POTATO TUBER PROTEIN AND ITS MANIPULATION BY CHIMERAL DISASSEMBLY USING SPECIFIC TISSUE EXPLANTATION FOR SOMATIC EMBRYOGENESIS

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ABSTRACT

Potato is a major part of the human diet in many countries of the world, providing substantial levels of carbohydrate, protein, and vitamins. This study examined the tuber protein content. In the first part of the research, total soluble protein (TSP) and patatin concentration were determined in periderm, cortex, and pith, in tubers of 20 important potato cultivars. TSP concentration was greater in periderm and lesser in cortex and pith tissues. Patatin was present in all tuber tissues but with the opposite pattern, less in periderm and greater in cortex and pith tissues. For intercultivar comparisons, a means of converting the specific tissue-based TSP and patatin data (dry weight) into a uniform weight whole tuber basis was developed. This relied on conversion factor values that were generated from percent weight tissue proportion and percent dry matter for each tissue layer. Cultivars with relatively more or less TSP and patatin in each tissue layer, and on a whole tuber basis, were identified. In the second part of the study, disassembly of chimeral (Russet Burbank) and putatively chimeral (Alpha, Bintje, Red Gold) tubers into their component genotypes was evaluated as a strategy for the production of intraclones with altered protein content. Explants were selected from tissue with greater or lesser protein levels and somatic embryogenesis was used to produce regenerants from each tissue source. Russeting was used as a phenotypic marker and TSP as a biochemical marker. Russet Burbank was confirmed as a periclinal chimera, although chimeral instability was evident, since some non-chimeral regenerants showed displacement of LI tunic cells with the russeting mutation into the pith. Red Gold was "uncovered" as an LII periclinal chimera (Red-Gold-Red). The value of chimeral disassembly in explaining an important component of somatic variation was clearly seen with this cultivar. The inconsistent TSP distribution in Russet Burbank intraclones proved that TSP was not distributed in a periclinal chimeral manner, as initially hypothesized. However, there was clear variation in protein content in the tubers of non-chimeral regenerants. Periclinal chimeral disassembly and somatic embryogenesis are potentially useful technologies for the production of improved intraclones of potato.

RÉSUMÉ

Dans pluseurs pays, la pomme de terre est une composante importante dans la diète humaine pour est une bonne source d'amidon, de protéines et de vitamines. Cette étude a été envissagé en protéine de ce tubercule. Dans la première partie, les concentrations de protéines solubles totaux (PST) et de patatine ont été déterminées dans le périderme, le cortex et le pith dans des tubercules provenant de 20 cultivars. La concentration de PST était plus élevée dans le périderme et basse dans le tissu du cortex et du pith. La patatine était présente dans tous les tubercules avec une distribution opposée à PST, était plus faible dans le périderme et plus élevée dans les tissus intérieurs. Pour des comparaisons inter-cultivar, une méthode pour convertir les données spécifiques de PST et de patatine (poids sèche) en données en fonction du poids total des tubercules a été développée. Pour ce faire, les valeurs de conversion ont été calculées à partir le pourcentage des proportions et le de matière sèche de chaque tissu. Dans la deuxième partie, le désassemblage de tubercules chimériques (Russet Burbank) et potentiellement chimériques (Alpha, Bintje, Red Gold) a été évaluée comme une stratégie de production de clones contenant des protéines altérées. Des explants de tissu ayant des concentrations élevées ou basses ont été sélectionnés pour l'embryogenèse somatique. Le caractère russeting a été utilisé comme marqueur phénotypique et le PST comme marqueur biochimique potentiel. Russet Burbank a été confirmé comme une chimère periclinale, malgré l'évidence d'instabilité chimérique, dans quelques des régénérants non-chimériques, des cellules tuniques LI avec mutations typiques du russeting ont été déplacées vers le pith. Red Gold a été déclaré comme une chimère périclinale de type LII (Red-Gold-Red). Avec ce cultivar, on voit clairement la valeur du désassemblage chimérique dans l'explication d'une composante importante de la variation somatique. L'inconsistance de la distribution du PST dans les tubercules intra-clones de Russet Burbank ont démontré que les distributions du PST ne suivaient pas la forme chimérique périclinale initialement postulée. Cependant, nous avons observé une variation dans le concentration du protéines dans les non–chimériques tubercules. Le désassemblage chimérique avec l'embryogenèse somatique sont des techniques potentiellement utiles pour la production d'intra-clones améliorés de pomme de terre.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ANOVA	analysis of variance
BSA	bovine serum albumin
cv., cvs.	cultivar (s)
Cys	cysteine
°C	centigrade degree
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FW	fresh weight
$g\ l^{-1}$	gram per litter
GMO	genetically modified organism
h	hour
ha	hectare
HHH	protein pattern (high, high, high)
HLL	protein pattern (high, low, low)
IAA	indole-3-acetic acid
kDa	kilodaltons
kg	kilogram
Leu	leucine
LHH	protein pattern (low, high, high)
LI	first histogenic layer (outer tunic)
LII	second histogenic layer (inner tunic)
LIII	third histogenic layer (corpus)
LLL	protein pattern (low, low, low)
LSD	least significance difference
Lys	lysine
M	molar
Met	methionine

mg g^{-1} DW	milligram per gram of tissue dry weight
min	minute
MS	Murashige and Skoog (1962) tissue culture medium
MW	molecular weight
N	nitrogen
NPN	non protein nitrogen
ppm	part per million
RAPD	random amplified polymorphic DNA
RGR	red-gold-red
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SE	standard error
SR_1	somatic regenerant (first generation)
SSR	single sequence repeat
TSP	total soluble protein
WHO	World Health Organization

CONTRIBUTIONS OF AUTHORS

This thesis has been written in the form of manuscripts to be submitted to scientific journals. This format has been approved by the Faculty of Graduates Studies as outlined in "Guidelines for Thesis Preparation".

This thesis contains five chapters (III to VII) representing five different manuscripts either already published, in press, or submitted for publication to refereed journals. I designed the experimental set-up, performed the experiments and the statistical analysis, and prepared the final manuscripts. All the experiments were done under the supervision of Dr. Danielle J. Donnelly, who contributed towards planning the experiments, provided valuable advice and suggestions, and reviewed the content of all parts of the thesis. Dr. Donnelly is a co-author on all five manuscripts.

Chapter IV involved a collaborative effort between different laboratories at McGill University and a Finnish University laboratory. Dr. Inteaz Alli (Food Science & Agricultural Chemistry Department, McGill University) supervised the SDS-PAGE work reported in this chapter. Dr. Tatiana Scorza (formerly of the Institute of Parasitology, McGill University) supervised the ELISA work and helped with data analysis. Dr. Ulla Seppälä and Dr. Timo Palosuo (Laboratory of Immunology, Helsinki University) provided the purified patatin protein and its polyclonal antiserum. All co-authors contributed by editing the final manuscript.

Chapter V was designed in cooperation with Dr. Venkatesh Sosle (Bioresource Engineering Department, McGill University). He also reviewed the statistical analysis and helped edit the final manuscript.

Chapter I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important vegetable and the fourth most important crop in the world, exceeded only by rice, wheat, and maize (Ahloowalia, 2001; Bamberg and del Rio, 2005). The crop represents roughly half of the world's annual output of all root and tuber crops and is part of the diet of half a billion people (Ewing, 1997). World potato production has increased at a much faster rate than other leading crops, in both developed and developing countries, over the past 20 years. In 2005, the total global potato crop covered more than 18 million ha and its production reached 323 million tons (FAO, 2006).

Potato is a source of dietary carbohydrate and highly nutritious protein (Kaldi, 1972; Markakis, 1975; Rexen, 1976; Desborough, 1985; Woolfe, 1987; Juliano, 1999; Buckenhüskes, 2005). Based on protein quality per hectare, potato could meet the protein requirement of more people than any other major crop (Niederhauser, 1993; Dale and Mackay, 1994). Potato is also considered an important source of vitamins and minerals such as vitamin C (ascorbic acid), vitamin B6, and potassium (Woolfe, 1987; Buckenhüskes, 2005).

The average protein content in a whole potato tuber is approximately 2% on a fresh weight (FW) basis and 10% on a dry weight (DW) basis (Desborough, 1985; Woolfe, 1987; Juliano, 1999). However, a wide range of crude protein content has been reported; from 5.1 to 16.1% (DW) among *Solanum* species and from 9.5 to 14% (DW) among *S. tuberosum* cultivars (Hoff et al., 1978; Snyder and Desborough, 1980). Protein distribution is not homogeneous in all tuber tissues. However, little information on specific tissue protein concentration is available.

The nutritional quality of potato tuber protein is well established (OECD, 2002). Potato protein contains substantial levels of essential amino acids. Lysine (Lys) and leucine (Leu) are the most abundant, while the sulfur containing amino acids methionine (Met) and cystine (Cys) are the least abundant (Hoff et al., 1978; Destéfano-Beltrán et al., 1991; Storey and Davies, 1992).

Compared with many other foods, potato makes a significantly nutritional contribution to the human diet. For example, potato contributes 3.4% of total household protein intake in the United Kingdom compared with fruit (1.3%), egg (4.6%), fish (4.8%), beef (5.7%), cheese (5.8%), white bread (9.8%), and milk (14.6%) (NFSC, 1983; Woolfe, 1987; Juliano, 1999). In developing countries where potato is the staple food, such as in the South America Andean and East Russian regions, 80-90% of the population is highly dependent on this single crop (Destéfano-Beltrán et al., 1991), and the percentage contribution of potato protein to total protein intake is much greater. Increasing potato tuber protein level would have great potential benefits in those countries where potato is an important constituent of the diet.

Potato improvement programs have focused on promoting pest and disease resistance (During et al., 1993; Ghislain et al., 1998; Hassairi et al., 1998), yield increase (Sonnewald et al., 1997), and enhanced abiotic stress tolerance (Zhu et al., 1996; Wallis et al., 1997). However, efforts to improve the nutritional content of potato have lagged. Traditional breeding methods to improve nutritional quality traits have involved the hybridization of parental clones, and the subsequent selection among large seedling populations for superior individuals with the desired combination of traits (Desborough and Lauer, 1977; Plaisted et al., 1994). However, traditional potato breeding has been a cumbersome task due to inherent biological factors including high heterozygosity, tetrasomic inheritance, and the sterility of many cultivars (Douches et al., 1996; Mackay, 2005). These difficulties require exceptionally large populations of potato seedlings to be screened for potential improvements.

Advances in genetic engineering technology have opened new possibilities to improve the nutritional value of potatoes. Approaches have involved the insertion and expression of genes encoding sulfur-rich protein in transgenic potato plants (Utsumi et al., 1994; Tu et al., 1998; Chakraborty et al., 2000) with partial results obtained to date. However, despite these efforts, there is a strong consumer-resistance against acceptance of genetically modified potato tubers. Some other alternatives for potato improvement may include selection of naturally occurring sports, induction of mutations, and in vitro production of somaclonal variants (Jain et al., 1998; Ahloowalia and Maluszynski, 2001; Jain, 2001).

A possible method of producing somaclonal variants could include disassembly of periclinal chimeral potato plants. Chimeral plants are composed of a mixture of tissues with different genotypes, resulting from mutations that have originated in one of the histogenic layers of the apical meristem (Tilney-Basset, 1986; Marcotrigiano, 1997; Hartmann et al., 2002). Chimeras are also known as genetic mosaics (Marcotrigiano, 1997; Marcotrigiano and Gadziel, 1997). Particularly in potato, chimerism has distinguished many new cultivars that differ phenotypically from their original cultivars (Miller, 1954; Howard, 1959; Klopfer, 1965 cited by Tilney-Bassett, 1986). In most cases, skin (periderm) colour and texture were the main altered tuber characteristics. The most often cited example is Russet Burbank, now the most widely grown potato cultivar in North America. Russet Burbank originated as a somatic mutation of Burbank in 1914 (Davis, 1992). Cv. Burbank is a thin and smooth-skinned long white potato while the periclinal chimeral cv. Russet Burbank, has a thick and russeted brown skin and an elongate-round shape.

Disassembly of chimeral potato tubers into their component genotypes has been overlooked as a potential method of modifying the nutritional content, such as the protein content, of potato cultivars. Knowledge of tuber tissue protein distribution is important in selecting explant tissues with relatively greater or lesser protein levels. Chimeral disassembly permits conservation of the original genotype, but may provide means to select for a small, potentially valuable change present in an entire tissue layer.

1.1. Thesis Outline

This thesis is comprised of a comprehensive literature review, five chapters presenting the results of this study in manuscript format, an overall conclusion, suggestions for future research, and a final section on the contributions to knowledge.

The Literature Review (Chapter II) serves to establish the main contributions of the thesis in relation to current knowledge. Main topics of the literature review included nitrogen composition and nutritional value of potato tubers, tuber storage proteins, and some methodologies for the nutritional improvement of potato, as genetic engineering, use of in vitro somaclonal variation and dissociation of periclinal chimeral tubers.

This study, described in Chapters III to VII, was divided into two main phases. In the first phase, field-grown tubers, both fresh and stored from 20 potato cultivars, were screened to determine the concentration and tissue-specific distribution of total soluble protein (TSP) (Chapter III and IV) and patatin, the major storage protein (Chapter IV). This was accomplished using Bradford, ELISA, and SDS-PAGE methods, following separation of the different tissue layers of the tuber; periderm, cortex, and perimedullary and pith areas together (pith). To facilitate interpretation of the TSP and patatin data set (Chapter IV) and enable intercultivar comparisons, a means of converting the specific tissue-based nutritional information (DW) into typical whole tuber information (FW) was developed (Chapter V). This was based on precise estimates of percent weight proportions of each tuber tissue layer for all 20 cultivars. Percent weight was calculated through volume and density of each component tissue.

The second phase began with a review of the main factors that cause variation in clonally propagated plants derived through tissue culture systems (Chapter VI). The impact of tissue culture-induced variation on the clonal integrity of cultivars was emphasized. The contribution of periclinal chimerism to somatic variation was evaluated through disassembly of chimeral (cv. Russet Burbank) and putatively chimeral (cvs. Alpha, Bintje, and Red Gold) tubers into their component genotypes (Chapter VII). TSP pattern was used as a biochemical marker and the russeting trait as a phenotypic marker. Chimeral disassembly through somatic embryogenesis from specific-tuber tissues with greater or lesser protein levels was expected to result in non-chimeral regenerated plants with altered protein levels. This strategy for production of intraclones has potential value in explaining some aspects of somaclonal variation and holds promise in the nutritional improvement of cultivated potato

The General Summary and Conclusions (Chapter VIII) integrated and summarized the findings from these five chapters. Future research suggestions were considered at the end of this chapter. Finally, the thesis concluded with the section Contributions to Knowledge (Chapter IX). References were listed at the end of the thesis.

1.2. Objectives

This thesis research focused on tuber protein content as an important nutritional component with potential for improvement in cultivated potato.

The study was divided into two main sections with the following objectives:

1) *Survey of protein content in potato tubers*

a. To determine total soluble protein (TSP) and patatin concentration in periderm, cortex, and perimedulla/pith tissues, in fresh and stored (6 months) tubers of 20 potato cultivars to identify tissues and cultivars with greater and lesser protein content.

b. To compare TSP content between field-grown tubers and microtubers of seven cultivars to evaluate the potential utility of microtubers as a model for studying protein in potato tubers.

2) *Chimeral disassembly through somatic embryogenesis*

a. To disassemble chimeral and putatively chimeral tubers into their component genotypes through somatic embryogenesis from specific tissue explants with greater or lesser protein levels.

b. To evaluate disassembly of periclinal chimeral potato tubers as a strategy for production of non-chimeral intraclones with modified tuber protein content.

1.3. Hypothesis

1) There are quantifiable differences in tuber protein content between cultivars and specific tissue layers. Cultivars with greater and lesser protein levels and tissues with greater and lesser protein concentrations can be identified.

2) Regenerated plantlets from somatic embryos derived from disassembled chimeral tuber tissue with greater or lesser protein level, may produce tubers with uniform protein distribution and protein levels consistent with the source tuber explant tissue (see Fig. 7.2).

Chapter II

LITERATURE REVIEW

2.1. Potato Crop

Potato (*Solanum tuberosum* L.) is an Andean tuber crop that was originally domesticated in South America, and started its worldwide dissemination after Columbus's voyages in the 16th century (Hawkes, 1990). Today, potato is one of the most important food crops in the world. Potato is grown in about 150 countries, of which two-thirds are developed (FAO, 2006). Consumption per capita in developing countries is rapidly increasing and has reached 14 kg per annum but is still far less than the European (86 kg) or North American (63 kg), contributing to expectations of continued world expansion (Ahloowalia, 2001).

Potatoes have a wide variety of uses around the world. They are grown for direct consumption, for processed food products (chips and French fries), for animal feed, and for industrial uses (primarily for starch and starch derivatives). Other uses of potato include as edible vaccines (Joung et al., 2004; Young-Sook et al., 2005) and, also, in the production of specific organic molecules such as palatinose (sucrose isomer isomaltulose), a sugar substitute (Börnke et al., 2002).

Potato is the most important vegetable crop in Canada. In 2005, potato production reached 4.4 million tons, grown on 165,000 ha of land (FAO, 2006). Potato is cultivated in most Canadian provinces. Prince Edward Island had the greatest production (1.18 million tons) followed by Alberta (0.79 million tons), Manitoba (0.72 million tons), New Brunswick (0.63 million tons), and Quebec (0.47 million tons) (CPP, 2005).

Potato is a rich source of energy and highly nutritious protein (Woolfe, 1987; Juliano, 1999; Buckenhüskes, 2005). It also contains vitamins (C, B1, B2, and B6) and minerals, such as potassium and phosphorus. Potato plants yield more weight in the form of stem tubers and produce more protein per unit area of land that any other major crop with the exception of soybean (Dale and Mackay, 1994). In those parts of the tropical developing world where potato competes as a food with other established root and tuber

crops, it is considered a source of high quality protein rather than carbohydrate energy (Woolfe, 1987; Juliano, 1999).

2.2. Nitrogen Composition and Nutritional Value of Potato Tubers

2.2.1. Nitrogenous constituents

The total nitrogen (N) of potato tubers occurs principally in the form of proteins (soluble and insoluble true proteins) and non-protein nitrogen (NPN) (Woolfe, 1987; van Es and Hartmans, 1987). Some 8 to 10% of the total nitrogen content of tubers is insoluble, which corresponds to the insoluble part of the protein-N fraction (Desborough, 1985).

2.2.1.1. Protein nitrogen

The proportion of protein N with respect to total N varies widely among *S. tuberosum* genotypes. A range of 29.5 to 51.2% was found among 11 *S. tuberosum* cultivars (Neuberger and Sanger, 1942) and 40 to 74% in 50 samples of *S. tuberosum* group Andigena (Li and Sayre, 1975).

The soluble proteins of potato are high-quality and contribute significantly to the nutritional value of the tubers (Desborough, 1985; Woolfe, 1987; Ewing, 1997). Soluble proteins comprise 90 to 92% of the total true protein (Woolfe, 1987). The average total protein content in potato is approximately 2% on a fresh weight (FW) basis and 10% on a dry weight (DW) basis (Desborough, 1985; Woolfe, 1987). However, wide ranges of crude protein content have been reported e.g. (DW) 5.1 to 16.1% among *Solanum* species, 9.5 to 14% among *S. tuberosum* cultivars (Hoff et al., 1978; Snyder and Desborough, 1980), 4.2 to 17.4% among diploid hybrids of Phureja-Tuberosum selections, and 6.9 to 11.0% among tetraploid hybrids of Andigena, Phureja, and Tuberosum selections (Desborough and Weiser, 1974; Desborough, 1985). Although the hybrids have protein of high nutritional quality, most lack the yield potential of commercial cultivars.

Protein concentration in the tuber tissue layers is not homogeneous. However, little information regarding protein tuber distribution is available in the literature. Average protein content in cv. Norchip tubers was similar in the cortex, and the outer and inner pith regions, being 6.0, 5.3, and 5.8% (DW) respectively (Desborough and Weiser, 1974). However, greater concentrations of protein were found in the cortex compared with the pith in the three potato cultivars Katahdin, Norking Russet, and Shepody (Munshi and Mondy, 1989). The pith region was significantly greater in NPN than the cortex area. Periderm contributed 2% (FW) of the total protein tuber content in the only reported mention of this (Munshi and Mondy, 1989).

The total protein content of potato tubers can vary, principally due to cultivar differences, duration of growth, maturation level, cultivation practices, climatic effects, growing season, and location (Woolfe, 1987). However, the composition of essential amino acids in the true protein of a specific cultivar is genetically determined and is little-affected by environmental conditions (Eppendorfer et al., 1979).

2.2.1.2. Non-protein nitrogen (NPN)

The NPN fraction constitutes from 40 to 60% of the total N. This fraction is not involved in the nutritional quality of the tuber (Desborough, 1985). It contains both organic and inorganic nitrogen. The organic nitrogen is composed of free amino acids and the amides asparagine and glutamine. These compounds account for a substantial part of the total fraction. Free amino acids constitute 22 to 35% of the total tuber amino acid content, while the amides are present in about equal amounts and together comprise approximately half the total free amino acids (Hoff et al., 1978). Other N-containing organic compounds are nucleic acids and alkaloids, specifically glycoalkaloids such as solanine and chaconine (van Es and Hartmans, 1987). The inorganic nitrogen fraction contains small amounts of nitrate and nitrite, ranging from 53 to 233 ppm and from 25 to 130 ppm respectively, which together comprise close to 1% of the total N (Munzert and Lepschy, 1983).

During the growth and development of potato tubers there are changes in the contents of crude and pure protein and of NPN. Maximal quantities of pure protein are

reached at earlier growth stages (75-120 days after emergence) and after decrease by 10-25% until senescence (Kolbe and Stephan-Beckmann, 1997). However, the organic fraction of NPN continues to increase in the tuber during the last stages of maturity (Kapoor and Li, 1983). Desborough (1985) found that young and small tubers have relatively high protein and very high nitrate contents and lower values of starch in comparison to older and larger tubers. At harvest, large tubers can have relatively high or low nitrogen concentrations, based on extreme differences in nutrient supply and weather conditions (affecting availability of assimilates) throughout the growth period.

2.2.2. Amino acid composition

Potato protein has a balanced amino acid composition. It is considered to be of high biological quality due to the substantial levels of essential amino acids (Buckenhüskes, 2005), which are often limiting in the human diet. Lys content is the greatest, being comparable to that of whole egg (van Gelder and Vonk, 1980), while the sulfur-containing amino acids such as Met and Cys are the least. To emphasize the essential amino acid concentration of potato protein, it was compared with that of other important food staples and whole egg (Table 2.1). The advantage of potato over cereal staples is its greater Lys content. In combination with other foods, potatoes can supplement diets that are limited in Lys. For example, wheat or rice with accompanying potatoes provides a better quality protein (Woolfe, 1987). An ideal combination is obtained with a 65% potato and 35% animal protein mixture, which gives well-balanced protein (Bajaj and Sopory, 1986).

