B.1.). Concentration has been reported to be specific for each primer and that the optimum varied from 1.5 to 4.5 mM (Park and Kohel, 1994). Our results confirmed that some primers, in this case OPF-18, need further optimization.

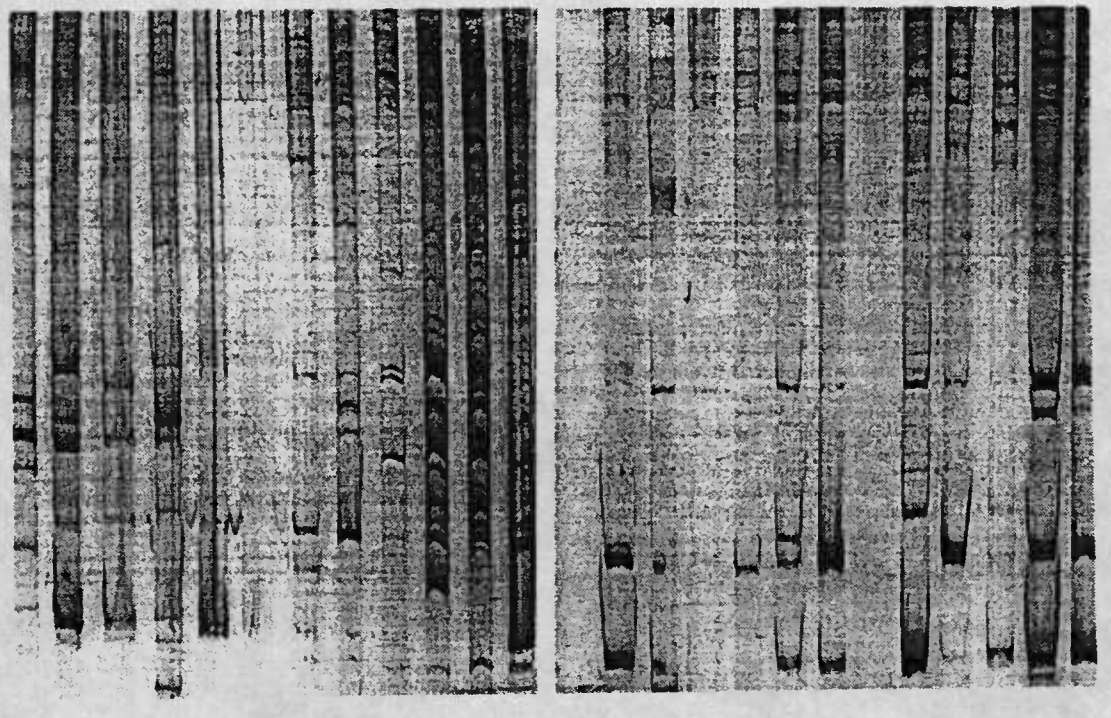

Fig. B.1. PCR conditions were altered in the process of amplification of strawberry DNA by OPF-18 primer. Two different $MgCl_2$ regimes were used- 2.5mM and 3.5 mM $MgCl_2$. 12 independent samples were PCR amplified. Lane 1 in the 2.5 mM profiles (left) correspond to lane 1 in the 3.5 mM profiles (right).

B.4.3. Primer annealing temperature

Increasing initial annealing temperature of OPF-18 primer using a "touchdown" PCR thermoprofile resulted in a smear of non-discernible bands migrating at around 300 bps on the gel. Primer annealing temperature doesn't offer much leeway for modification with 10-mer primers since the melting point of the duplex is quite low. MgCl2 concentration is one component that can be varied easily.

B.4.4. Reaction volume and genome size

Genome size can play an important role in RAPD-PCR. When the reaction volume was increased by 3 times a marked reduction in the number of amplification products was seen. Amplification profiles were more reproducible for major bands. Increasing reaction volume without increasing DNA concentration in the mixture may have lead to a less competitive reaction resulting in optimum amplification (Fig. B.2.). DNA associated enzymes, like restriction enzymes and polymerases, have been shown to act spuriously and non-specifically under high concentrations. Hence, the amount of polymerase could contribute to the variation.

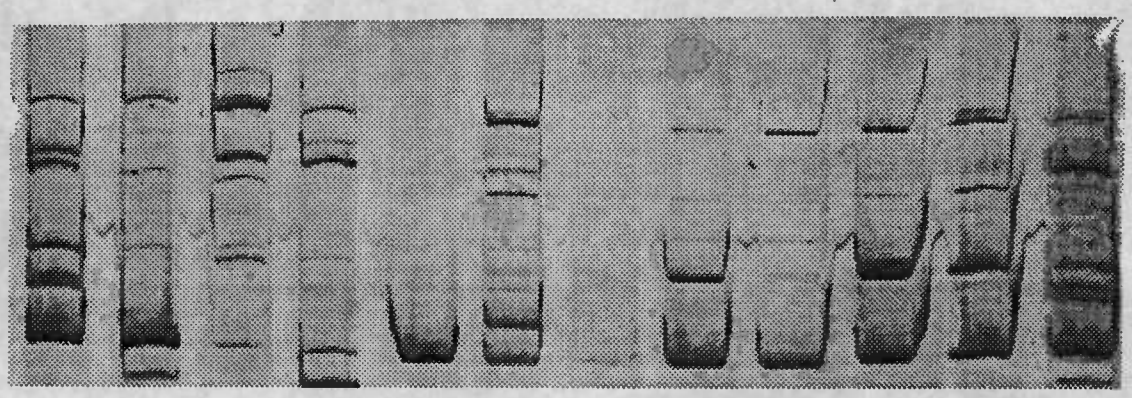

Fig. B.2. PCR conditions were altered in the process of amplification of strawberry DNA by OPF-18 primer. DNA profiles generated by OPF-18 primer in a 3X reaction volume. 12 independent samples were PCR amplified with the OPF-18 primer.

B.4. 5. Polymerase concentration

Decreasing the concentration of Amplitaq DNA polymerase from 1U to 0.5 U reduced the number of amplification products. This may indicate that increased amounts of polymerase result in non-specific amplification events. Hence, a RAPD-PCR reaction needs to be optimized for the specimen being used as well as for primers and the amount of DNA template and themocycle profiles.

B.5. REFERENCES

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