Wide ranges in the content of the true protein essential amino acids were found in 40 potato genotypes; from 4.62 to 10.82 and 0.19 to 2.69 mg g^{-1} (DW) for Lys and Met, respectively (Desborough and Weiser, 1974). However, analysis of amino acids from 34 *S. tuberosum* cultivars showed that, although the cultivars covered a wide range of protein from 0.37 to 1.24 g/100 g (FW), there was little variation in amino acid composition among them (van Gelder and Vonk, 1980). Low values of Met were found among 45 wild *Solanum* species (Hoff et al., 1978).

Potato protein has an adequate ratio of total essential amino acids to total amino acids and a balance among individual concentrations to meet the needs of infants and small children. According to the energy and protein requirements described by the World Health Organization (WHO, 1985), as little as 100 g of potato tuber can supply a significant percentage of the daily protein requirements for childhood growth. For example, 100 g (one small tuber) can supply 10-12% of the daily protein needs of children aged 1-5 years, respectively. For adults, depending upon body weight and sex, the same amount of potato can supply 3-6% of the daily protein requirements (Woolfe, 1987).

Most amino acids in the NPN are representative of true protein. However, the NPN contains lesser amounts of essential amino acids than the true protein (Kapoor et al., 1975; Wolfe, 1987). Even though N is contributed to the diet by the free amino acid pool, this is relatively less important nutritionally than the essential amino acids from the true protein. The amino acid composition of the NPN fraction is subject to many influences and is not a stable nitrogen component of potato (Desborough, 1985). Particularly, amides are strongly affected by mineral nutrition, cultivar, soil, and climatic conditions. In contrast, the amino acid pattern of individual proteins appears to be genetically determined and cannot be influenced by fertilizers or other growth factors (Eppendorfer et al., 1979).

2.3. Potato Tuber Storage Proteins

Potato tuber storage proteins are numerous compared with those of grains and legumes, where only one or a few storage proteins occur in seed endosperm. Biological value, as a useful measure of potato protein quality ranges from 70 to 81 on a scale of 100 (Juliano, 1999). Potato proteins are also of interest as ingredients for prepared foods because they exhibit functional properties such as their foam-forming and stabilizing capacity (Ralet and Guéguen, 2000; van Koningsveld et al., 2002).

Several types of potato protein have been isolated. The first separation of potato tuber proteins was based mainly on their solubility and classified into tuberin, albumin, globulin, glutelin, and prolamine (Lindner et al., 1960). Similar protein fractions were

reported by Kappor et al. (1975), who determined protein content of potato tubers to be albumin (49%), globulin (26%), glutelin (9%), prolamine (4%), and a residue (9%). The albumin fraction is greater than the globulin fraction, ranging from 49-75% and 23-36%, respectively (Seibles, 1979; Gorinstein et al., 1988). Albumin contributes to the digestibility of potato proteins.

Current classification of proteins is based on molecular mass as found by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and chromatographic techniques (Lindner et al., 1981; Gorinstein et al., 1988; Rajapakse et al., 1991). Classification of potato proteins is into three classes: 1) Patatin, 2) Protease inhibitors, and 3) Other proteins with high-molecular weight (Pots et al., 1999a; Ralet and Guéguen, 1999).

2.3.1. Patatin

Patatin protein has been detected in tubers of all cultivated varieties that have been examined, including the South American genotypes Andigena and Phureja. Patatin is a highly homologous group of 43 kDa glycoprotein isoforms with great nutritional value (Racusen and Foote, 1980; Racusen and Weller, 1984). Patatin is the major storage protein in potato tubers, although the actual amount described in tubers varies substantively. Patatin accounted for > 20% (Racunsen and Foote, 1980), 40 to 45% (Paiva et al., 1983), and even 40 to 60% of the total soluble proteins (Pots et al., 1999a). Patatin is localized in cell vacuoles, in which it accumulates during tuber development after passage through the endoplasmic reticulum and Golgi complex (Sonnewald et al., 1989).

Patatin accumulates in relatively large amounts in tubers, and in much lesser concentrations in stolons and roots. However, under certain conditions it can be induced to accumulate to high levels in other organs such as stems and petioles (Paiva et al., 1983; Hannapel et al., 1985). This accumulation occurs under environmental and hormonal conditions that interfere with the normal tuberization process, such as the removal of tubers and axillary buds. Accumulation of patatin can also be induced in leaves that have been incubated in high concentrations of sucrose (Rocha-Sosa et al., 1989). Patatin accumulation has been observed during the tuberization process, accounting for 5-7% of

the total protein in 1-g tubers increasing to 25% in 25- to 30-g tubers. Even though patatin does not exhibit strict tuber-specific expression, its close correlation with early events in tuber development and relative abundance distinguish it as a possible marker for tuberization (Hannapel, 1990).

Genes encoding patatin have been mapped genetically and physically (Park et al., 1983; Mignery et al., 1984; Stiekema et al., 1988; Twell and Ooms, 1988; Ganal et al., 1991). Patatin is encoded by a multigene family consisting of approximately 10-15 genes per monohaploid genome in potato (Mignery et al., 1988; Twell and Ooms, 1988). All patatin genes show high homology (85-98%) in their coding sequence (Park et al., 1983; Mignery et al., 1984; Pikaard et al., 1987; Stiekema et al., 1988; Twell and Ooms, 1988), except for the 5'-upstream untranslated region. The promoter sequences revealed two different classes of patatin genes, Class I and Class II. These classes differ in the absence (Class I) or presence (Class II) of a 22-base pair insertion just 5' to the translation initiation codon (Pikaard et al., 1987) and complete divergence of the sequences upstream of position 87 (Rocha-Sosa et al., 1989). Class I and II patatin genes diverge completely in their pattern of expression (Pikaard et al., 1987; Mignery et al., 1988). Class I patatin genes encode the major patatin isoforms in tubers, whereas Class-II genes encode the root form of patatin. In addition, Class I patatin genes are sucrose-inducible to accumulate in large amounts in leaves and stem explants, but Class II do not appear to be sucrose-inducible (Rocha-Sosa et al., 1989; Ganal et al., 1991).

Patatin isoforms are immunologically identical (Paiva et al., 1982; Park et al., 1983; Ganal et al., 1991) and have homologous NH_2-terminal amino acid sequences (Park et al., 1983). Patatin can be separated into four isoform pools, representing 62, 26, 7, and 5% of the total amount of patatin, respectively (Pots et al., 1999b). All isoforms of the patatin family contained proteins with two molecular masses of approximately 40.3 and 41.6 kDa; these differences reflect glycosilation patterns. Patatin is a highly structured molecule, in which structural integrity is maintained around pH 6 and up to 28°C. Due to the identical immunological responses and the high degree of homology within the gene families, patatin is studied as a group without the need to examine individual isoforms.

The amino acid sequence of patatin is 366 amino acids long without extended hydrophilic nor hydrophobic clusters (Stiekema et al., 1988). Positive and negative charges of the side-chains are randomly distributed over the sequence. Patatin has an estimated molecular mass on SDS-PAGE of 43 kDa (Racusen and Weller, 1984).

Unlike most other storage proteins, patatin may have a role in plant defense mechanisms. Patatin has enzymatic functions in lipid metabolism, such as lipid acyl hydrolase (LAH, esterase) and acyl transferase (wax synthase) activities (Galliard, 1971; Wardale, 1980; Racusen, 1984; 1985; Andrews et al., 1988; Anderson et al., 2002). These activities seem to be involved in the resistance reaction induced by attack by a pathogen, being important for the rapid degradation of cell membranes and, thus, rapid degradation of certain metabolites (Hirschberg et al., 2001). A further type of hydrolytic activity has been described for patatin, as a β-1,3 glucanase (Tonón et al., 2001). This glucanase may contribute to plant defense against fungal pathogens by digesting specific β-1,3 glucans in hyphal cell walls (Shewry and Lucas, 1997; Shewry, 2003). Other physiological properties, including antioxidant function, have also been associated with patatin (Al-Saikhan et al., 1995; Liu et al., 2003).

2.3.2. Protease inhibitors

Protease inhibitors account for up to 25% of the soluble proteins in potato tubers (Ryan, et al., 1987; Birk, 2003). These proteins are considered defensive chemicals in plant tissues that are both developmentally regulated and induced in response to insect and pathogen attack (Ryan, 1990). Potato protease inhibitors are divided into different groups: protease inhibitor I (PI-I, 10 kDa protein), protease inhibitor II (PI-II, 20 kDa protein), and the carboxipeptidase inhibitor group together with several other polypeptide inhibitors of serine proteases (22-25 kDa) (Ryan, 1990; Birk, 2003). However, Pouvreau et al. (2001) re-classified protease inhibitors into seven different families: potato inhibitor I, potato inhibitor II, potato cysteine protease inhibitor, potato aspartate protease inhibitor, potato Kunitz-type protease inhibitor, potato carboxipeptidase inhibitor, and a last group considered as "other serine protease inhibitors".

Potato protease inhibitors are developmentally regulated in a coordinated fashion during tuber growth (Paiva et al., 1983). PI-I, PI-II and potato cysteine protease inhibitors are the most abundant in potato tubers and are present from the very earliest stages of tuber development until the onset of sprouting (Rodis and Hoff, 1984; Walsh and Strickland, 1993; Pouvreau et al., 2001). PI-I is an effective inhibitor of Cys proteases, including papain, ficin, and chymopapain, and PI-II inhibits serine proteases, such as trypsin, chymotrypsin, subtilisin, oryzin, and elastase (Plunkett et al., 1982).

PI-II has been studied widely in potato tubers. It represents approximately 5% of the TSP (Balandin et al., 1995). It is encoded by a gene family, which contains about twenty members per tetraploid genome (Peña-Cortes et al., 1992). Until now, this gene family has been found only in the Solanaceae (Beekwilder et al., 2000). Studies of the PI-II gene family revealed that it exhibited a complex pattern of expression subject to both developmental and environmental regulation (Keil et al., 1989). PI-II mRNA constitutively accumulates to high levels in developing tubers and young floral buds of healthy, non-stressed potato plants (Lorberth et al., 1992). It also accumulates in the leaves after mechanical wounding, insect attack, fungal elicitor or bacterial infection (Peña-Cortes et al., 1992). These stresses triggered the transcriptional activation of the PI-II gene family, not only in the damaged leaves but also in distal, non-damaged ones (Sanchez-Serrano et al., 1990). PI-II gene expression can be induced by plant hormones like abscisic acid (ABA) and methyl jasmonate as part of the wound signal transduction pathway and by plant cell wall fractions, chitosan, and sucrose (Sanchez-Serrano et al., 1986; Kim et al., 1991; Peña-Cortes et al., 1992). The PI-II genes might also be regulated by naturally occurring and synthetic auxins, gibberellins (GAs), and the ethylene-releasing compound ethephon (Kernan and Thornburg, 1989; Taylor et al., 1993a; Jacobsen and Olszewski, 1996).

Different potato tuber proteins of 22, 23, and 24 kDa were purified by Suh et al. (1990). All three inhibited serine proteases. The 22 and 23 kDa tuber proteins also inhibited both trypsin and chymotrypsin, while the 24 kDa protein only inhibited trypsin activity (Suh et al., 1991). Transcription expression of 22 kDa Kunitz-type potato protease inhibitor (KPP1) is developmentally-regulated in tubers and environmentally-regulated in leaves (Suh et al., 1990). KPP1 is localized in cell walls, with some

detectable levels in the plasma membrane of cells in both tubers and non-wounded upper leaves from wounded potatoes. KPP1 is translated in the endoplasmic reticulum of the cytoplasm, processed and targeted to the cell wall where it is stored as a mature protein (Suh et al., 1999).

2.3.3. Other proteins

Other proteins of high molecular weight comprise 20 to 30% of the TSP (Pots et al., 1999a, Ralet and Guéguen, 1999). These proteins are mainly represented by enzymes and kinases involved in starch synthesis that include sucrose synthase, ADP-glucose pyrophosphorylase (AGPaseB and AGPaseS), granule bound starch synthase (GBSS), branching enzyme (BE), and plastidic starch synthase (STP) (Gerbrandy and Doorgeest, 1972; Bánfalvi et al., 1996; Marshall et al., 1996).

2.4. Tissue Layers within the Potato Tuber and their Relative Volume Contribution

Protein distribution in the different tuber tissues is a major focus of this study. Therefore, it is important to describe the structure of potato tubers. Tuber structure reflects its stem origin but is influenced by extensive radial growth. In cross section, mature tubers have four clearly distinguishable areas (Fig. 2.1). These areas are the periderm, cortex, perimedullary, and pith tissues (Reeve et al., 1969; Peterson et al., 1985). The periderm replaces the epidermis during tuber expansion and comprises the outermost layer of the tuber. It is usually thicker at the stolon than at the bud (rose) end, although its thickness varies considerably depending on cultivar and growing conditions (Diop and Carveley, 1998). The region immediately inside the periderm extending inwards to the vascular ring is the cortex layer. This area originally was divided into two parts: outer cortex (next to the periderm, not more than 2 mm thick) and inner cortex, considered as a layer of storage parenchyma (between outer cortex and vascular ring) (Artschwager, 1924). Total cortical layer thickness varies as well, but it is negligible at the eyes and point of stolon attachment. Beneath the cortex is the vascular ring comprised of xylem and phloem. Inside the vascular ring, there is another layer of storage

parenchyma called perimedullary tissue or outer medulla. It represents the major part of the tuber and, like the cortex, contains starch grains as reserve material. Towards the centre is the pith, which consists of a small central core with arms of medullary parenchyma radiating from it. The pith cells are relatively lower in starch, higher in water content, and more translucent than the other tissues.

Despite clear differences in the tissue-proportions of potato tubers, few volume-proportion estimates are available in the literature for specific tuber tissue layers. Neuberger and Sanger (1942) determined with a simple method the percentage contribution of each tissue layer in the two cultivars Majestic and King Edward. It was done by dissecting the potato tubers into different parts, separating the tissues, and weighing them. The periderm amounted to 1.5-5%, of the total fresh weight, the cortex 35-45%, and the outer and inner medulla (pith) the remaining percentage. Percentage contributions of the major areas of whole tubers was calculated by Chapell (1958; cited by Woolfe, 1987). Tissue percent proportions for small potatoes were 2.8, 52.2, 31.3, and 13.7% for periderm, cortex, outer medulla (perimedullary area), and pith respectively. While for large potatoes it was 2.8, 37, 40, and 20.2% for these tissues. More recently, Liu and Xie (2001) calculated the specific tissue volumes for microtubers of two cultivars using an ellipsoid formula. Volume proportions of individual tissues differed slightly for each cultivar. In cv. Mira, volume proportions for cortex, perimedulla, and pith were 32, 67, and 1.5%, while for cv. E-Potato 1 these were 29, 68, and 3%, respectively. Periderm volume proportions were not determined.

Variation between estimates for the tuber tissue-proportions may be attributed to differences in tuber shape and size between cultivars, age, and growing conditions (microtubers, field-grown tubers). Difficulties in defining the exactly tissue boundaries may also account for this variation.

2.5. Genetic Improvement of Potato to Increase Tuber Protein Level

The cultivated potato, as one of the most important world food crops, demands continued genetic improvement to meet the needs of a changing world. The high biological value of potato protein and its potentially high yields per unit of area of land

have attracted scientific interest for years.

Attempts to improve the protein levels of potato tubers have included traditional breeding methods through the hybridization of parental clones, and the subsequent selection among large seedling populations for superior individuals with the desired combination of traits (Desborough and Lauer, 1977; Plaisted et al., 1994). Single plant selections were then propagated vegetatively and evaluated for relevant agronomic and quality attributes. This breeding approach has resulted in the development of some elite clones with increased protein levels. However, to the best of our knowledge, none of these have been released as a new cultivar.

One of the major difficulties associated with traditional potato breeding relates to the tetraploid nature of potato in conjunction with high heterozygosity and the sterility of many selections (Douches et al., 1996; Mackay, 2005). These difficulties require exceptionally large populations of potato seedlings to be screened in order to recover superior individuals. Consequently, the initial selection for many desirable characters has often been inefficient and time consuming. Some other alternatives to traditional breeding efforts for potato improvement include genetic engineering, use of somaclonal variation, and dissociation of chimeral plants (Dunwell, 2000; Ahloowalia and Maluszynski, 2001; Jain, 2001).

2.5.1. Genetic engineering

Genetic engineering has been used in potato cultivar improvement programs because of the relative ease of potato transformation and its clonal mode of multiplication (Destéfano-Beltrán, 1991; Ghislain et al., 1998). The most widely used technology has been genetic transformation using *Agrobacterium tumefaciens*. Most of these studies have focused on resistance to different pest, virus, and fungal diseases. Some examples include resistance to Colorado potato beetle (*Leptinotarsa decemlineata)* (Perlak et al., 1993), potato tuber moth (*Phthorimaea operculella*) (Davidson et al., 2002), potato leafroll luteovirus (PLRV) and potato virus Y (PVY) (Hassairi et al., 1998), soft rot (*Erwinia carotova)* (During et al., 1993), late blight *(Phytophthora infestans)* (Cornelissen and Melchers, 1993; Osusky et al., 2004), and black scurf *(Rhizoctonia*

solani) (Broglie et al., 1991). Many transgenic potato releases have been approved by the European Commission and Joint Research Centre to investigate the expression and stability of the modified traits, and the general agricultural value of these modified lines (Biotechnology and GMOs, 2006).

On the other hand, less attention has been directed to improve the nutritional value of potato tubers. Attempts to enhance tuber nutritional composition have centered on improvements to essential amino acid composition of the proteins. One approach was the expression of synthetic genes encoding proteins rich in essential amino acids as the HEAAE-DNA (High Essential Amino Acid Encoding DNA) and HEAAE II Tetramer (Destéfano-Beltrán et al., 1991). However, while detectable levels of these synthetic proteins were observed, the potato protein content was not significantly increased. Another approach to improve tuber proteins involved the insertion and expression of gene (s) encoding essential amino acid-rich protein in potato plants. Genes of storage proteins, such as glycinin from soybean (Utsumi et al., 1994; Hashimoto et al., 1999), and Brazil nut 2S protein (BN2S) from Brazil nut *Bertholletia excelsa* (Altenbach et al., 1989, Tu et al., 1998), are two examples used for this purpose. Expression levels of glycinin proteins in the transgenic potato tubers were detected. However, there were not significant differences between the transgenic and control tubers. In transformed potato tubers with BN2S genes, the Met content was further enriched, but significant decrease in Cys content occurred. This reduced the apparent usefulness of the BN2S protein as a means of improving the nutritional quality of potato plants.

Recently, the seed albumin gene *AmA1* (from *Amaranthus hypocondriacus*), was successfully introduced and expressed in a late blight-resistant diploid potato cv. A16 (Chakraborty et al., 2000). Transgenic potato plants expressed significantly increased total protein content, with an increase in most of the essential amino acids. Protein content ranged from 14.6 to 16.6 mg g^{-1} tuber (DW) in transgenic plants compared with 11.1 mg g^{-1} tuber for the original diploid potato, which corresponded to an increase of 30-48% in protein level. Multicentric field trials on this transgenic line have been conducted to asses the nutritive value and agronomic performance. The resultant enhanced protein potato cultivar is under approval for new cultivar release.

Many other improvements in potato nutritional value are expected using recombinant DNA technology. However, strong resistance among consumers to accept genetically modified plants continues.

2.5.2. Somaclonal variation

Development of plant biotechnology has led to the application of in vitro techniques for crop improvement. Somaclonal variation is a term introduced by Larkin and Scowcroft (1981) to describe genetically novel shoots or plantlets derived from tissue culture systems. The utility of somaclonal variation to plant improvement results from the ability to isolate improved variants without loss of horticultural quality, from well-established cultivars (Evans et al., 1984; Jain, 2001). However, it is not always known if this variation arises from genetically variant cells that are present prior to culture (pre-existing mutated cells) or if variant cells are induced by the culture process itself due to environmental stress and/or chemical mutation from exposure to growth medium ingredients (Skirvin et al., 1994; 2000).

2.5.2.1. Origin of somaclonal variation

Pre-existing variation

Cell division is a controlled event that normally yields identical copies of the parental cell. However, mutations can arise during this process. Mutated cells either die or cease to divide, but sometimes these cells may become part of a meristem and grow to constitute a significant part of the plant body, developing into chimeras of various complexities (Hartmann et al., 2002) (see section 2.5.3. Chimeral plants). Regeneration of whole plants from these tissues can yield individuals which differ from the source plant (Skirvin et al., 2000).

Vegetatively propagated clonal cultivars are known to accumulate mutations over time that come about through microenvironment effects on plant apical and lateral shoot meristems. If a chimeral cultivar is propagated through callus and adventitious shoot or embryoid formation, then chimeral disassembly can occur and the cultivar status is

irrevocably altered (Skirvin et al., 1994; 2000). Variation in regenerated plants can originate from within the source tissue in several ways (Fig. 2.2A). Variation can result from explants containing a mixture of individual cells with different genotypes (mix of cells derived from different histogenic layers LI & LII, LII & LIII or LI, LII & LIII) or when explants come from different cell layers of the chimeral plant. Either leads to a mixed population of regenenerants; some that look like the source plant and others like the mutant genotype.

Tissue culture induced variation

When explants are grown in vitro, the tissue culture environment itself appears to modify normal controls of cell division and chromosome distribution to result in somaclonal variation. It is suggested that the tissue culture environment "resets" or "reprograms" plant genomes to yield plants with altered genotypes (McClintock, 1984). Somaclonal variation is associated with indirect tissue culture systems that involve a callus phase. The process of accumulation of mutations in this system is said to result from asynchrony between nuclear and cell division that occurs in callus. High variation is expected in regenerated plants from adventitious shoots or somatic embryos formed on callus (Fig. 2.2B).

The use of excessive growth regulators, length of time in culture, number of subcultures, and mutation events that result from in vitro selection pressure are also among the factors inducing somaclonal variation (Skirvin et al., 1994; Jain, 2001). If meristems that are initiated in callus accumulate mutations in vitro in the same way as in the field, adventitious chimeral shoot tips could arise. These could have transient sectorial or mericlinal chimeral arrangements or the stable periclinal arrangement. These shoots may appear identical to the source plant tissue, unless the genes involved affect some obvious phenotypic trait. The genetic risk associated with these adventitious culture systems varies with the species involved. The risk is estimated to be relatively low (1-3%) for adventitiously regenerated plants (Skirvin et al., 2000). However, off-types are usually visually assessed and real numbers of clonal variants may be far greater.

2.5.2.2. Epigenetic variation

Confounding pre-existing and culture-induced somatic variation is a complex of epigenetic characteristics associated with culture-induced phenotype. It includes a suite of environmentally-dependent anatomical and physiological changes characteristic of in vitro-grown plants (Donnelly and Tisdall, 1993). These result from exposure to the culture environment, which imposes: saturated atmosphere, low medium water potential, low light level, low rate of gas exchange, high and constant temperature, presence of sugars and exogenous growth regulators in the medium. Some of the many features of the culture-induced phenotype include: miniaturization, mixotrophic nutrition, reduced epicuticular and cuticular wax deposition, reduced and altered trichome population, and altered stomatal function (Donnelly and Tisdall, 1993; Kaeppler et al., 2000). All of these features affect acclimatization of ex vitro transplants. However, the new tissues formed ex vitro exhibit the control phenotype in response to the climate outside of the culture containers. The culture-induced phenotype is transient and quickly outgrown.

2.5.2.3. Somaclonal variation in potato

One of the first well-documented reports of somaclonal variation in potato involved leaf mesophyll protoplast-derived (protoclones) of cv. Russet Burbank (Shepard et al., 1980). Variation was extensive and included changes in growth habit, tuber shape and size, skin color, photoperiod requirements, and maturation date. Some of them (20 out of 800 tested) also showed greater resistance to late blight (*Phytophthora infestans*) (Secor and Shepard, 1981). Similar variation was observed in protoplast-derived regenerants in later studies with other potato cultivars (Sree-Ramulu et al., 1983; Creissen and Karp, 1985). However, although many of these protoclones were described as having some agronomic trait exceeding the parental cultivar, most displayed too many accompanying undesirable changes to merit continued breeding efforts.

Despite the potential utility of somaclonal variation in potato, it has seen limited use in potato breeding programs, due to general disagreement on its potential to improve commercially important characteristics such as yield. Many investigations into

somaclonal variation have been reported with interesting findings, despite the absence of commercial releases. For example, Rietveld et al. (1991) obtained somaclonal variation in cv. Superior for commercially important traits occurring at frequencies useful for breeding purposes. Selected somaclones exhibited desirable improvements in yield, vigor, tuber number, and shape, and most of them showed phenotypic stability over more than two consecutive tuber generations and maintained their horticulturally desirable characteristics. Other studies of potentially useful somaclonal variants derived through in vitro selection have focused on resistance to diseases. Some examples have included somaclonal variants with resistance to scab *Streptomyces scabies* (Thompson et al., 1986), protoclonal variants of cv. Crystal with resistence to *Erwinia* soft rot (Taylor et al., 1993b), regenerated plants from stem-derived callus of cv. Desirée with resistance to *Verticilium dahliae* (Sebastini et al., 1994), gametoclones of 3 potato genotypes with resistance to four species of root knot nematodes (*Meloidogyne* spp.) (Grammatikaki et al., 1999).

Attempts to detect somaclonal variation through protein based or molecular techniques have lead to mixed results. Isozyme variation was found in some regenerated somaclones from stem internodes of three potato cultivars (Binsfield et al., 1996). Altered band profiles in 2 of 40 somaclones of cv. Skirma were detected using four ISSR primers (Albani and Wilkinson, 1998), and mixoploidy and plant chimeras were observed among somaclones of cv. Bintje (Jelenic et al., 2001).

It has been estimated that the somaclonal variation rate is 1-3% per culture cycle (Skirvin et al., 2000), while others believe it can be greater than 10% per cycle (Larkin et al., 1989). A statistical approach to somaclonal variation rate in plant tissue culture was evaluated by Côte et al. (2001). They concluded that: 1) variation rate increase can be expected as an exponential function of the number of culture cycles, and 2) after a given number of culture cycles, a percentage of variable off-types can be expected. To be of practical value, the expression of variation among new plants derived in vitro should be frequent enough to enable selection of desirable traits, and the selected lines should perform well under a range of environments (Karp, 1995; Duncan, 1997). Increasing the number of parameters under evaluation during in vitro or ex vitro screening will increase the opportunity to select material with improved characteristic(s). Once in vitro selection

has been performed field selection can follow (Duncan, 1997). Many desirable traits of potato should be screened directly in the field, including yield and tuber type.

Reconsideration of the potential importance of somaclonal variation for crop improvement has increased lately, due to heightened awareness of genetically modified organisms (GMOs) and the public's concern with their real and perceived safety (Skirvin et al., 2000; Jain, 2001). Somaclonal variation remains a potential tool to introduce variation into a potato breeding program.

2.5.2.4. Use of in vitro somaclonal variation

Only a small percentage of identified somaclonal variants have ever been released as new cultivars. Some of them were recently reviewed by Jain (2001) and include a Cavendish banana resistant to wilt (*Fusarium*), wheat cv. He Zu No.8 with greater yield, maize cv. Yidan 6 as a variant for grain and forage use, rice cv. Dama resistant to *Picularia* and with improved cooking quality, celery cv. UC-TC resistant to *Fusarium*, tomato cv. DNAP17 resistant to *Fusarium* and cv. DNAP9 with high solid content, flax cv. Andro tolerant to salt and heat, pepper cv. Bell Sweet with yellow fruit, *Haemerocallis* cv. Yellow Tinkerbell with dwarf stature and short flowers, among others.

For potato, development of new cultivars from somaclonal variation has been modest; only one cultivar release, the cv. White Baron, a variant of cv. Danshakuimo (Irish Cobbler) which does not turn brown after peeling (Arihara et al., 1995). Other important desirable traits have been selected in potato, although these somaclonal variants have not been released as new cultivars. They include resistance to *Fusarium solani*, *F. oxyporum, Phytophthora infestans, Alternaria solani* (Jain et al, 1998; Critinzio and Testa, 1999), and salt tolerance (Ochatt et al., 1999).

Only a few studies have used somaclonal variation to attempt to improve potato tuber protein quality. Tubers regenerated from leaf explants of cv. Superior showed high variation in electrophoretic protein band pattern (Smith, 1986). As a strategy to increase the Met levels in potato, regeneratedt plants from protoplast-derived calli were grown in the presence of the amino acid analogue, ethionine (Languille et al., 1998). In six of the

48 protoclones selected, tubers produced significantly increased free Met content, up to 2.66 times the control level.

Somaclonal variation, although difficult to direct and manipulate, represents one potential way for nutritional improvement of the potato crop. It can offer a rapid and easily-accessible source of variation for use in breeding programs and novel variants can arise that may not be achieved by conventional methods. In addition, although selected somaclones require extensive field testing, some desirable traits can be screened during the in vitro phase.

2.5.3. Chimeral plants

Potato tubers, like all dicot plant stems, are composed of distinct tissue layers derived from defined histogenic layers in the shoot meristem (Tilney-Basset, 1986; Lineberger, 2005). Each histogenic layer can be distinguished by the planes of cell division, according to the *Tunica-Corpus* theory (Schmidt, 1924). In the embryogenic shoot, the tunica (tunic) consists of two histogenic layers covering the inner corpus. The outermost histogenic layer (LI) forms the outer covering (epidermis) of the plant. The plane of cell division in the tunic is principally anticlinal (at right angles to the long axis of the organ). The second histogenic layer (LII) gives rise to most inner leaf tissue mesophyll and cortical tissues, and is responsible for the formation of the mature sexual reproductive cells (gametes) and derived structures. This region develops from anticlinal and periclinal (tangential) divisions. The third histogenic layer or corpus (LIII) gives rise to some inner mesophyll of leaves, vascular bundles, as well as most of the central stem tissue such as perimedulla and pith. In the corpus, the planes of cell division are in all directions (mass meristem). The tunic enlarges in surface area and the corpus in volume.

Sometimes, mutations can originate in one of the histogenic layers of the apical meristem, resulting in plants composed of tissues of more than one genotype. These plants are called chimeras (Tilney-Basset, 1986; Hartmann et al., 2002) or genetic mosaics (Marcotrigiano and Gradziel, 1997). Chimeras are classified based on the position and extent of the mutant sectors in the shoot apical meristem: sectorial, mericlinal, and periclinal chimeras (Fig. 2.3). Sectorial chimeras consist of a sector of

mutant tissue present in the three histogenic layers of the meristem. These chimeras are rare and unstable. Mericlinal chimeras possess a mutated sector in some, but not all the layers of the meristem. They can develop from sectorial chimeras or are generated spontaneously. These chimeras are also unstable and revert to periclinal chimeras or the nonmutated (wild-type) form. Periclinal chimeras are the most common; the mutated tissue includes one or two (but not all three) complete histogenic layers. Generally, the mutated layer is the outer tunic (LI) that develops into the epidermis. The plant phenotypically presents the characteristics of the outside layer, as the inner tissue is not visible. Periclinal chimeras are relatively stable and can be maintained vegetatively through axillary growth including stem cuttings (where growth is from axillary buds), grafting, or budding, but not necessarily through adventitious growth (where growth is from leaf, root, or stem cuttings without axillary buds) (Marcotrigiano, 1990).

The persistence of chimeras is largely dependent on the localization of the mutant cell(s) in the plant and the organization of the shoot apical meristem (Tilney-Basset, 1986). The stability of periclinal chimeras is well-know. Many plant sports with unique and improved characteristics are stable, and have been propagated horticulturally by cuttings for centuries. Chimeras, developed through spontaneous mutation, are common among fruit, vegetable, and ornamental species. Some examples of recognized chimeral plants include pear cv. Max Red Bartlett a sport of the old green cv. Barlett (Reimer, 1951); apple cv. Bridgham a sport of cv. Delicious (Dayton, 1969); and blackberry cv. Thornless Evergreen a periclinal chimera of thorny *Rubus laciniatus* (McPheeters and Skirvin, 1983).

2.5.3.1. Potato chimeras

Particularly in potato, periclinal chimerism has given rise to many new cultivars that are phenotypically different from their original cultivars (Miller, 1954; Howard, 1959; Klopfer, 1965 cited by Tilney-Bassett, 1986). Altered tuber characteristics, especially skin (periderm) color and texture have resulted from periclinal chimerism. For example, cv. Golden Wonder, with a thick brown russeted skin originated from cultivar Langworthy with a thin white smooth skin (Crane, 1936). Similarly, cv. Russet Burbank,

the most popular potato cultivar in North America, originated as a periclinal somatic mutation of Burbank in 1914 (Davis, 1992). Cv. Burbank is a thin, smooth-skinned long white potato while cv. Russet Burbank has a thick, slightly rough reticulated skin commonly termed "netted" as in Netted Gem, a common synonym for Russet Burbank (see Fig. 7.1). Some other examples of potato chimeras were reviewed by Klopfer (1965 cited by Tilney-Basset, 1986). The latter listed many other russeted potato sports that are recognized as periclinal chimeras. In each case, the observed chimera had the mutation in LI affecting the periderm and LII and LIII layers were apparently wild-type.

2.5.3.2. Dissociation of periclinal chimeras into their component genotypes

One possible explanation for some observed somaclonal variation, and a potential means of potato improvement, is through the dissociation (disassembly) of chimeral plants. This methodology provides an opportunity for cultivar improvement by benefiting from the different genetic tissues present in clonal cultivars (Jain, 2001). For example, separation of periclinal chimeral tissues has produced non-chimeral, genetically homogeneous plants composed of one of the genotypes of the chimera, as done when chimeral plants were unstable and difficult to maintain (Abu-Qaoud et al., 1990). For seed-propagated species, dissociation was useful when the desired genotype was not present in the cell layer (LII) that gives rise to the gametes and when it was not economical to perpetuate the chimera vegetatively (Tilney-Basset, 1986; Macotrigiano and Gradziel, 1997). Major reasons for chimeral dissociation include its utility in verification of the chimeral composition of a plant used for developmental analysis or as a patented cultivar (Howard, 1970; Tilney-Basset, 1986; Macotrigiano and Gradziel, 1997). Less attention has been dedicated to using chimeral separation as a potential tool for cultivar improvement.

Some chimeral dissociation techniques include radiation treatments, propagation by adventitious roots, development of adventitious shoots, and development of somatic embryoids. Ionizing radiation has been widely used for chimeral separation, especially in ornamental plants (Pereau-Leroy, 1974; Marcotrigiano, 1997). This procedure kills the central mother cells of the apical meristem and forces regeneration of new meristems

from less damaged surrounding cells, causing either chimeral dissociation or rearrangement of cell layers. However, this technique is clearly mutagenic. Chimeral dissociation through mutagenesis with X-rays was used by Howard (1964a) to investigate potato chimeras, such as Red King (a bud-sport from the variety King Edward VII produced by a mutation in LI), Bonte Furore and Bonte Urgenta (both also from mutations in LI), "Miller's Purple" sport (from a mutation in LII), Yellow Rode Star and Yellow Urgenta (both from mutations in both LI and LII). From Red King, Bonte Furore, Bonte Urgenta, and "Miller's Purple" sports, it was possible to obtain by X-ray treated plants which were homogeneous for either LI or LII of the original chimeras. However, no changes occurred in the two irradiated sports which had mutations in both LI and LII.

Adventitious roots from stem cuttings have an "internal" origin; they typically arise from LIII derivatives. The entire root of a periclinal chimera has the genotype of the corpus of the shoot apical meristem (Tilney-Bassett, 1986; Macotrigiano and Gradziel, 1997). Plant regeneration from root cuttings is not a mutagenic technique, but the cultivar should be able to regenerate roots that later will produce adventitious shoots. In addition, callus formation should be avoided to decrease chances of regenerated plants with somaclonal variation. Adventitious shoot formation from root cuttings has been used to study the chimeral structure and separate chimeral genotypes of some fruit, such as pear (Chevreau et al., 1989). In potato, Howard (1964b; 1970) generated shoots from plant roots that produced non-chimeral tubers with the LIII genotype.

The most common technique that has been used to dissociate chimeral potato plants involves production of in vivo adventitious shoots (Marcotrigiano, 1990). Adventitious shoots were induced by surgically removing all terminal and axillary shoot buds (disbudding). Asseyeva (1927) developed the "eye-excision" method to reveal the chimeral nature of potato cultivars. This disbudding technique induced the development of adventitious shoots from internal tissues, specifically from the perimedullary area and pith. This method was used in many studies to verify the periclinal chimeral composition of different potato cultivars (Crane, 1936; Howard, 1959; 1970). While inconsistencies were observed in the phenotypes of non-chimeral tubers of regenerated plants, these were mainly explained as "faulty experimentation".

The use of plant tissue culture techniques is well suited to the dissociation of plant chimeras (Jonhson, 1980; Abu-Qaoud et al., 1990; Marcotrigiano and Gradziel, 1997). A classic example is the development of pure thornless blackberry from chimeral source plants (McPheeters and Skirvin, 1983; 1989). Thornless Evergreen is a thornless mutant of the thorny Evergreen blackberry (*Rubus laciniatus*). Thornless Evergreen is a periclinal ("hand in glove") chimera in which a layer of mutant (thornless) cells surrounds a core of wild type (thorny) tissue. Chance separation of genotypes in this chimeral blackberry (Thornlees Evergreen) by in vitro propagation showed that most regenerants were thornless, others were chimeral like the source tissue and some were genetically thornless derived from the mutant LI histogenic layer (McPheeters and Skirvin, 1983; Skirvin et al, 1994). Following field-selection among a population of thornless plants, a commercially non-chimeral plant was selected and named, cv. Everthornless (McPheeters and Skirvin, 2000).

Dissociation would be more efficient if derivatives of the three histogenic layers are separated and if somatic embryoids are induced to form from single cells. Direct somatic embryogenesis (Fig. 2.2), where callus is minimal or not present, represents a new and alternative "cleaner" method for dissociation of chimeral plants into their component genotypes. By definition, regenerated plants from somatic embryos would all be non-chimeral and reflect the cell variation present in the explant, as opposed to new variation introduced by mutation or adventitious growth during the tissue culture process. This strategy for chimeral disassembly is investigated in this thesis. In addition, tissue culture technology does not face the same negative public image and concerns of genetic engineering technologies. Therefore, it is more likely that people will accept plant modification from this source (Jain, 2001).

Table 2.1. Essential amino acid composition of protein from potato, cereals (wheat, rice, oat), legume (bean), and whole egg.

Essential amino acid		POTATO[a]	WHEAT[b]	RICE[b]	OAT[b]	BEAN[b]	WHOLE EGG[c]
		---------- [g/16 g N] ----------					
Histidine	(His)	2.0	2.1	2.4	2.1	2.9	2.4
Isoleucine	(Ile)	3.9	3.8	3.8	3.8	4.2	5.6
Leucine	(Leu)	5.9	7.0	8.2	7.2	7.7	8.3
Lysine	(Lys)	6.0	1.9	3.7	3.7	7.2	6.2
Methionine + Cysteine	(Met + Cys)	(1.5 + 1.5) 3.0	4.2	3.7	4.5	1.9	5.0
Phenylalanine + Tyrosine	(Phe + Tyr)	(4.3 + 3.5) 7.8	7.4	8.8	8.3	7.9	9.1
Threonine	(Thr)	3.9	2.7	3.4	3.4	4.0	4.0
Tryptophan	(Trp)	1.4	1.1	1.3	1.3	1.0	1.0
Valine	(Val)	5.1	4.3	5.8	5.1	4.6	5.0

a Average concentration reported by various authors (Kaldy and Markakis, 1972; Rexen, 1976; López de Romaña et al., 1981).
b Cited in Woolfe, 1987
c WHO, 1973.

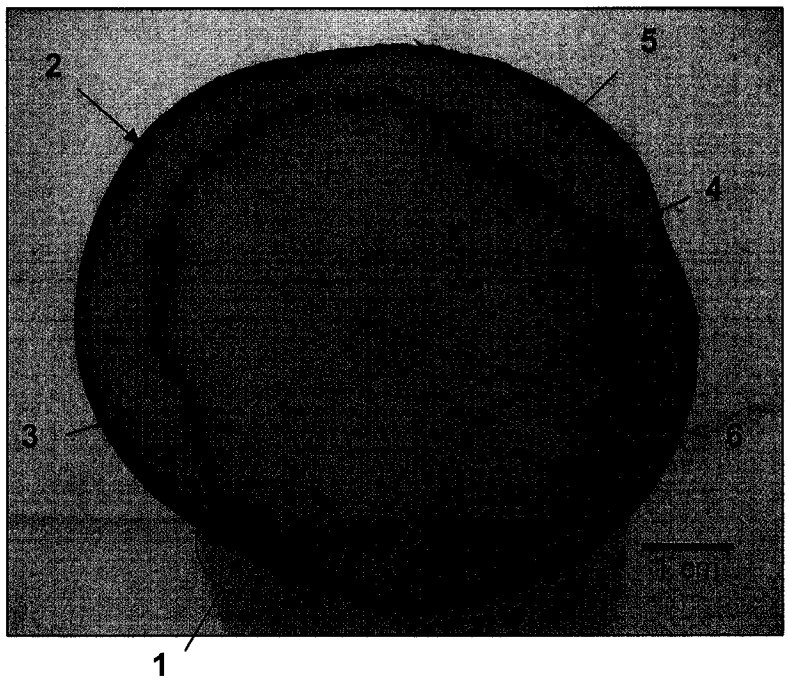


Figure 2.1. Cross section of mature potato tuber showing internal structure. 1) "eye" containing buds in axil of scale leaves, 2) periderm, 3) cortex, 4) vascular ring, 5) perimedullary zone, 6) pith. In all research described in this thesis, the perimedullary and pith regions were treated as a single unit (pith).

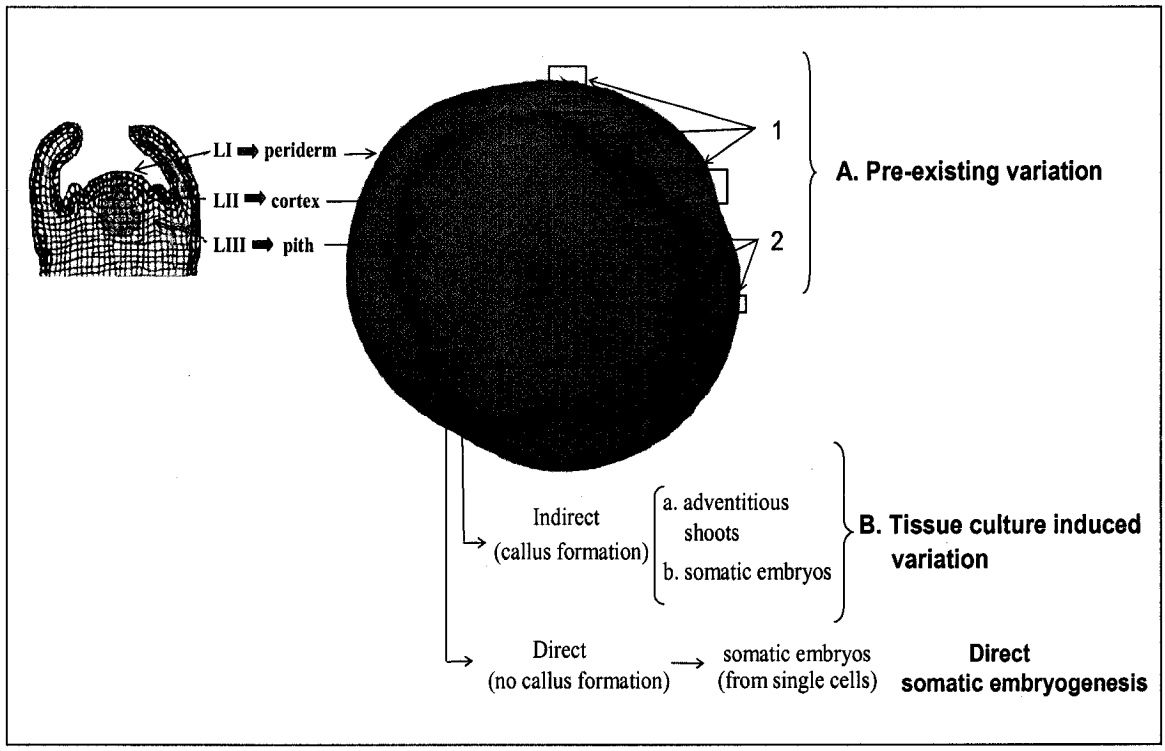
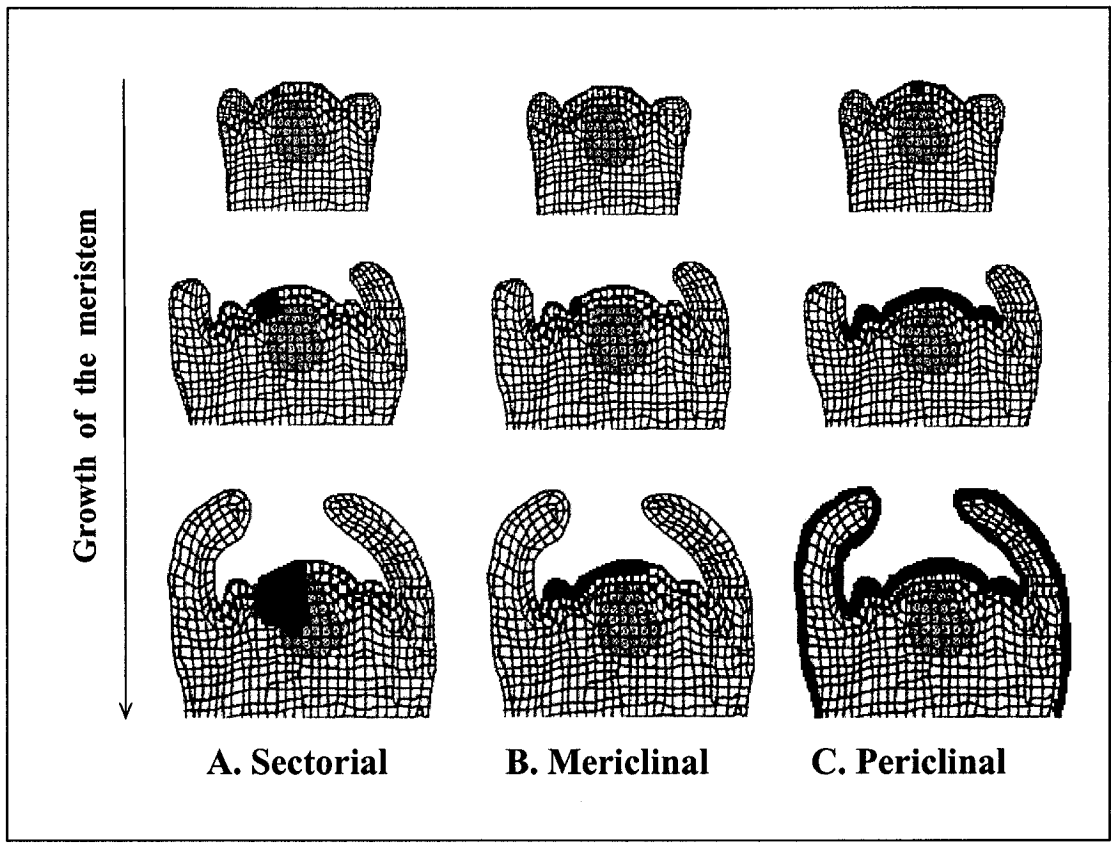


Figure 2.2. Origin of somaclonal variation from a periclinal chimeral potato tuber. **A.** Pre-existing variation. 1. Variation results when explants include tissues derived from two or three histogenic layers, such as LI & LII, LII & LIII or LI, LII & LIII. 2. Variation results from explants taken from different cell layers of the chimeral plant, and as a consequence with different genotypes. The explants could be from LI, LII, or LIII separately. **B.** Tissue culture induced variation. High variation is likely in regenerated plants from indirect culture systems (adventitious shoots and somatic embryos) involving a callus formation phase. Direct somatic embryogenesis (without callus) is expected to regenerate plants with characteristics similar to the explant genotype. The phenotype of the regenerants may or may not look like the chimeral cultivar.



Modified from Lineberger, 2005

Figure 2.3. Classification and development of chimeras. **A.** Sectorial. The mutated tissue involves a sector of the meristem that extends to all three histogenic layers. This chimeral type is unstable and reverts either to a mericlinal or periclinal chimera. **B.** Mericlinal. Cells carrying the mutated gene occupy only a part of the outer layer of the meristem. This type is unstable and reverts to a periclinal chimera, the non-mutated form (wild-type), or may continue to produce mericlinal shoots. **C.** Periclinal. The mutated tissue occupies one, or more than one, entire histogenic layer that is genetically distinct from another layer. This type is the very stable "hand-in-glove" arrangement.

CONNECTING STATEMENT FOR CHAPTER III

Chapter III consists of the manuscript entitled "Concentration and distribution of total soluble protein in fresh and stored potato tubers" prepared by E. Ortiz-Medina and D.J. Donnelly. This manuscript was presented in the form of a poster for the *XXVIth International Horticultural Congress (IHC2002), Potatoes - Healthy Food for Humanity: International Developments in Breeding, Production, Protection and Utilization* held in Toronto, August 11-17, 2002. The accompanying manuscript was published in the Congress Proceedings in Acta Horticulturae (2003) 619:323-328.

Although protein has been widely studied in many potato cultivars, little information is known about the protein distribution within the tuber tissues. This chapter describes the total soluble protein (TSP) content of 20 field-grown potato cultivars, including the most important cultivars grown in North America. TSP is reported for different tuber tissue layers (periderm, cortex, and perimedulla/pith), at the time of tuber harvest (fresh) and after 6 months of storage. For a subset of seven cultivars, TSP concentration and its tissue-specific distribution were compared between fresh field-grown tubers and in vitro-grown tubers (microtubers).

Chapter III

CONCENTRATION AND DISTRIBUTION OF TOTAL SOLUBLE PROTEIN IN FRESH AND STORED POTATO TUBERS

E. Ortiz-Medina and D.J. Donnelly[1]

3.1. Abstract

Total soluble protein (TSP) concentration and its distribution in different tissues was determined for 20 cultivars of both fresh and stored potato tubers from the 2000 and 2001 growing seasons. A subset of 7 cultivars was used to compare the concentration and distribution of TSP between fresh field-grown tubers and microtubers. TSP concentration was quantified separately in three tissue layers (periderm, cortex, and perimedulla/pith) using the Bradford method. In most cultivars, the TSP concentration on a dry weight (DW) basis was significantly greater in the periderm compared with the cortex and pith. The TSP concentration in fresh field-grown tubers ranged from 38 to 73 mg g^{-1} DW in the periderm compared with 30 to 49 mg g^{-1} DW in the cortex and pith. After 6 months of tuber storage, TSP concentration was affected in half of the cultivars, decreased (mean of 16%) in five cultivars and increased (mean of 18%) in four cultivars. While the relative TSP concentration in the tissues tended to be distributed in a similar pattern for each cultivar, whether fresh or stored, concentrations were greater in microtubers than in fresh field-grown tubers; possibly a function of the readily available nitrogen in the tissue culture medium. These results suggest avenues for identifying and selecting genotypes with increased protein concentration and improved nutritive value.

3.2. Introduction

Potato (*Solanum tuberosum* L.) protein has a high nutritional value (Kapoor et al., 1975; Racusen and Foote, 1980; Woolfe, 1987, Juliano, 1999) and is composed of three classes of soluble protein; patatin (40-60% of all buffer-extractable proteins), protease inhibitors (20-30%), and other proteins with high-molecular weight (20-30%) (Pots et al.,

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1999a; Ralet and Guéguen, 1999). Estimates of protein quantity among *S. tuberosum* cultivars vary widely, from 9.5 to 14% on a dry weight (DW) basis (reviewed by Desborough, 1985). In some cultivars (Norchip), similar protein concentration occurred in the cortex and pith (Desborough and Weiser, 1974), while in others (Katahdin, Norking Russet, and Shepody), the concentration of protein was greater in the cortex than in the pith (Munshi and Mondy, 1989). For most cultivars, it is not known how protein is distributed within tuber tissue layers or how quantity and distribution are affected seasonally or by storage.

Studies on tuber storage have primarily focused on features affecting tuber processing rather than protein. However, nitrogenous compounds such as proteins and free amino acids are affected during storage (Pots et al., 1999a; Peshin, 2000). Only two studies have examined protein levels in microtubers (Rajapakse et al., 1991; Désiré et al., 1995) although these have potential utility as a model for studying protein in potatoes (Coleman et al., 2001).

The objectives of this study were 1) To quantify the total soluble protein (TSP) concentration and its distribution within the tuber tissues (periderm, cortex, and perimedullary/pith) in fresh and stored (6 months) field-grown tubers of 20 cultivars, and 2) to determine if TSP concentration or distribution is affected in microtubers from a subset of 7 cultivars.

3.3. Materials and Methods

Experiment 1.

Potato tubers of 20 important cultivars grown in North American were used in this experiment, including Alpha, Atlantic, Belleisle, Bintje, Conestoga, Goldrush, Green Mountain, Kennebec, Norland, Onaway, Ranger Russet, Red Gold, Red Pontiac, Russet Burbank, Sebago, Shepody, Superior, Tobique, Tolaas, and Yukon Gold. Tubers of all cultivars were field-grown except for Alpha, where greenhouse-grown minitubers were used. Tubers were provided by the Bon Accord Elite Seed Potato Centre (Bon Accord, NB, Canada) in the autumn of 2000 and 2001. Storage did not involve anti-sprouting treatments and took place in the dark at 4°C at a relative humidity of 80-95%. Storage duration was 6 months.

Information on each cultivar (origin, botanical features, tuber characteristics, agricultural utilization, susceptibility to diseases, etc) can be found in the Canadian Potato Varieties descriptions at the Canadian Food Inspection Agency (CFIA) website http://www.inspection.gc.ca/english/plaveg/potpom/var/indexe.shtml.

Experiment 2.

Plantlets of the cvs. Atlantic, Green Mountain, Kennebec, Norland, Red Pontiac, Russet Burbank, and Shepody were obtained in vitro from the Plant Propagation Centre, Fredericton, N.B., Canada. These were micropropagated from nodal segments in 25 x 150 mm test tubes containing 10 ml of solidified MS basal salt medium (Murashige and Skoog, 1962) without growth regulators. Medium pH was adjusted to 5.7 before autoclaving. Growth room conditions were 65 μmol m^{-2} s^{-1} cool white fluorescent light at 21°C with 16/8 h day/night cycle. Microtubers were induced from layered plantlets by following the procedures of Leclerc et al. (1994).

For both experiments, tuber samples were randomly taken from three tissue layers; periderm, cortex, and pith. The periderm was removed in strips using a potato peeler and the cortex and pith were separated and cut into small pieces (1x1 cm) to total approx. 1-3 g FW per sample. Samples were immediately frozen under liquid nitrogen and lyophilized at -50°C in a freeze-dryer (SNL216V, Savant Instruments Inc, NY, USA), then ground and stored at -20°C until analysis.

TSP was extracted from 5 mg DW of stored sample with 10 ml of 0.1 N NaOH, pH 12.8 (Jones et al., 1989). Protein content was determined by the Bradford (1976) method using bovine serum albumin (Bio-Rad Laboratories, ON, Canada) as standard at 595 nm spectrophotometer (Ultrospec 4300 pro, Biochrom, UK) and reported in mg g^{-1} DW of tuber tissue.

3.3.1. Statistical analysis

Experiment 1 was carried out in a factorial randomized complete design involving three factors: condition (fresh and stored), cultivars (20), and tissue layers (periderm, cortex, and pith); 2 x 20 x 3. The experimental unit was one tuber per cultivar with seven replicates. The experiment was performed twice (tubers from 2000 and 2001 seasons).

Similar data was observed in both years and data were combined after applying Barlett's test for homogeneity of variance. Data was analyzed by ANOVA (Analysis of Variance) using the General Lineal Model of SAS 9.1 (SAS Institute Inc., 2003) with significance at the 0.05 level.

Experiment 2 was conducted once. There were seven biological replicates (one tuber) per cultivar and three samples/tissue layer/tuber. Data was analyzed by ANOVA (Analysis of Variance) using SAS 9.1 (SAS Institute Inc., 2003) with significance at the 0.05 level. Pearson correlation coefficients were calculated to analyze the relationship of protein content distribution between microtubers and field-grown tubers in each tissue layer.

3.4. Results and Discussion

All factors (storage, cultivars, tissues) and the interaction between them significantly affected TSP content (Table 3.1). TSP concentration was significantly greater in the periderm (38 to 73 mg g^{-1} DW) than in the cortex or pith (30 to 49 mg g^{-1}) in fresh tubers of most cultivars (Fig. 3.1). After 6 months of storage, the differences between tissues were less. In half of the cultivars, TSP concentration was affected by storage, mostly in the periderm. TSP concentration decreased (mean of 16%) in five cultivars (Tolaas, Red Gold, Kennebec, Atlantic, and Belleisle), and increased (mean of 18%) in four cultivars (Yukon Gold, Ranger Russet, Goldrush, and Onaway). Similar cultivar-specific differences have previously been reported during storage. For example, over 1 year of storage, a gradual decrease in protein levels occurred in cvs. Bintje and Désiré that was ascribed to increased proteolytic enzyme activity (Pots et al., 1999a). In contrast, protein concentration increased at the first signs of dormancy-breaking after 22 weeks of storage in microtubers of cv. Désirée (Désiré et al., 1995).

TSP concentration was also affected significantly by cultivar and cultivar interaction with tissue (Table 3.1). The cultivars with the greatest and lowest concentrations in the periderm in both fresh and stored tubers were Tolaas and Alpha, respectively. Only three cultivars (Shepody, Norland, and Sebago) had the same TSP concentration in all three tissue layers. The greater fraction of extractable protein in the

periderm, relative to internal tissues, may reflect the presence of plant defense proteins such as deposits of cubic protein crystals found in the phellogen and pelloderm layers (Peterson et al., 1981). These crystals consisted of a single 85 kDa polypeptide, an inhibitor of cysteine proteases, and seemed to increase in the presence of viral infections (Rodis and Hoff, 1984; Bergey et al., 1996).

The TSP concentrations in microtuber tissues tended to be significantly greater in all three tissue layers compared with the field-grown tubers (Fig. 3.2). The mean increase in the periderm, cortex, and pith were 37, 48, and 29%, respectively. However, the pattern of protein distribution was similar in microtubers compared with the field-grown tubers; greater in the periderm than in the cortex and pith tissues. In addition, protein content in microtuber tissues was positively correlated with those of field-grown tubers, periderm ($r = 0.656$), cortex ($r = 0.638$), and pith ($r = 0.711$). The elevated TSP concentrations found in microtubers may reflect the readily available nitrogen in the tissue culture medium. However, nitrogen fertilizer applications in the field have not always resulted in increased tuber protein concentrations, although yield and tuber N levels increased (Eppendorfer et al., 1979; Eppendorfer and Eggum, 1994). As a model system for tuber protein research, microtubers appear to be similar. One apparent system-dependent artifact is the greater TPS level found in microtubers.

This study showed that among the 20 cultivars tested there were differences in the total soluble protein content of both fresh and stored tubers. Microtuber tissue protein concentrations were consistently greater but distributed in a similar way to field-grown tubers. These results offer possible avenues for identifying and selecting genotypes with higher protein concentrations and, perhaps, nutritive value.

Table 3.1. ANOVA summary analysis of TSP in the three main factors (storage, cultivar, tissue) and their interactions.

Variable	Degrees of Freedom	Sums of squares	Means Square	F	P
Storage (S)	1	513.40	513.40	8.13	0.0044*
Cultivar (Cv)	19	20139.85	1059.99	16.78	<0.0001*
Tissue (T)	2	32801.98	16400.99	259.57	<0.0001*
S x Cv	19	5648.38	297.28	4.71	<0.0001*
S x T	2	972.15	486.08	7.69	0.0005*
Cv x T	38	14225.14	374.35	5.92	<0.0001*
S x Cv x T	38	4170.17	109.74	1.74	0.0038*
Error	1457	92059.83	63.18		
Total	1576	167778.39			

* Significant at $P<0.05$ level.

Figure 3.1. Total soluble protein content in three tissue layers of fresh and stored (6 months) field-grown tubers of 20 potato cultivars. The data represent the mean values ± SE of the combined tubers from the 2000 and 2001 field seasons (n=14).

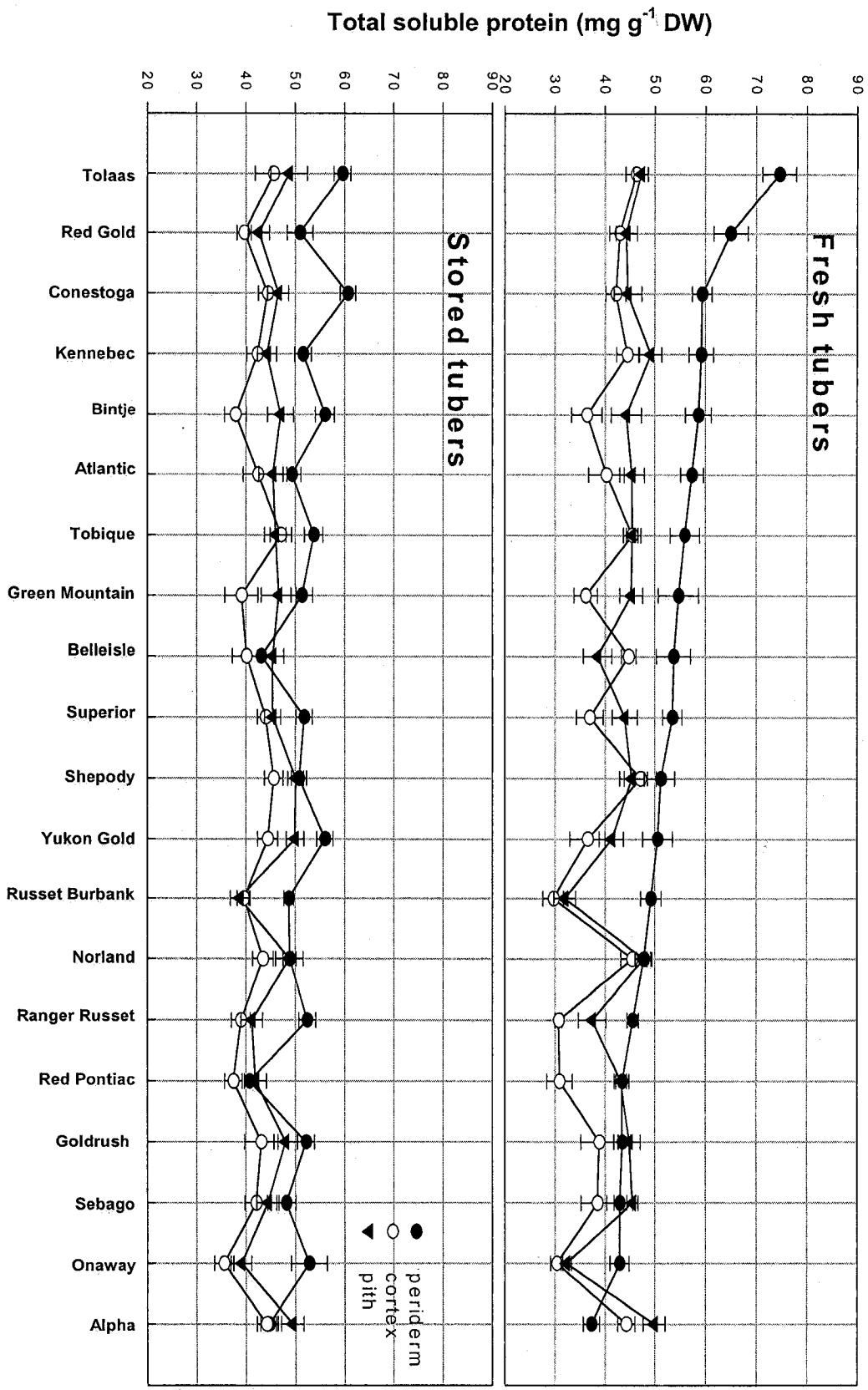
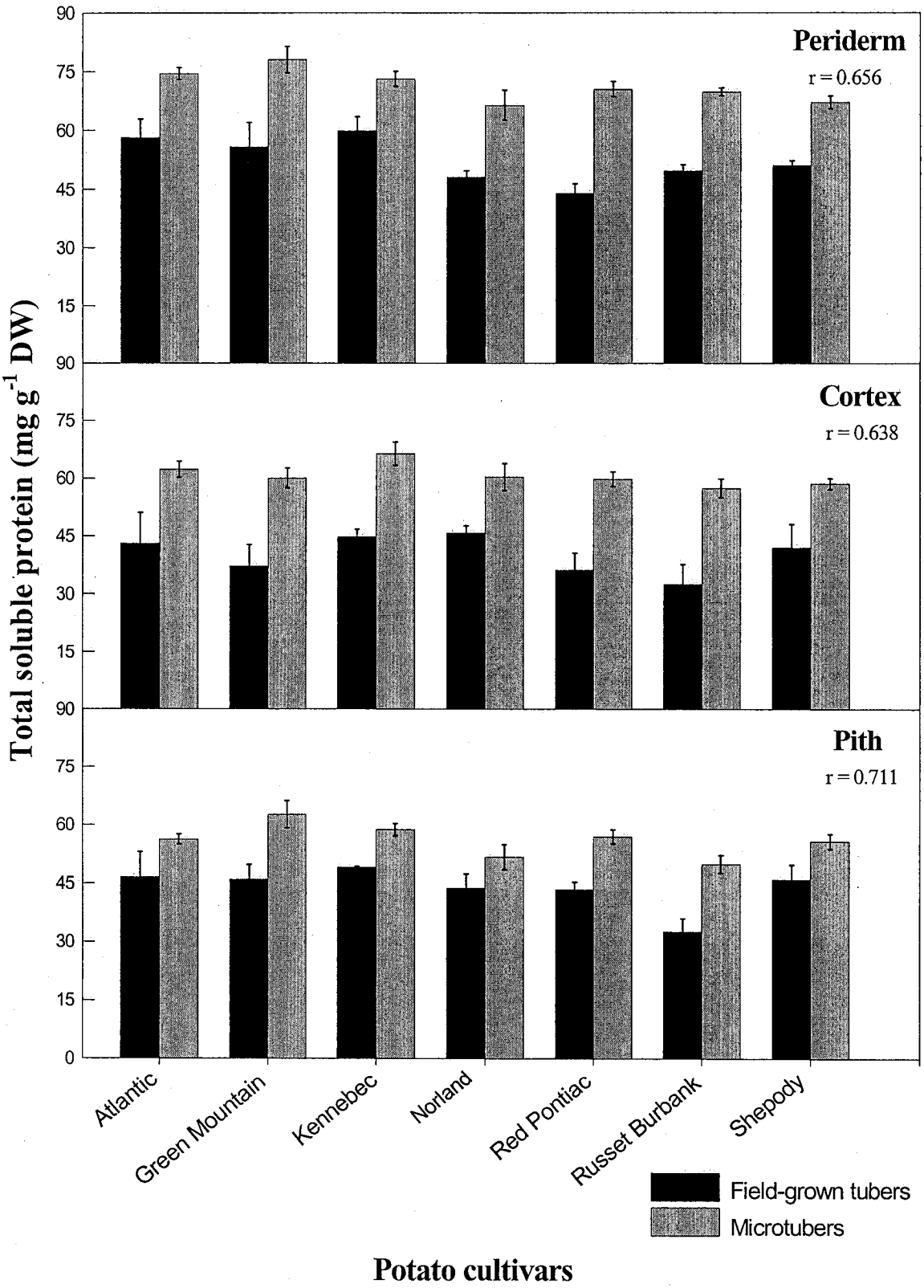

Total soluble protein (mg g-1 DW)
Fresh tubers
Stored tubers
periderm
cortex
pith
Tolaas
Red Gold
Conestoga
Kennebec
Bintje
Atlantic
Tobique
Green Mountain
Belleisle
Superior
Shepody
Yukon Gold
Russet Burbank
Norland
Ranger Russet
Red Pontiac
Goldrush
Sebago
Onaway
Alpha
Potato Cultivars

Figure 3.2. Comparison of the total soluble protein concentration and its distribution in three tissue layers of fresh field-grown tubers (2000 and 2001 seasons) and microtubers of 7 potato cultivars. Data represent the mean value ± SE from 7 tubers (3 samples/tissue layer/tuber). Pearson correlation coefficients (r) between field-grown tubers and microtubers in each tissue layer ($P<0.05$).


Periderm
r = 0.656
Cortex
r = 0.638
Pith
r = 0.711
Total soluble protein (mg g-1 DW)
90
75
60
45
30
15
0
Atlantic
Green Mountain
Kennebec
Norland
Red Pontiac
Russet Burbank
Shepody
Field-grown tubers
Microtubers
Potato cultivars

CONNECTING STATEMENT FOR CHAPTER IV

Chapter IV consists of the manuscript entitled "Tissue-specific distribution of patatin in fresh and stored potato tubers" prepared by E. Ortiz-Medina, T. Scorza, I. Alli, U. Seppälä, T. Palosuo, and D.J. Donnelly. This manuscript was submitted for publication in American Journal of Potato Research.

The concentration and distribution of total soluble protein (TSP) in different tuber tissues of 20 cultivars was reported in Chapter III. TSP distribution was generally greater in the periderm and relatively less in the cortex and pith. This chapter reports the tuber tissue distribution of patatin, the major storage protein, in relation to TSP. Patatin concentration was determined in the same tissue layers (periderm, cortex, and perimedulla/pith) for the same 20 potato cultivars, using indirect ELISA. On a subset of four cultivars, SDS-PAGE was used to compare proteins within each tissue layer.

Chapter IV

TISSUE-SPECIFIC DISTRIBUTION OF PATATIN IN FRESH AND STORED POTATO TUBERS

E. Ortiz-Medina[1], T. Scorza[2], I. Alli[3], U. Seppälä[4], T. Palosuo[4] and D.J. Donnelly[1]

4.1. Abstract

Tissue-specific distribution of total soluble protein (TSP) and patatin were examined in fresh and stored field-grown tubers of 20 potato (*Solanum tuberosum* L.) cultivars by means of indirect ELISA. TSP concentration (mg g^{-1} DW) was greater in the periderm compared with cortex and pith tissues of most cultivars. In fresh tubers, TSP concentration in the periderm ranged from 50 to 123 mg g^{-1} DW, in the cortex from 39 to 62 mg g^{-1} DW, and in the pith from 39 to 85 mg g^{-1} DW. An opposite pattern was obtained for patatin; lower concentration in the periderm compared with internal tuber tissues for all cultivars. In fresh tubers, patatin as a percentage of the TSP (% patatin) in the periderm ranged from 24 to 51%, in the cortex from 41 to 79%, and in the pith from 40 to 69%. Patatin concentration was significantly affected by 6 months of storage in most cultivars although no clear trend was observed. The presence of patatin was shown by SDS-PAGE in all three tissues of the four cultivars examined, as one or two overlapping bands of 40-45 kDa. The intensity of the bands differed between tissues, cultivars, and fresh versus stored tubers, suggesting changes in expression and probable protein turnover during storage. Other proteins of high (70-116 kDa), low (20-25 kDa), and very low (< 16 kDa) molecular weights were detected in some, but not all cultivars. Cultivars with the greatest patatin concentration in all tissues were Red Gold, Conestoga, and Kennebec; whereas those with the least patatin concentration were Sebago, Onaway, and Alpha. In conclusion, potato cultivars varied widely in TSP and patatin

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concentrations, although most cultivars had greater TSP and lesser patatin in the periderm compared with internal tuber tissues.

4.2. Introduction

Patatin is considered the major storage protein in potato tubers, accounting for more than 40% of the total soluble protein (TSP) (Paiva et al., 1983; Park et al., 1983; Mignery et al., 1984; Pots et al., 1999a; Ralet and Guéguen, 1999). Patatin is actually a group of homogeneous glycoproteins with a molecular mass of $\approx$40 kDa (Park et al., 1983). Four isoforms have been identified (Pots et al., 1999b), with sequences that are highly homologous (85-98%) and immunologically identical (Park et al., 1983; Mignery et al., 1984). Patatin is predominantly found in tubers, with lesser concentration in other plant organs including stolons, roots, and flowers (Höfgen and Willmitzer, 1990).

Unlike other storage proteins, patatin is involved in plant defense induced by pathogen attack. It exhibits lipid acyl hydrolase and acyl transferase activities against insect pests by affecting lipid metabolism (Racusen, 1984; Andrews et al., 1988; Anderson et al., 2002) as well as Beta-1,3-glucanase activity in response to fungal pathogens such as *Phytophthora infestans* (Tonón et al., 2001). Antioxidant properties are also associated with patatin (Al-Saikhan et al., 1995; Liu et al., 2003). Patatin, localized in the cell vacuoles in an inactive form, is translocated to the cytosol following pathogen attack or mechanical wounding, and becomes enzymatically active under basic conditions (Sonnewald et al., 1989; Hirschberg et al., 2001).

Although many biological functions of patatin are recognized, and its nutritional importance in tubers is widely accepted (Seibles, 1979; Woolfe, 1987), the tissue-specific distribution of patatin and its relation to TSP tissue distribution have not yet been described. Most studies have reported patatin on a whole tuber basis, although periderm tissue was discarded and patatin analysis restricted to whole peeled tubers or internal tuber sections (Racunsen and Foote, 1980; Paiva et al., 1983; Racunsen, 1983; Bohac, 1991; Pots et al., 1999a). Immunocytochemical techniques have identified patatin in the vacuoles of starch-storing parenchyma cells, but not in cell walls, intercellular spaces or periderm tissues (Sonnewald et al., 1989). In spite of these findings, the presence of

patatin in the periderm tissue has sometimes been assumed. For example, tuber sections of periderm and cortex showed greater antioxidant activity (ascribed to patatin in addition to other components) compared with separated sections of cortex or pith (Al-Saikhan et al., 1995). Similarly, the characteristic tissue-specific distribution of TSP pattern found in potato tubers, with a relatively greater concentration in the periderm and lesser concentration in the cortex and pith (Ortiz-Medina and Donnelly, 2003) suggests a similar patatin distribution.

The objective of this study was to determine the concentration and distribution of patatin in different tuber tissue-layers (periderm, cortex, and perimedullary/pith) of 20 potato cultivars. These were investigated at the time of harvest and following 6 months of storage, because little information is available on how storage conditions affect patatin levels. Patatin concentrations expressed as a percentage of the total soluble protein in the partitioned tubers were also calculated

4.3. Materials and Methods

4.3.1. Plant material

Potato tubers of 20 cultivars were used in this study: Alpha, Atlantic, Belleisle, Bintje, Conestoga, Goldrush, Green Mountain, Kennebec, Norland, Onaway, Ranger Russet, Red Gold, Red Pontiac, Russet Burbank, Sebago, Shepody, Superior, Tobique, Tolaas, and Yukon Gold. Tubers freshly harvested from the field (19 cultivars) or greenhouse minitubers (Alpha only) were utilized. These cultivars were analyzed previously for TSP by Ortiz-Medina and Donnelly (2003). Freshly harvested tubers and tubers stored for 6 months at 4°C in the dark at relative humidity of 80 to 95%, with no anti-sprouting treatment, were examined.

4.3.2. Sample preparation

Tuber samples from each cultivar were randomly taken from each of three different tissue layers: periderm, cortex, and pith (perimedullary and pith together). The tubers were partitioned in the following way: periderm samples were taken in superficial strips with a sharp knife or potato peeler, taking care to avoid cutting into cortex tissue.

Cortex, and pith tissues were separated using the vascular ring as a demarcation line. Tissue samples were cut into small pieces (approx. 1 cm^2) and immediately frozen in liquid nitrogen and lyophilized (SNL216V, Savant Instruments Inc, NY) at –50°C. After freeze-drying, the samples were ground into a powder with a mortar and pestle and stored in plastic vials at -20°C until analysis.

4.3.3. Protein extraction and determination

Sodium phosphate buffer (pH 7.0) was used for TSP extraction. Soluble proteins were extracted from 30 mg DW of stored samples with 5 ml of cold (4°C) sodium phosphate buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4 containing 2 mM of $K_2S_2O_5$) 0.1 M, pH 7.0. The crude homogenate was centrifuged at 14,000 *g* for 10 min at 4°C. Total soluble protein was determined by the Bradford (1976) method, using bovine serum albumin (BSA; Bio-Rad Laboratories, ON, Canada) as a standard. The results were reported in mg g^{-1} DW of tuber tissue.

4.3.4. Indirect ELISA development for patatin determination

Patatin concentrations were measured by Indirect ELISA using purified patatin as a standard. Patatin was purified from cv. Bintje according to Seppälä et al. (1999), and polyclonal IgG antibodies were produced against patatin samples in rabbits. Titration assays were performed to optimize coating antigen and antiserum concentration for the greatest sensitivity with ELISA. The coating antigen concentration ranged between 1:200 and 1:100,000 and the antiserum ranged between 1:500 and 1:4000. Microtiter plates (Costar EIA/RIA plate, polystyrene, 96 wells) were coated with TSP extracts (100 µl/well) from each tuber sample, diluted in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was then washed with PBS (0.05% Tween 20), and blocked with 200 µl/well of FCS/PBS (10% Fetal Calf Serum + PBS pH 7.2) for 1 h at 37°C. Following washing, antiserum diluted with FCS/PBS (1:2000) was added (100 µl/ well) and incubated for 1 h at 37°C followed by washing. Goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase diluted with FCS/PBS (1:3000) was added to the plates (100 µl/ well) and incubated for 1 hr at 37°C. Substrate and chromogen solution

(0.4 mg ml^{-1} *o*-phenylenediamine dihydrochloride, 0.4 mg ml^{-1} urea hydrogen peroxide and 0.05 M phosphate-citrate, pH 5.0) (Sigma *Fast*-OPD, P 9187) were used to develop the reaction (100 µl/ well). After 15 min, the reaction was stopped with 2 M sulfuric acid (50 µl/well). The developed colour was read at 492 nm in an ELISA microplate reader (Synergy HT, Bio-Tek Instr. Inc. VT, USA). The results were reported in mg g^{-1} DW of tuber tissue.

4.3.5. Statistical analysis

TSP and patatin determination was carried out in a factorial randomized complete design involving three factors: condition (fresh and stored), cultivars (20), and tissue layers (periderm, cortex, and pith); 2 x 20 x 3. The experimental unit was one tuber per cultivar (3 samples/tissue layer/tuber) with three replicates. Data was analyzed by ANOVA (Analysis of Variance) using the General Lineal Model of SAS 9.1 (SAS Institute Inc., 2003) with significance at the 0.05 level. Means comparison between tissue layers was performed for patatin concentration expressed as a percentage of the TSP by the Least Significant Difference test ($P<0.05$).

4.3.6. SDS-PAGE electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl-sulphate (SDS) using 12% acrylamide gels (Laemmli, 1970). Proteins in the gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R250 and destained overnight with methanol:acetic acid:water (2:1:7). Estimation of molecular weight (MW) was done using standard proteins (SDS-PAGE Molecular Weight Standards, Broad range, Bio-Rad). Purified patatin was used as a control.

Protein extracts were analyzed on three tissue layers (periderm, cortex, and pith) of fresh and stored tubers from four potato cultivars: Alpha, Red Gold, Shepody, and Tolaas. Changes in the relative density of protein bands were visually examined using two different gels for each sample. The relative molecular weight of the polypeptides was estimated from their migration in the gels in relation to the standard proteins.

4.4. Results

4.4.1. Total soluble proteins

For all 20 potato cultivars, the concentrations of TSP were greater in tuber tissues when compared to our previously reported results (Chapter III) (Fig. 4.1). Sodium phosphate buffer was more efficient than the previously used sodium hydroxide buffer to extract tuber TSP. Distribution of protein in the different tissues confirmed earlier findings; cultivars had significantly more TSP (mg g^{-1} DW) in the periderm compared with the cortex or pith tissues (Table 4.1; Fig. 4.1). Among the 20 cultivars, TSP concentration of periderm, cortex, and pith tissues ranged from 50 to 123, 39 to 62, and 39 to 69 mg g^{-1} DW, respectively. Storage for 6 months did not affect the TSP concentration in individual cultivars or with the interaction with tissues (Table 4.1). However, significant differences on TSP concentration between cultivars were observed. Cultivars with relatively greater or lesser TSP concentration in all their tissues were identified. Tobique and Conestoga had the greatest TSP concentration and Alpha and Shepody the least.

4.4.2. Patatin

Patatin was detected in all 20 cultivars and distributed differently in all three tissues within partitioned tubers (Fig. 4.2). As seen with TSP concentration, patatin concentration in periderm was significantly different compared with the cortex and pith tissues in fresh tubers. However, these differences were not as extreme as that observed with TSP. Significant differences between cultivars were also observed. For instance, cvs. Red Gold and Conestoga had greater patatin concentration in all tissues, while Sebago, Onaway, and Alpha exhibited the lesser. These results were not completely consistent with the cultivars containing the greatest and least TSP concentrations.

Storage for 6 months significantly affected the patatin concentration of potato tubers (Table 4.2, Fig. 4.2), resulting in an increase or decrease in patatin levels in stored cultivars with differential effects between tuber tissues. In 10 cultivars, the concentrations of patatin increased in one or two tissues (*cortex*: Shepody, Red Pontiac and Russet Burbank; *pith*: Green Mountain and Kennebec; *periderm and cortex*: Conestoga, Tolaas,

Tobique, and Yukon Gold; *cortex and pith*: Red Gold). In contrast, patatin concentrations decreased in one or two tissues in eight cultivars (*periderm*: Atlantic, Belleisle, Goldrush, Superior; *periderm and cortex*: Norland and Sebago; *periderm and pith:* Bintje and Ranger Russet) and in two cultivars, its concentration was similar for all three tissues following storage (Onaway and Alpha). In stored tubers not differences in patatin concentration between tissues were observed.

Patatin, calculated as a percentage of the TSP (% patatin) for each tissue, was significantly less in the periderm than in the cortex and pith (Table 4.3). After storage, the differences were more consistent. The percentage patatin ranged from 20 to 57%, 41 to 80%, and 40 to 83% in periderm, cortex, and pith, respectively.

4.4.3. Analysis of proteins - SDS-PAGE electrophoresis

SDS-PAGE patterns for the four potato cultivars were similar enough to allow direct comparison (Fig. 4.3). The number of protein bands indicated cultivar-specific variations between tissue layers in fresh and stored tubers. However, some specific bands were found to be similar in all cultivars.

Soluble proteins were classified into four tentative groups based on the molecular weight of the protein bands: a) high molecular weight (70-116 kDa), b) medium molecular weight, corresponding mainly to the patatin family (40-45 kDa), c) low molecular weight (20-25 kDa), and d) very low molecular weight (< 16 kDa). The relative abundance of the proteins in each tissue was interpreted according to the intensity of the bands.

Not all four soluble protein groups were present in the cultivars tested. Cv. Red Gold showed many clear major bands corresponding to high molecular weight proteins (Fig. 4.3B) of apparent molecular weights 116, 97, and 88 kDa. These were present in all three tissues of fresh and stored tubers. Similar bands were also seen in cv. Tolaas although they were not as distinct (Fig. 4.3D).

SDS-PAGE electrophoresis confirmed the presence of patatin in all four cultivars and in all three tissue layers; detected as one or two overlapping protein bands between 40-45 kDa. Differences in patatin band intensity were also observed between cultivars and tissue layers. In fresh tubers of cvs. Alpha and Shepody, patatin was less abundant in

periderm compared with cortex and pith tissues (Fig. 4.3A, C). Following storage, only trace amounts of patatin were detected in all three tissues of cv. Alpha while few changes were observed in cv. Shepody. In fresh tubers of cvs. Red Gold and Tolaas, patatin was present at relatively high levels in all three tissues, being greatest in the periderm (Fig. 4.3B, D). Following storage, few changes were observed in cv. Red Gold but increased levels of patatin occurred in all tissues of cv. Tolaas. While Western blots were not done, the presence of patatin was clearly confirmed and quantified by the ELISA immunoassay technique.

Tissues of all four cultivars showed bands in the low molecular weight protein group (20-25 kDa). However, their intensity varied between cultivars and tissues. In cvs. Alpha, Red Gold, and Shepody, the bands were clearly visible in fresh tubers, and were less intense in periderm compared with cortex and pith tissues. Storage decreased the intensity of this protein group in cv. Alpha but not in cvs. Red Gold or Shepody. In fresh tubers of cv. Tolaas, this protein fraction was less abundant in all three tissues, compared to the other three cultivars, but increased following storage. The fourth protein group, composed of protein bands of molecular size < 16 kDa, was clearly present in the cortex and pith of the two cvs. Alpha (Fig. 4.3A) and Shepody (Fig. 4.3C) and less distinct in Red Gold (Fig. 4.3B).

4.5. Discussion

4.5.1. Total soluble proteins

It is clear that TSP concentration in the periderm contributed substantially to the total tuber protein content despite the relatively small proportion of whole tuber volume occupied by this tissue. This contribution has been neglected in many tuber protein studies where protein analysis was limited to internal tuber sections or whole peeled tubers (Seibles, 1979; Ahldén and Trägårdh, 1992; Désiré et al., 1995; Espen et al., 1999a) but reported on a whole tuber basis. In our study, where TSP concentration was measured on a specific tissue basis, conversion factor values are needed to convert these measurements to a uniform weight whole tuber basis for intercultivar comparisons (Chapter V; Ortiz-Medina et al., 2007b). Relative protein quantity and its distribution

within the tuber becomes particularly important when select tuber tissues are used for food purposes (Pots et al., 1999a). From a nutritional point of view, using the complete tuber (periderm included) in any food process is likely to increase the final nutritional content of the product.

Similar cultivar-specific differences in TSP concentration following storage have been previously reported in whole tubers (Désiré et al., 1995; Okeyo and Kushad, 1995; Espen et al., 1999a; Kumar et al., 1999; Pots et al., 1999a; Peshin, 2000) and in partitioned tubers (Ortiz-Medina and Donnelly, 2003), but at different intervals of storage. TSP variation was associated with the disappearance or appearance of specific polypeptides (Désiré et al., 1995; Espen et al., 1999a) coinciding with the termination of dormancy, as seen in cv. Désirée at 5.5 months of storage (Désiré et al., 1995). However, changes in tuber protein composition have been related to many other physiological and biochemical events in stored potato such as metabolic enzyme activities, synthesis or breakdown of starch, fluctuations in respiration rate or plasma membrane function (van der Plas, 1987; Brisson et al., 1989; Espen et al., 1999b).

4.5.2. Patatin

The clear tissue-specific pattern of % patatin; low, high, high in periderm, cortex, and pith respectively, contrasts significantly with the tissue-distribution pattern found for TSP. The relatively greater concentration of TSP found in the periderm is, therefore, attributable to other proteins. The relatively low percentage of patatin in periderm seems counter-intuitive, considering its proposed role in defense. Instead, protease inhibitors, another class of antipathogenic proteins, may be represented in this tissue. Indeed, a 22-kDa Kunitz-type protein has been found in periderm cell walls (Suh et al., 1999), and protein crystals consisting of an 80-85 kDa protease inhibitor were located in the subphellogen layer (Rodis and Hoff, 1984; Walsh and Strickland, 1993). Structural glycoproteins such as the extensin family of hydroxyproline-rich glycoproteins (HRGPs) (Sabba and Lulai, 2005) may also account for the TSP content in the periderm.

The patatin percentage in TSP of tuber tissues ranged from 24-80%. This is greater than expected based on whole tuber estimates and represents a much broader range than previously reported for whole tubers. For example, reports include: > 20%

(Racusen and Foote, 1980), 40-45% (Paiva et al., 1983), and 40-60% of all buffer-extractable tuber proteins (Pots et al., 1999a; Ralet and Guéguen, 1999). A combination of factors, including differences between cultivars, protein extraction techniques, and tissues examined (whole vs. partitioned tubers) account for these differences.

Cultivar-specific differences in patatin concentration following storage were significantly evident but without any clear trend. These findings support earlier observations on changes in patatin concentration (increase and decrease) in whole tubers of cvs. Bintje, Désiré, and Elkana during different storage intervals of up to 47 weeks (Pots et al. 1999a). However in other studies, only reduced patatin levels at the time of tuber sprouting have been reported, following 31 weeks of storage in cv. Kennebec (Racusen, 1983), and a complete loss of patatin after 72 weeks of storage in Russet Burbank tubers (Kumar et al., 1999). The decline in patatin concentration has been associated with an increase in proteolytic enzyme activity at the end of tuber dormancy, resulting in the breakdown of this protein (Brierley et al., 1996). Differences in dormancy period between the cultivars in our study could account for variation in the patatin concentration following 6 months storage.

In spite of the significant effect of storage on the tissue distribution of patatin, the fact that changes were observed in one or two tissues, but not in all three tissues of any one cultivar is of interest. Specific-tissue conditions appear to affect patatin concentrations differentially.

4.5.3. Analysis of proteins - SDS-PAGE

Protein banding patterns from specific tissues of all four cultivars enabled the tentative classification of tuber proteins into different groups. The presence of proteins of high molecular weight (first group) was limited. These proteins are mainly represented by enzymes and kinases involved in starch synthesis (Gerbrandy and Doorgeest, 1972; Marshall et al., 1996) and contribute 20-30% of the tuber TSP (Marshall et al., 1996; Pots et al., 1999a).

The presence of patatin (second group) as one of two bands may indicate singular isoforms (Racunsen and Foote, 1980; Park et al., 1983; Pots et al., 1999b), assuming that differences in glycosylation have resulted in minor changes in the mobility of the patatin

isoforms on SDS-PAGE (Sonnewald et al., 1989; Pots et al., 1999b). The high band intensity variation suggests that patatin levels may be determined by tuber tissue, genotype and/or environmental conditions (as storage). Patatin isoforms fractioned by electrophoretic charge differed sufficiently among cultivars to suggest utility in cultivar identification (Park et al., 1983; Bohac, 1991; Kormut´ák et al., 1999).

Protease inhibitors of different families were represented (third and fourth protein group). In contrast to patatin, protease inhibitors are a more heterogeneous group of proteins, differing significantly in molecular sizes (Pouvreau et al., 2001). Great variation in the protein-banding pattern of these groups was observed. Protein inhibitors (20-25 kDa) were detected in all tissues, but according to their band intensity, these proteins were more abundant in cortex and pith tissues. In addition, storage increased the amount of these proteins contrary to the reduction in the protease inhibitor (20-22 kDa) concentration reported after 5 months storage (Pots et al., 1999a). In general, protein banding patterns showed the differences between cultivars/tissues in fresh and stored tubers, suggesting changes in gene expression within cultivars and tissues and protein turnover during storage.

Patatin was found in the periderm tissue in fresh and stored tubers of all 20 cultivars using ELISA and was detected in all 4 cultivars examined by SDS-PAGE electrophoresis. These results contrast with those reported by Sonnewald et al. (1989), who showed this protein was exclusively found in vacuoles of parenchyma cells, but not in periderm cells. This discrepancy may result from differences in the maturity levels of sampled periderm. Immature phellem cells within the periderm may contain soluble proteins, such as patatin, that are not present once these cells are fully suberized and have lost their cytoplasm, as these cells are non-living at maturity. In addition, periderm samples may contain varying numbers of phelloderm cells that constitute the innermost tier of the periderm or subphellogen (Reeve et al., 1969). The phelloderm layer is composed of parenchyma cells that show transitional characteristics between periderm and cortex tissues such as accumulation of some starch grains and storage proteins (patatin included) in lower concentrations (Lulai and Freeman, 2001). Apart from periderm developmental considerations, "contamination" of the periderm samples with

some patatin-containing parenchyma cells from the cortex may occur, even when extreme care is used in excision.

4.6. Conclusions

Concentration and distribution of patatin as a percentage of TSP is reported for the first time in partitioned tubers (periderm, cortex, pith) from fresh and stored tubers of 20 cultivars. Among cultivars, TSP concentration was generally greater in the periderm compared with the internal tissues. In contrast, % patatin was consistently less in the periderm compared with the cortex and pith tissues, suggesting that tissue-specific expression of patatin seems highly regulated in tissue layers. Storage of tubers for 6 months affected the patatin but not TSP concentration.

Protein banding patterns showed differences between cultivars/tissues in fresh and stored tubers, suggesting cultivar-specific gene expression and protein turnover during storage. Differences in patatin isoforms could be useful for varietal identification or for monitoring the genetic stability of plants after long-term storage as previously suggested (Park et al., 1983; Bohac, 1991; Kormut´ák et al., 1999). Patatin was found in the periderm in all cultivars by ELISA and SDS-PAGE.

Cultivars with relatively high or low TSP and patatin contents in all tuber tissues were identified. As a result, this study provides useful information for potato breeders and nutritionists interested in genotypes with enhanced nutritional value for the food and nutraceutical industries.

Table 4.1. ANOVA summary analysis of TSP in the three main factors (storage, cultivar, tissue) and their interactions.

Variable	Degrees of Freedom	Sums of squares	Means Square	F	P
Storage (S)	1	1128.35	1128.35	5.72	0.0178*
Cultivar (Cv)	19	21136.49	1112.45	5.64	<0.0001*
Tissue (T)	2	101365.29	50682.64	256.78	<0.0001*
S x Cv	19	2756.58	145.08	0.74	0.7792
S x T	2	66.70	33.35	0.17	0.8447
Cv x T	38	13709.91	360.79	1.83	0.0048*
S x Cv x T	38	1986.64	52.28	0.26	1.0000
Error	178	35132.95	197.38		
Total	297	199488.67			

* Significant at $P<0.05$ level.

Table 4.2. ANOVA summary analysis of patatin in the three main factors (storage, cultivar, tissue) and their interactions.

Variable	Degrees of Freedom	Sums of squares	Means Square	F	P
Storage (S)	1	331.56	331.56	2.75	0.0987*
Cultivar (Cv)	19	17470.00	919.47	7.64	<0.0001*
Tissue (T)	2	2611.46	1305.73	10.85	<0.0001*
S x Cv	19	5364.71	282.35	2.35	0.0020*
S x T	2	546.53	273.26	2.27	0.1063*
Cv x T	38	4568.20	120.22	1.00	0.4802*
S x Cv x T	38	2667.36	70.19	0.58	0.9745
Error	179	21546.56	120.37		
Total	298	56041.69			

* Significant at $P<0.05$ level.

Figure 4.1. Distribution of total soluble protein (TSP) (mg g^{-1} DW) in three tissue layers (periderm, cortex, and pith) of fresh and stored (6 months at 4°C) tubers from 20 potato cultivars. Proteins were extracted with sodium phosphate buffer 0.1 M (pH 7.0). Values are expressed as means ± SE (n=3) for each tissue.

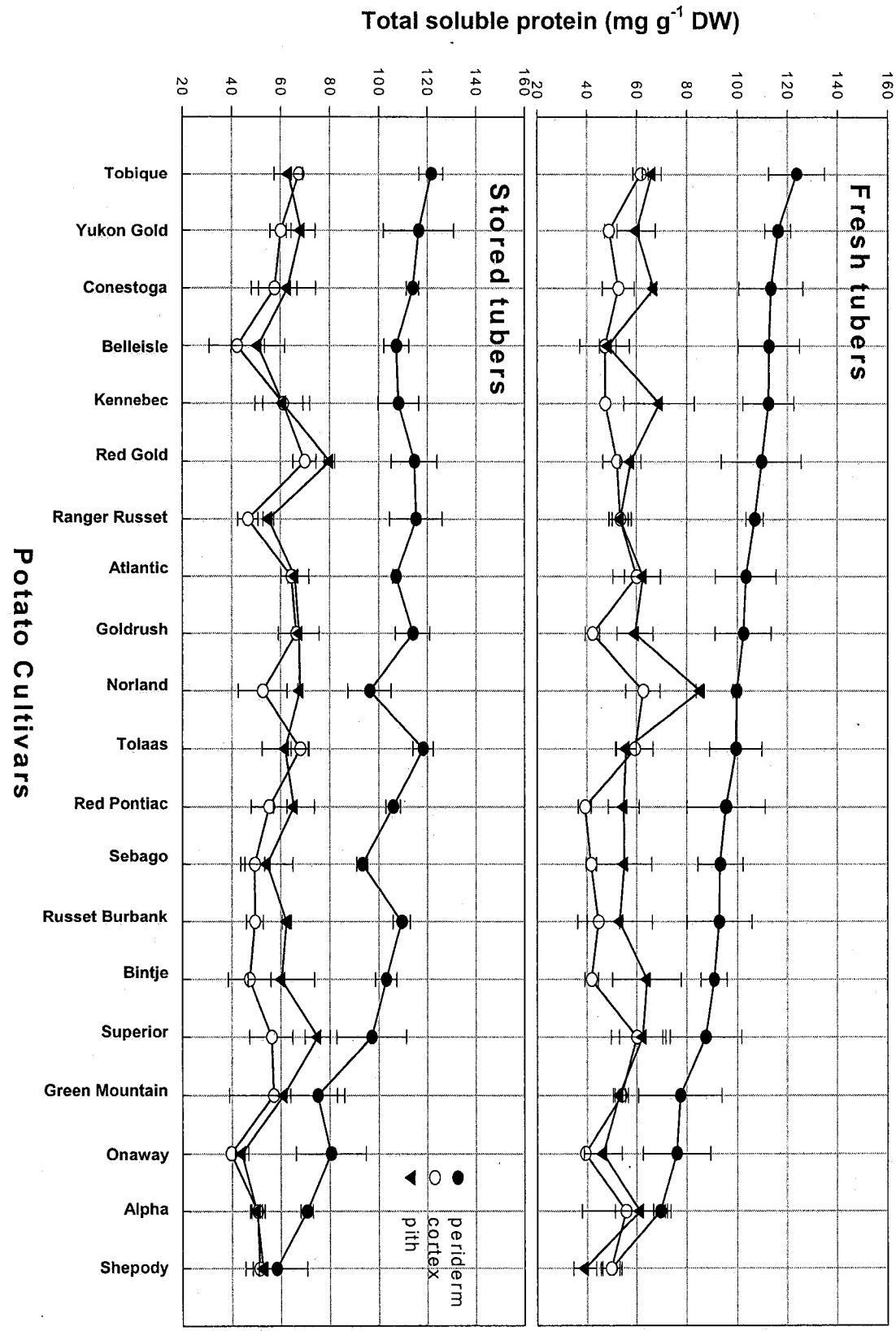
Total soluble protein (mg g-1 DW)
Fresh tubers
Stored tubers
periderm
cortex
pith
Potato Cultivars
Tobique
Yukon Gold
Conestoga
Belleisle
Kennebec
Red Gold
Ranger Russet
Atlantic
Goldrush
Norland
Tolaas
Red Pontiac
Sebago
Russet Burbank
Bintje
Superior
Green Mountain
Onaway
Alpha
Shepody
20
40
60
80
100
120
140
160

Figure 4.2. Distribution of patatin (mg g^{-1} DW) in three tissue layers (periderm, cortex, and pith) of fresh and stored (6 months at 4°C) tubers from 20 potato cultivars. Values are expressed as means ± SE (n=3) for each tissue.

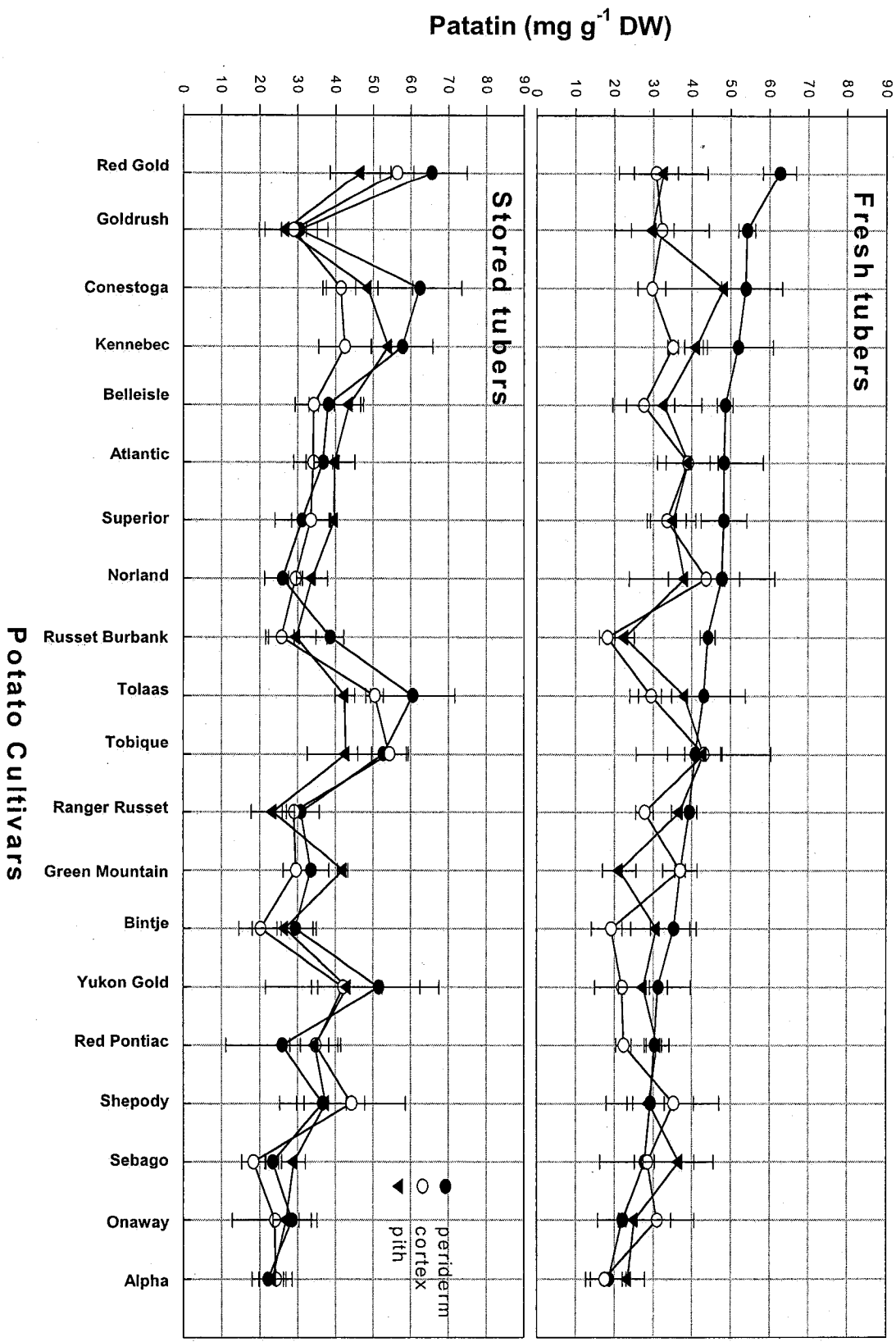
Patatin (mg g-1 DW)
Fresh tubers
Stored tubers
0
10
20
30
40
50
60
70
80
90
Red Gold
Goldrush
Conestoga
Kennebec
Belleisle
Atlantic
Superior
Norland
Russet Burbank
Tolaas
Tobique
Ranger Russet
Green Mountain
Bintje
Yukon Gold
Red Pontiac
Shepody
Sebago
Onaway
Alpha
periderm
cortex
pith
Potato Cultivars

Figure 4.3. SDS-PAGE analysis of total soluble protein from different tissue layers of fresh and stored tubers of four potato cultivars. **A.** Alpha, **B.** Red Gold, **C.** Shepody, and **D.** Tolaas. The patatin band region is indicated with brackets. ***M*** Molecular markers, ***p.*** periderm, ***c*** cortex; ***pt*** pith, ***PAT*** purified patatin. Molecular size of standards (kDa) are shown on the left.

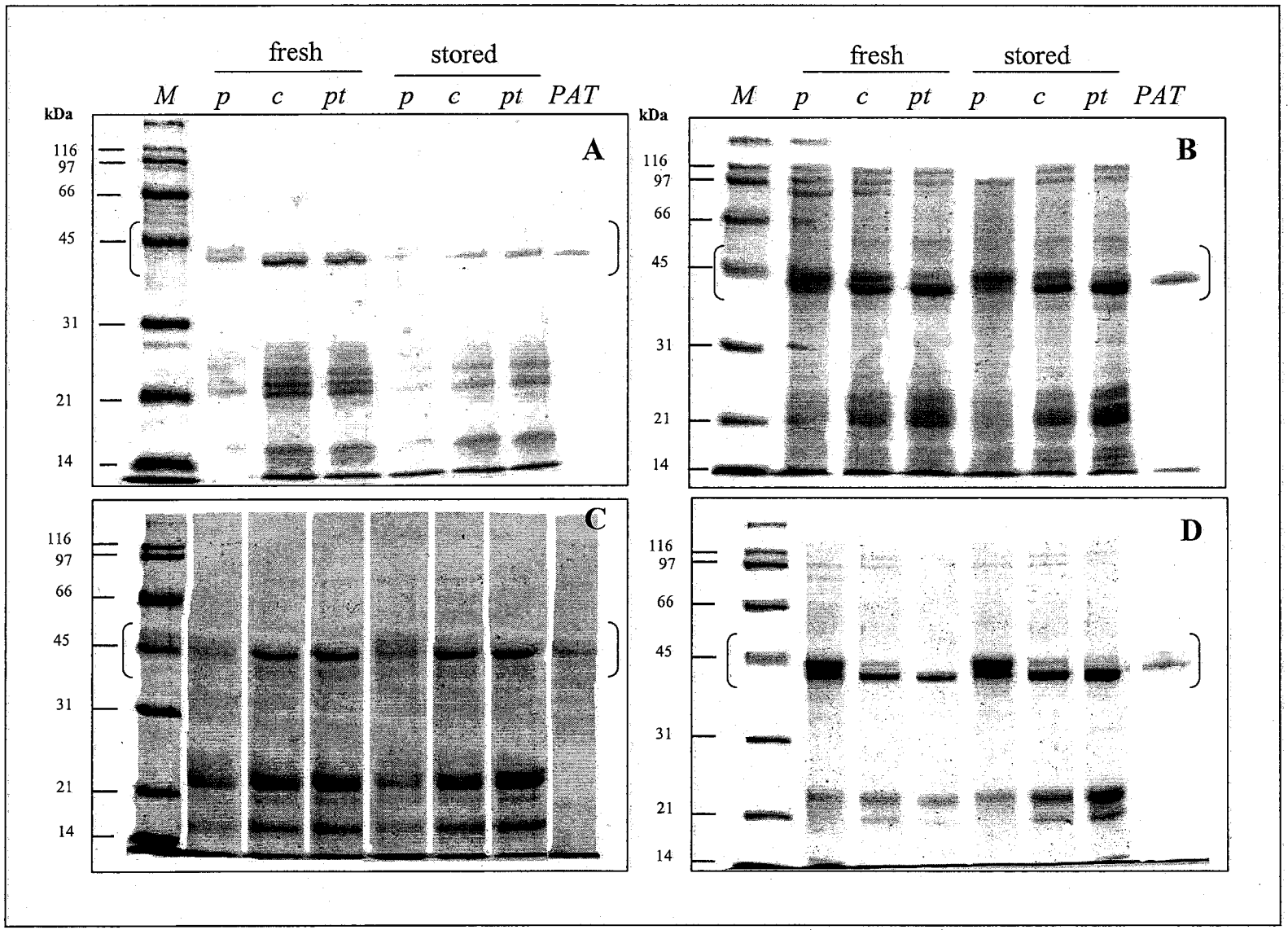
fresh
stored
M
p
c
pt
p
c
pt
PAT
kDa
116
97
66
45
31
21
14
A
B
C
D

Table 4.3. Patatin concentrations expressed as a percentage of the total soluble protein (% patatin) in tubers partitioned into three tissue layers (periderm, cortex, and pith) in fresh and stored (6 months at 4°C) tubers from 20 potato cultivars. Values are expressed as means ± SE (n=3).

CULTIVAR	% PATATIN					
	Fresh tubers			Stored tubers		
	periderm	cortex	pith	periderm	cortex	pith
Tobique	28.6 ± 2*	69.8 ± 23	64.9 ± 6	40.3 ± 6**	80.6 ± 6	67.8 ± 13
Yukon Gold	26.8 ± 6*	45.0 ± 12	45.9 ± 9	20.1 ± 1**	70.1 ± 19	63.2 ± 11
Conestoga	40.2 ± 8	56.3 ± 6	67.3 ± 13	52.0 ± 5	64.6 ± 11	58.9 ± 10
Belleisle	23.9 ± 6*	58.4 ± 14	67.6 ± 16	22.5 ± 4**	75.6 ± 5	81.8 ± 3
Kennebec	42.8 ± 4*	74.0 ± 2	59.5 ± 2	47.9 ± 9**	69.7 ± 9	82.9 ± 6
Red Gold	51.4 ± 5	59.0 ± 9	56.8 ± 16	41.5 ± 8	77.0 ± 8	58.3 ± 8
Ranger Russet	29.7 ± 5*	51.9 ± 2	68.8 ± 8	26.8 ± 3**	62.2 ± 2	42.7 ± 8
Atlantic	37.2 ± 2*	65.0 ± 11	62.6 ± 7	28.8 ± 3**	53.1 ± 6	60.6 ± 7
GoldRush	36.4 ± 10*	76.4 ± 23	50.2 ± 8	26.7 ± 3**	43.6 ± 11	39.9 ± 7
Norland	35.9 ± 14*	69.8 ± 5	44.6 ± 11	27.0 ± 4**	55.9 ± 3	50.0 ± 5
Tolaas	42.8 ± 8	49.7 ± 7	68.5 ± 17	48.7 ± 7**	74.3 ± 3	68.8 ± 2
Red Pontiac	31.6 ± 6*	57.2 ± 14	56.6 ± 5	24.5 ± 11**	63.0 ± 10	53.0 ± 5
Sebago	29.8 ± 2*	68.4 ± 24	66.5 ± 13	25.1 ± 2**	37.1 ± 4	53.3 ± 3
Russet Burbank	33.6 ± 2	40.8 ± 11	42.6 ± 3	42.8 ± 3	52.1 ± 6	47.6 ± 11
Bintje	36.7 ± 4	45.8 ± 10	48.1 ± 11	28.5 ± 4	42.7 ± 10	44.1 ± 11
Superior	49.8 ± 2	55.9 ± 7	56.5 ± 8	32.1 ± 6**	59.7 ± 7	52.9 ± 2
Green Mountain	39.9 ± 2*	68.6 ± 7	40.2 ± 7	43.4 ± 5**	52.0 ± 5	68.9 ± 1
Onaway	29.1 ± 2*	78.8 ± 20	54.2 ± 17	35.5 ± 5**	60.5 ± 23	61.3 ± 5
Alpha[a]	31.4 ± 5	31.2 ± 7	38.2 ± 6	26.6 ± 5**	48.1 ± 7	46.6 ± 6
Shepody	53.7 ± 18	67.6 ± 17	67.0 ± 1	57.9 ± 12	78.4 ± 18	71.0 ± 9

Periderm means are significantly different within a row for fresh (*) and stored (**) tubers at the 5% level according to Least Significant Difference test (LSD). [a] minitubers

CONNECTING STATEMENT FOR CHAPTER V

Chapter V consists of the manuscript entitled "Intercultivar comparisons of potato tuber protein using specific tissue weight proportions" prepared by E. Ortiz-Medina, V. Sosle, and D.J. Donnelly. This manuscript was submitted for publication in American Journal of Potato Research.

Chapter III and IV reported the TSP and patatin content, and their relative distribution in the tuber tissues of 20 potato cultivars, on a specific tissue basis (mg g^{-1} DW). This chapter reported the proportional contribution, as percent weight of each specific tuber tissue, for the 20 cultivars. The percent weight and percent dry matter for each tissue were used to generate conversion factor values. These values can be applied to any nutrient data reported on a specific tissue basis to estimate the whole specific tissue content, and by summation, the content of a whole typical tuber of 100 g FW. For illustration purposes, these conversion factors were applied to the data set described in Chapter IV. This enabled estimates of the TSP and patatin content in each specific tissue and in a whole typical tuber of 100 g FW for each cultivar. Intercultivar comparisons were reported for the 20 potato cultivars used in the study.

Chapter V

INTERCULTIVAR COMPARISONS OF POTATO TUBER PROTEIN USING SPECIFIC TISSUE WEIGHT PROPORTIONS

E. Ortiz-Medina[1], V. Sosle[2] and D.J. Donnelly[1]

5.1. Abstract

Potato cultivars have a distinctive tuber shape and size and as a consequence their internal tissue proportions differ. To obtain a better understanding of the contribution of the different layers of tuber tissue to the whole tuber, proportional volume (% volume) and weight (% weight) of periderm, cortex, and perimedullary/pith (pith) tissues were estimated for 20 field-grown potato cultivars. Weight estimations were based on the volume (calculated through an ellipsoid formula) and density of each component tissue. Variation in the % weight of specific tuber tissues was observed between cultivars. Percent weight of periderm ranged from 0.8-3.4%, cortex from 26-43%, and pith from 54-73%. Percent weight values together with percent dry matter for each tissue provided conversion factor values that were tabulated for the 20 cultivars. These conversion values were applied to a data set of TSP and patatin measurements reported on a specific tissue DW basis (Chapter IV, Ortiz-Medina et al. 2007a). This enabled TSP and patatin estimations for each specific tissue and for typical whole tubers of 100 g fresh weight. Average TSP contributions of periderm, cortex, and pith were 2.6, 34.1, and 63.3%, respectively. However, average patatin contribution with respect to TSP for the same tuber tissues was 1.0, 20.4, and 35.7%, respectively. Total protein content in a whole tuber, by summation of protein content of each individual tissue, permitted a more precise estimation of the nutritional value of different cultivars and enabled intercultivar comparisons. Tobique and Norland contained the greatest TSP concentration, while Red Pontiac and Belleisle the least. However, Tobique and Atlantic contained the greatest patatin content and Russet Burbank and Alpha the least. Patatin as a percentage of TSP in

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the whole tuber ranged from 67 to 35%, with the pith contributing the greatest amount followed by cortex and periderm. Useful applications of the conversion factor values generated from specific tissue percent weight estimations are discussed.

5.2. Introduction

Each potato cultivar has a relatively characteristic tuber shape and size at maturity as described in different catalogues of potato varieties. Tuber shape can be round, oval, long, short-oval, long-oval, elongated, oblong, etc. (Netherlands Catalogue of Potato Varieties, 1997). Similarly, the tissue layers that comprise the tubers of each cultivar, including the periderm, cortex, perimedulla and pith tissues are different in shape and proportion.

A few volume and weight estimates are available in the literature for specific tuber tissue layers. Neuberger and Sanger (1942) determined in a simple way the percentage contribution of each tissue layer by dissecting the potato tubers into different parts, separating the tissues, and weighing each tissue individually. On the other hand, Chapell (1958; cited in Woolfe, 1987), estimated the percentage volume of specific tissues in small- and large-sized tubers, but the method used to calculate these values was not clearly stated. More recently, specific tissue (cortex, perimedullary, and pith) volumes were calculated for fresh microtubers of the two cvs. Mira and E-Potato 1 (Liu and Xie, 2001). This was done using an ellipsoid formula for volume calculations. However, field tubers are much larger and far more variable in shape and tissue proportions, than the relatively tiny and more uniform microtubers. Tubers of different cultivars have different volumes and proportional weights for each tissue.

There are many applications for which weight and volume estimates for tuber component tissues of important cultivars would be useful. For example, in extrapolations of fresh or dry weight data from different tuber areas for a long list of nutritional compounds (proteins, vitamins, pigments, glycoalkaloids, etc). These estimates could be used to rapidly convert specific tissue concentration data for compounds that may not be distributed evenly in different tuber tissues into relatively accurate whole tuber estimates.

This conversion facilitates intercultivar comparisons that may be biochemically and nutritionally more useful than the absolute concentration data.

The objective of this study was to estimate the total tuber volume and the proportional volume and weight of each specific tissue layer (periderm, cortex, and pith) in field-grown potato tubers of 20 cultivars. A further objective was to illustrate the application/utility of these estimates, using data on total soluble protein (TSP) and patatin concentrations expressed as mg g^{-1} (DW) on a tissue-specific basis (Chapter IV, Ortiz-Medina et al., 2007a). Specific tissue weight estimates provided a means to convert tissue concentration data into values for total TSP and patatin in each tissue and in a typical whole tuber of 100 g FW for each cultivar. This enabled intercultivar comparisons of TSP and patatin for these 20 important potato cultivars.

5.3. Materials and Methods

5.3.1. Plant material

Potato tubers of 20 cultivars were used in this study, including Alpha, Atlantic, Belleisle, Bintje, Conestoga, Goldrush, Green Mountain, Kennebec, Norland, Onaway, Ranger Russet, Red Gold, Red Pontiac, Russet Burbank, Sebago, Shepody, Superior, Tobique, Tolaas, and Yukon Gold. All tubers were field-grown except for Alpha, where greenhouse-grown minitubers were used. Freshly harvested tubers were provided by the Bon Accord Elite Seed Potato Centre (Bon Accord, NB, Canada). All data were collected on randomly selected fresh tubers, soon after harvest.

5.3.2. Sample measurements

Weight and dimensions of six tubers of each cultivar were recorded. For each tuber, three dimensions were measured for volume calculations, including length, width (average of two measurements), and height (Fig. 5.1A). Measurements were made using a digital Vernier caliper (resolution 0.025 mm). Tubers were cut into regular-sized slices through cross and longitudinal sections. Four slices (two from each section) were taken for volume and tissue density calculations. For total slice volume estimation, length, width, and slice thickness (height) were measured (Fig. 5.1B). Due to the irregularity of

surface areas for periderm, cortex, and combined perimedullary with pith (pith) tissues, three sets of measurements of the periderm, between the periderm and vascular ring (cortex), and within the vascular ring (pith) were taken from these tissues (Fig. 5.1C). These measurements were used to calculate specific tissue areas as an average of three elliptical surfaces. Density of cortex and pith were calculated from two cylindrical plugs taken from each slice. The weight, diameter, and height of each plug were measured (Fig. 5.1C).

5.3.3. Calculations

The tuber volume was calculated for each cultivar by using measurement data in the formula for an ellipsoid, $V=1/6\pi lwh$. Where V is the tuber volume, l is the length from the stolon-end to the rose-end, w is the width (average of two measurements), and h is the height. The volume of a tuber slice was calculated as an elliptical-based cylinder, $Vs=1/4\pi lwh$. Where Vs is the total slice volume, l is the length, w is the width, and h is the height of the slice. Pith volume (Vpt) and total slice volume without periderm (Vs-p) were calculated the same way. Periderm volume (Vp) was estimated from $Vp= Vs - (Vs-p)$, and cortex volume (Vc) from $Vc=Vs-(Vp+Vpt)$. Volume values of each tissue were then used for the calculation of weight-tissue contribution to the total tuber weight.

Total tuber density was calculated by the relation $\sigma_t=W/V$, where σ_t is the density of the tuber, W is the total weight of the tuber, and V is the total volume of the tuber. Density of cortex and pith were calculated from the cylindrical plug data of each tissue applying the same formula. The total weight of the tuber was represented for all the weight-tissue constituents as: $TW= V_p\sigma_p + V_c\sigma_c + V_{pt}\sigma_{pt.}$ Where TW is the total weight of the tuber, V is volume, and σ is density of the p-periderm, c-cortex, and pt-pith.

Moisture content of the tissue-samples was calculated by the equation: $M_{tl} = ((W_f - W_d)/W_f) \times 100$. Where M_{tl} is the percentage moisture content of the tissue layer, W_f is the fresh weight of the sample, and W_d is the dry weight of the sample. Dry tissue-samples were obtained from lyophilized (24-30 h) and subsequently oven-dried samples (8 h at 100°C). Dry matter content of tissue-samples was derived from the moisture content values. Percent dry matter content was multiplied by % weight for each tissue, and used to tabulate specific tissue conversion factors for each cultivar.

5.3.4. Estimates of TSP and patatin content of individual tuber tissues on a whole tuber basis

To illustrate the utility of these conversion factors for rapid intercultivar comparisons of tuber nutrients, a data set of TSP and patatin concentration in specific tissues from the same 20 cultivars (reported in mg g^{-1} DW) was used (data from Chapter IV; Ortiz-Medina et al., 2007a). This yielded accurate estimations of the total TSP and patatin contents of each tissue layer on a whole tuber FW basis and in a typical tuber of 100 g FW for each cultivar. Intercultivar comparisons of whole tuber TSP and patatin were then possible.

5.3.5. Statistical analysis

Volume and weight proportions for each tissue layer were calculated for the 20 cultivars. The experimental unit was one tuber per cultivar (1 sample/ tissue layer/ tuber) with six replicates. Analysis of variance (ANOVA) was done using SAS 9.1 (SAS Institute Inc., 2003). TSP and patatin data estimates for total tuber were analyzed and Least Significant Difference test was conducted for means comparisons (LSD, $P \leq 0.05$).

5.4. Results and Discussion

5.4.1. Proportion of tuber tissues

Significantly differences in the weight and volume for each specific tuber tissue were noted (Table 5.1). Pith constituted the greatest proportion of tuber volume (average of 64%), followed by cortex (average of 34%), and periderm (average of 2%). Percent weight of each tissue was similar to the percent volume values. Despite this similarity, tissue-weight values were considered to be more accurate for estimating the solid composition of each tissue layer because this value included the density of each tuber tissue.

Significant cultivar-specific differences occurred in the % weight of each tuber tissue: periderm (0.8-3.4%, avg = 1.87%), cortex (26-43%, avg = 33.84%), and pith (54-73%, avg = 64.29%) (Table 5.1). Tubers of different cultivars had different proportional weights for each tissue. However, no relationship was found between % weight of tissue

and tuber shape, size, maturity or other cultivar characteristics. Percent weight values for each tissue multiplied by % dry matter content for that tissue resulted in the specific tissue dry matter for a typical tuber of 100 g FW (Table 5.2). These values were calculated for all cultivars.

5.4.2. Use of conversion values for intercultivar comparisons of TSP and patatin content

Conversion factors from Table 5.2 were used to estimate TSP and patatin content in each tuber tissue on a FW basis and in a whole tuber of 100 g FW for all 20 potato cultivars (Fig. 5.2). For example, in cv. Tobique, the TSP values for the periderm, cortex, and pith were 123.61, 61.57, and 66.04 mg g^{-1} DW (Fig. 4.1. fresh tubers). These values were multiplied by their respective conversion factors 0.372, 9.120, and 15.547 from Table 5.2. In resulted that in a typical whole Tobique tuber of 100 g FW there is 45.98, 561.49, and 1033.53 mg TSP in the periderm, cortex, and pith, respectively (Fig. 5.2).

There were significant differences in the total TSP and patatin content for specific tissues of the 20 cultivars (Fig. 5.2). While TSP concentrations in periderm were significantly greater than in cortex and pith tissues for most of these cultivars (Chapter IV, Ortiz-Medina et al., 2007a), the total content of this tissue made a small proportion of the tuber when estimated on a whole tuber FW basis. TSP content in the periderm ranged from 15 to 62 mg, in cortex from 215 to 638 mg, and in pith from 579 to 1027 mg in a whole tuber of 100 g FW. Patatin content ranged from 5 to 33, 126 to 445, and 263 to 666 mg for the same tissues, respectively. These results show clearly that the protein distribution within specific tuber tissues varies considerably between cultivars.

Intercultivar comparisons of TSP and patatin content in whole tubers of 100 g FW are shown in Fig. 5.3. Significant differences were found between cultivars; a 2-fold difference occurred between cultivars with the greatest and least TSP and patatin values. Cultivars Tobique (1634.1 mg) and Norland (1617.1 mg) had the greatest TSP contents, while Red Pontiac (977.1 mg) and Belleisle (820.3 mg) were among a small group of cultivars with relatively low TSP content. Patatin did not follow the same tissue distribution pattern as TSP. It was generally true that cultivars with the greatest and least TSP levels also had greatest or least patatin levels. However, cultivars in the median range of total tuber TSP varied in patatin content. Tobique (1071.2 mg) and Atlantic

(968.3 mg) had the greatest and Russet Burbank (452.3 mg) and Alpha (421.8 mg) the least total patatin values. Total protein content on a whole tuber basis, obtained by the summation of the protein content of each individual tissue, allows for a better comparison of the nutritional value of different cultivars.

Patatin as a percentage of the TSP, and its tissue-specific distribution is shown for fresh tubers of 100 g FW of all cultivars (Fig. 5.4). Cultivar-specific differences were apparent. Total % patatin ranged from 67 and 66% in Shepody and Tobique down to 41 and 35% in Russet Burbank and Alpha. This represents a wider range than the 40-60% (Pots et al., 1999a; Ralet and Guéguen, 1999) or 40-45% (Paiva et al., 1983) previously reported for whole tubers.

The specific-tissue conversion factors generated in Table 5.2 can be used to estimate the content of other nutritional compounds in these cultivars, such as vitamins, glycoalkaloids, minerals, etc. For example, unevenly distributed materials whose concentration is known on a specific-tissue DW basis, can be converted into whole 100 g FW tuber values and compared between cultivars. However, if the tissue concentration data were obtained after specific conditions that modified the moisture content of the tuber tissues (such as after time in storage), it becomes necessary to determine the dry matter content values of each tissue and generate new conversion factors as was done for Table 5.2. On the other hand, if data were available on a whole tuber FW or DW basis, and the material is evenly distributed and sampled proportionately, it is possible to estimate the specific tissue content using the calculated % weight values of Table 5.1.

5.5. Conclusion

Fresh potato tubers of different cultivars varying in size and weight were used to determine the % weight of each tuber tissue, including the periderm, cortex, and pith. Calculated % weight values together with % dry matter content for each tissue provided conversion factor values that were used to estimate the TSP and patatin content in each tuber tissue and (by summation) in a typical whole tuber of 100 g FW for 20 cultivars. These estimates facilitated intercultivar comparisons on a whole tuber basis, giving nutritional information more useful than the absolute concentration data of each tissue.

Tobique and Norland were identified as the cultivars with the greatest and Belleisle with the least TSP and patatin content.

It is suggested that the specific-tissue conversion tables obtained in this paper can be used to estimate the content of other nutritional compounds that are unevenly distributed throughout the tuber tissues in these cultivars. For that, a simple approach is to evaluate the concentration in each individual tissue, as was done for TSP and patatin (Chapter IV, Ortiz-Medina et al., 2007a). After that, these data can be converted to a whole tuber FW basis using the specific-tissue weight proportion values. This will give an accurate estimation of the compound in the whole tuber of a cultivar of interest and facilitates nutritional comparison with other cultivars. This knowledge is important, particularly for cultivars used in the food processing industry (Keijbets, 2005).

Figure 5.1. Schematic representation of the procedure used for tuber sectioning, measurement, and volume and density calculations from specific tissue layers of potato tubers. **A.** Longitudinal section of potato tuber showing the measurements of tuber dimensions for volume calculation. **B.** Cross and longitudinal tuber slices showing the measurements for slice volume calculation. **C.** Different length measurements for cortex and pith areas used for surface area calculation. Measurements of cortex and pith sections for their tissue-density estimation. X.S. = cross section, L.S. = longitudinal section, l = length, w = width, d = diameter, h = height.

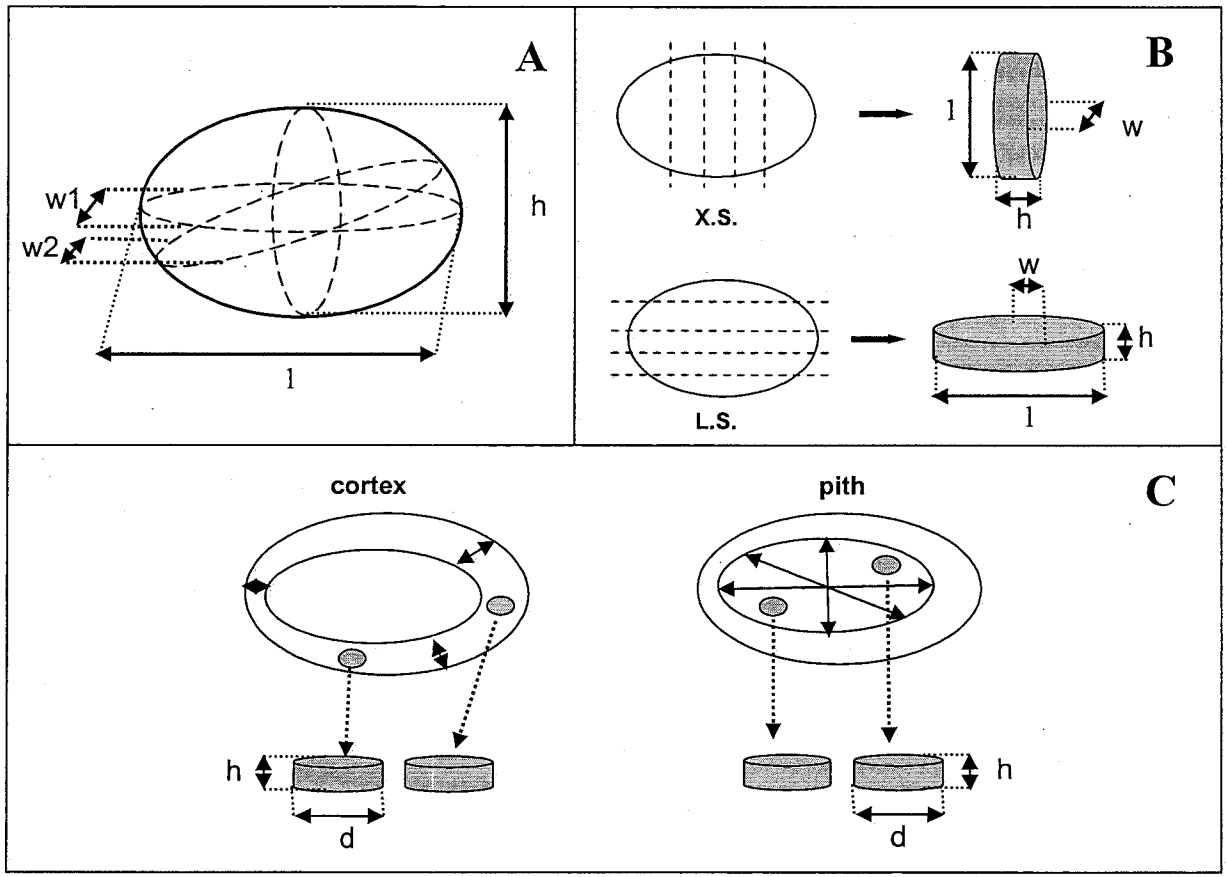

A
w1
w2
h
l
B
X.S.
l
w
h
L.S.
w
h
l
C
cortex
pith
h
d
h
d

Table 5.1. Tuber fresh weight (g), calculated tuber volume (cm^3) and proportion of volume (% volume) and weight (% weight) of individual tissues (periderm, cortex, and pith) of tubers of 20 potato cultivars. Values are the means ± SE (n=6).

CULTIVAR	Tuber fresh weight (g)	Calculated tuber volume (cm^3)	Proportion of the potato tuber					
			% volume*			% weight*		
			periderm	cortex	pith	periderm	cortex	pith
Alpha[a]	39.69 ± 2.8	45.88 ± 3.7	1.81	35.69	62.51	2.15	35.56	62.29
Atlantic	134.20 ± 12.3	116.33 ± 10.3	2.67	37.90	59.43	2.77	37.86	59.37
Belleisle	99.53 ± 7.4	98.32 ± 9.1	2.64	25.87	71.49	1.29	26.23	72.48
Bintje	77.11 ± 10.8	71.49 ± 10.1	2.71	43.00	54.29	1.56	43.51	54.93
Conestoga	95.00 ± 8.7	86.89 ± 8.0	1.45	30.70	67.85	1.37	30.73	67.90
Goldrush	88.36 ± 4.7	78.50 ± 3.5	1.97	35.29	62.74	2.08	35.25	62.67
Green Mountain	119.72 ± 5.4	112.47 ± 11.2	2.53	34.58	62.90	1.89	33.30	64.81
Kennebec	136.25 ± 10.8	138.87 ± 12.7	1.83	29.67	68.50	1.00	29.92	69.08
Norland	125.49 ± 9.5	117.00 ± 9.5	3.68	42.34	53.99	3.37	42.47	54.16
Onaway	48.03 ± 4.0	46.46 ± 3.5	1.54	30.65	67.81	1.75	30.59	67.66
Ranger Russet	52.07 ± 1.3	52.00 ± 4.8	2.27	31.86	65.87	1.95	31.69	66.36
Red Gold	72.71 ± 6.0	53.17 ± 3.8	2.52	39.36	58.12	3.00	39.16	57.83
Red Pontiac	155.97 ± 14.6	139.54 ± 16.2	2.39	29.45	68.16	1.77	29.64	68.59
Russet Burbank	112.02 ± 7.0	99.43 ± 4.5	2.45	39.78	57.77	2.78	39.64	57.57
Sebago	101.55 ± 10.9	99.49 ± 12.7	1.59	30.40	68.01	1.41	31.09	67.51
Shepody	147.97 ± 10.1	131.76 ± 10.6	1.58	32.21	66.21	1.38	32.28	66.34
Superior	99.80 ± 9.0	91.18 ± 9.0	2.52	41.48	56.01	1.99	41.70	56.31
Tobique	114.53 ± 10.7	101.34 ± 9.5	1.97	32.53	65.49	1.90	32.56	65.54
Tolaas	109.98 ± 18.5	99.10 ± 18.6	1.64	26.37	71.99	1.04	26.53	72.43
Yukon Gold	171.10 ± 18.7	155.29 ± 19.0	0.97	27.14	71.88	0.85	27.18	71.97
Average			*2.14*	*33.81*	*64.05*	*1.87*	*33.84*	*64.29*

[a]Tubers of all cultivars were field-grown except for Alpha, where greenhouse-grown minitubers were used.

* Significant differences between tissue layers ($P<0.05$).

Table 5.2. Dry matter content of specific tuber tissues (periderm, cortex, and pith) and in a typical tuber of 100 g FW for 20 potato cultivars. Dry matter per 100 g FW values resulted from multiplying the % weight by the % dry matter for each tissue and were used as conversion factors for estimation of TSP and patatin content in each tissue layer.

CULTIVAR	Periderm		Cortex		Pith	
	dry matter (%)	dry matter per 100 g FW[b]	dry matter (%)	dry matter per 100 g FW[b]	dry matter (%)	dry matter per 100 g FW[b]
Alpha[a]	16.77	0.361	22.28	7.923	19.04	11.853
Atlantic	16.42	0.455	27.22	10.306	22.23	13.198
Belleisle	17.27	0.223	17.37	4.556	16.50	11.951
Bintje	16.87	0.263	23.62	10.272	21.02	11.542
Conestoga	16.07	0.220	21.64	6.646	18.60	12.623
Goldrush	24.46	0.509	28.22	9.945	22.07	13.824
Green Mountain	17.46	0.330	23.11	7.695	19.11	12.379
Kennebec	15.38	0.154	26.76	8.006	20.10	13.885
Norland	13.04	0.439	24.07	10.219	20.22	10.951
Onaway	18.85	0.330	24.80	7.582	21.63	14.629
Ranger Russet	19.35	0.377	22.31	7.070	22.49	14.917
Red Gold	18.69	0.561	26.09	10.218	21.28	12.301
Red Pontiac	15.38	0.272	22.67	6.717	18.36	12.587
Russet Burbank	14.48	0.403	23.56	9.336	20.49	11.791
Sebago	15.22	0.215	21.49	6.681	21.90	14.777
Shepody	22.06	0.305	25.91	8.363	24.38	16.174
Superior	17.53	0.348	22.98	9.579	18.99	10.694
Tobique	19.59	0.372	28.02	9.120	23.73	15.547
Tolaas	17.55	0.182	20.09	5.330	17.60	12.748
Yukon Gold	18.68	0.159	23.40	6.356	20.52	14.762
Average	*17.56*	*0.32*	*23.78*	*8.10*	*20.51*	*13.16*

[a]Tubers of all cultivars were field-grown except for Alpha, where greenhouse-grown minitubers were used.
[b]These values can be used as conversion factors for other nutritional compounds reported on a tuber tissue-specific DW basis.

Figure 5.2. Total soluble protein (TSP) and patatin content estimates for specific tuber tissues (periderm, cortex, and pith) in a typical tuber of 100 g FW for 20 potato cultivars. Values are the means ± SE, n=3. Significant differences were found between tissue layers ($P<0.05$).

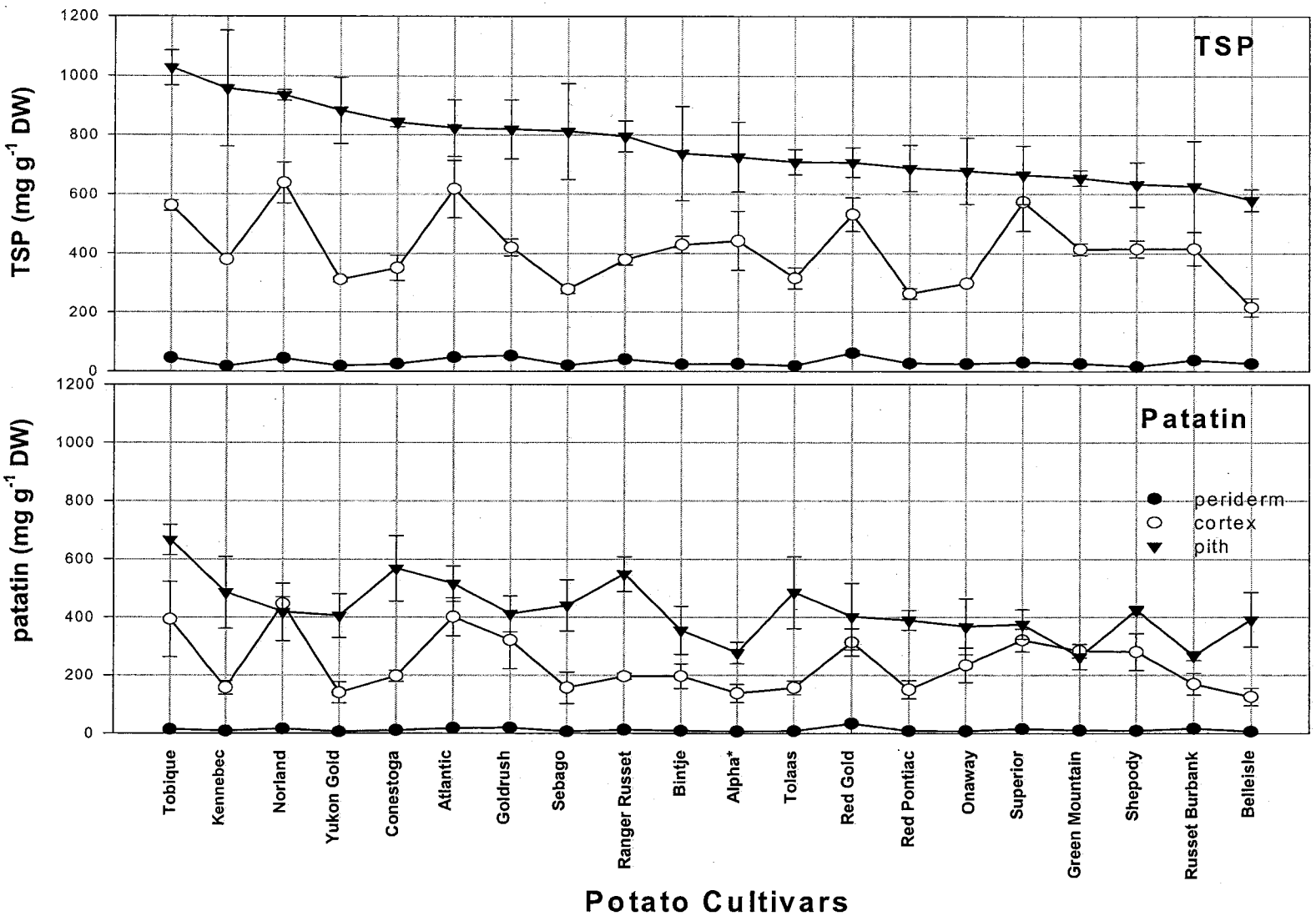
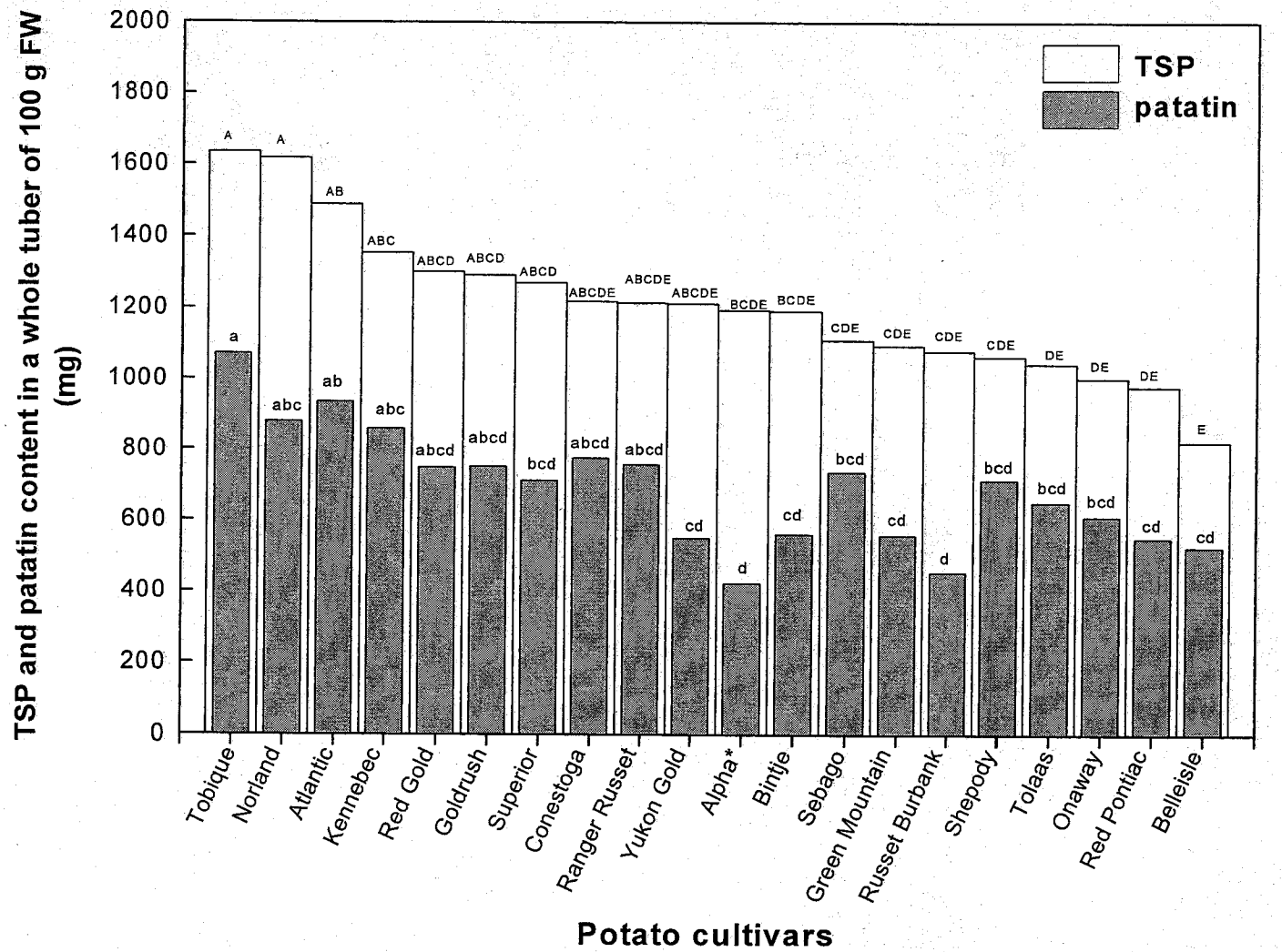

TSP
TSP (mg g-1 DW)
1200
1000
800
600
400
200
0
Patatin
patatin (mg g-1 DW)
periderm
cortex
pith
Tobique
Kennebec
Norland
Yukon Gold
Conestoga
Atlantic
Goldrush
Sebago
Ranger Russet
Bintje
Alpha*
Tolaas
Red Gold
Red Pontiac
Onaway
Superior
Green Mountain
Shepody
Russet Burbank
Belleisle
Potato Cultivars

Figure 5.3. Total soluble protein (TSP) and patatin content calculated for whole tubers of 100 g FW for 20 potato cultivars. Mean differences in TSP concentration between cultivars are represented by capital letters, while mean differences in patatin concentration are represented by small letters ($LSD_{0.05}$).

*Alpha minitubers


TSP and patatin content in a whole tuber of 100 g FW (mg)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
TSP
patatin
A
A
AB
ABC
ABCD
ABCD
ABCD
ABCDE
ABCDE
ABCDE
BCDE
BCDE
CDE
CDE
CDE
CDE
DE
DE
DE
E
a
abc
ab
abc
abcd
abcd
bcd
abcd
abcd
cd
d
cd
bcd
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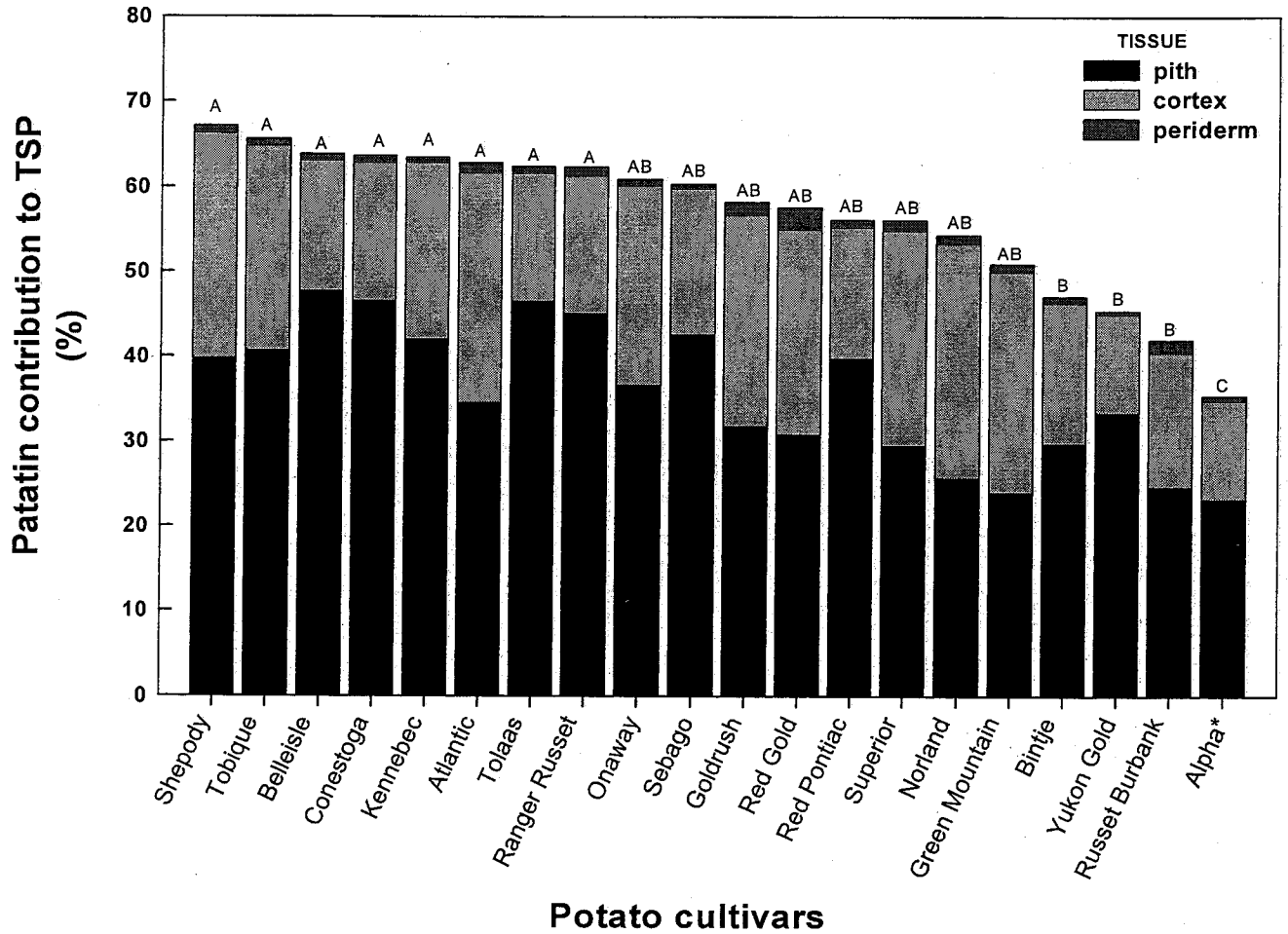
d
bcd
bcd
bcd
cd
cd
Tobique
Norland
Atlantic
Kennebec
Red Gold
Goldrush
Superior
Conestoga
Ranger Russet
Yukon Gold
Alpha*
Bintje
Sebago
Green Mountain
Russet Burbank
Shepody
Tolaas
Onaway
Red Pontiac
Belleisle
Potato cultivars

Figure 5.4. Patatin as a percentage of the total soluble protein (% patatin), and its tissue-specific contribution in fresh tubers of 20 potato cultivars. Mean differences for total % patatin between cultivars are represented by letters ($LSD_{0.05}$).

* Alpha minitubers


Patatin contribution to TSP (%)
80
70
60
50
40
30
20
10
0
TISSUE
pith
cortex
periderm
A
A
A
A
A
A
A
A
AB
AB
AB
AB
AB
AB
AB
AB
B
B
B
C
Shepody
Tobique
Belleisle
Conestoga
Kennebec
Atlantic
Tolaas
Ranger Russet
Onaway
Sebago
Goldrush
Red Gold
Red Pontiac
Superior
Norland
Green Mountain
Bintje
Yukon Gold
Russet Burbank
Alpha*
Potato cultivars

CONNECTING STATEMENT FOR CHAPTER VI

Chapter VI consists of the manuscript entitled "Micropropagation and genetic risk: securing clonal fidelity" prepared by E. Ortiz-Medina and D.J. Donnelly. The content of this chapter was presented orally by Dr. D.J. Donnelly at *The International Workshop on True-To-Typeness of Date Palm Tissue Culture-Derived Plants* held in Marrakech, Morocco, 23–25 May, 2005. This manuscript was published in *Proceedings of The international Workshop on True-to-Typeness of Date Palm Tissue Culture-Derived Plants*. A. Zaid (Ed). 2005: 45-53.

Chapter VI is a review of the factors implicated in causing variation in clonally propagated plants derived through micropropagation systems. The major emphasis is on the impact of tissue culture-induced variation on the clonal integrity of genotypes. As vegetatively-propagated clones accumulate mutations over time all clonally-propagated cultivars are chimeral to some extent. Therefore, intraclonal variation may arise in some cases from the disassembly of chimeral plants into their component genotypes.

Chapter VI

MICROPROPAGATION AND GENETIC RISK: SECURING CLONAL FIDELITY

E. Ortiz-Medina and D.J. Donnelly[1]

6.1. Abstract

Genetic risk associated with single-node culture and axillary micropropagation systems can usually be controlled in culture. Axillary shoot multiplication can occasionally be confounded by adventitious shoot proliferation. This is more prevalent for some cultivars in commercial situations. For many plant species, axillary shoot culture systems are not an option. The genetic risk associated with adventitious culture systems varies with the plants involved; relatively low (1 to 3% per regeneration cycle) for adventitious shoots and much greater (up to 10% per regeneration cycle) for adventitious somatic embryoids. Shoots or embryoids may show variation that reflects normal source-tissue variation. In chimeral species, somaclonal variation results from disassembly of the component genotypes and may approach 100% of regenerants, completely undermining attempts of tissue culturists to achieve clonal fidelity. How can clonal fidelity be maintained when adventitious tissue culture systems are employed? This can only be done through rigorous choice of methodology, understanding of the type of variation inherent in the system, especially chimeral status of the explant, and careful screening of propagules. It will take a collaborative approach among plant anatomists, tissue culturists, and molecular geneticists to solve clonal fidelity issues.

6.2. Introduction

Micropropagation technology is at work in laboratories all over the world due to the advantages over conventional methods of propagation. Micropropagation is used to increase a diverse range of vegetatively-propagated plants; many of them are fruit species

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of temperate orchards and tropical plantations, ginger, potato, bulbous species and other rhizomes and geophytes, many types of vegetables and spices, trees for forestry, and a long list of ornamental species (reviewed by Rana and Raina, 2000). Clonal fidelity is the single most critical issue faced by propagators. It has biological and commercial implications. Understanding the forces that work against clonal fidelity challenges our knowledge of plant anatomy and genetics and has the potential to impact on many aspects of the commercial plant industry.

In vitro propagation is achieved through different methods, depending on the species and the commercial choices made. The full range of factors that affect clonal integrity in different types of culture systems is not completely understood. It is known that variation is inherent within the explant, and the frequency of variant propagules is affected by choice of pre-culture and culture techniques. Some of these choices, and their impact on clonal fidelity, are reviewed. Possible strategies to modulate variation are proposed.

6.3. Clonal Fidelity in Single Node Cuttings and Axillary Shoot Multiplication Systems

Propagation from single-node cuttings or axillary shoots has been used for a large number of plant species. These methods are believed to be least susceptible to mutations and phenotypic variation due to the presence of preexisting meristems within the explant, from which all in vitro growth derives (George, 1993; Pierik, 1997; Kane, 1996; 2005). For example, commercial micropropagation of potato involves single-node cuttings, while for most temperate fruit species, including apple, blackberry, blueberry, cherry, grape, raspberry, strawberry, etc. axillary shoot multiplication is used. Cultivars of potato or temperate fruit species are available from North American germplasm repositories. Requesting laboratories receive duplicate or triplicate test tubes of specific pathogen tested (SPT) plantlets. Often, the original explants were meristem tips, dissected following thermotherapy of virus-infected source (stock) plants. Therefore, the distributed plantlets are meristem tip-source clones. When SPT source plants are

available, the explants are apical or lateral shoot buds or single-node cuttings, and distributed plantlets are shoot tip-source clones.

Potato micropropagation facilities in North America use media devoid of growth regulators, relying on single-node cuttings rather than axillary shoot multiplication for increase in plantlet numbers. Despite this supremely cautious approach to micropropagation, routine practices among the germplasm repositories that supply the propagation facilities may result in the distribution of intraclonal variants. An explanation for this involves the method by which germplasm repositories regularly "audit" their cultivar collections (Fig. 6.1). Every 1-several year(s), representative potato plantlets from a few test tubes are planted into the field for a grow-out test. Visual ratings of stem and tuber characteristics, and especially yield and maturity factors, are evaluated. If these plants are considered true-to-cultivar, cuttings are taken for transfer to the greenhouse, where plants undergo a pathology check, for presence of virus. If plants are healthy, shoot tip explants are used for tissue culture. If the clone is now virus-infected, plants receive thermotherapy before meristem tip explants are placed into culture. One or a limited number of shoot tip- or meristem tip-source clones are then used to represent the cultivar in the germplasm repository. The audit procedure relies on experienced nurserymens' and growers' subjective decisions on trueness-to-cultivar, for a limited number of plants, usually at one geographic location. This imposes local field selection pressure, based on performance in that geographic local. In vitro selection pressure follows, for acceptable performance in culture. The cycle repeats at site-specific intervals over decades. For old cultivars like Russet Burbank, held in several repositories in North America, this process repeated at several geographic locations over half a century has resulted in the emergence of suspected intraclonal differences.

So, how does the maintenance of germplasm affect plant genetics? If a cultivar is represented by fields of plants, accumulating genetic mutations with each field season, the older the cultivar, the greater the range of genetic variation that has accumulated within the clone. However, in the current reality, a cultivar may be represented by one or a few meristem tip- or shoot tip-source clones. The amount of inherent genetic variation that has accumulated in the clone is reduced during the pre-micropropagation process. For example, in the past, it was possible for potato breeders to identify superior plants

from large field-grown populations of clonal members. These intraclonal variants, (strains or geographical clones) were renamed as new cultivars, for their superior performance in specific regions (Leever et al., 1994). Will breeders still be able to do this when the repository sends them a cultivar represented by germplasm that has received repeated cycles of geographic selection and meristem tip culture?

How different is the clonal germplasm maintained in different locations? Ten clones of potato cultivar Russet Burbank that had been geographically isolated for 25 years or more (some for >60 years), or subject to systematic selection (by breeders) were gathered for a yield comparison (Love et al., 1992). Yields in Idaho (mid-western U.S.), were not the same for all the clones, and the first alarm bells were heard over possible emerging intraclonal differences. Ten years later, a comparison in Eastern Canada showed that yield and maturity factors between 11 of these clones were not substantially different (Coleman et al., 2003). Nevertheless, geographical biases were evident in chemical maturation rates and storage performance; and some phenotypic differences were apparent. Although Single Sequence Repeats (SSR) and Random Amplified Polymorphic DNA (RAPD) analysis could not detect DNA polymorphisms to distinguish these intraclonal strains, more sensitive techniques may resolve these differences in the future.

It is extremely rare to hear of "variants" or "off-type" plants, among temperate fruit species micropropagated through axillary shoot multiplication. However, this does occur. Occasionally, certain cultivars, for which the media employed are not ideal, may have a tendency to form callus at the base of axillary shoot cultures. Where this occurs, adventitious shoots may become mixed and difficult to distinguish from the axillary shoots. For example, strawberry cultures may contain a mixture of adventitious and axillary shoots, unless callus is stringently removed at each subculture. In a recent North American law suit, dozens of commercial strawberry growers were compensated when a provincial certification agency distributed a micropropagated strawberry cultivar that fruited abnormally. It is not a simple matter, even for certification authorities, to avoid these litigious situations. Commercial laboratories may not have the experience, or may not take the time, to optimize "generic" medium formulations for the needs of individual cultivars. Technicians working in laminar air flow units may not have the training to

distinguish adventitious shoot clusters or may feel too pressured, to harvest as many shoots as possible per culture cycle, to rogue the adventitious shoots.

Economic pressures to maximize the productivity of axillary shoot multiplication systems sometimes leads to excessive use of growth regulators (especially certain auxins, such as 2,4-Dichlorophenoxyacetic acid and cytokinins), or the practice of "pulsing"; increasing the growth regulator level for one or more monthly culture cycles followed by decreasing the concentration. Prolonged use of elevated levels of growth regulators are suspected of causing mutations. However, there is not a clear relationship between growth regulator concentration and frequency of somaclonal variation (van Harten, 1998). Still, it is common to see recommendations to limit growth regulator exposure by reducing the total number of culture cycles following explantation. For example, in commercial strawberry production, 8-10 months of culture (8-10 subcultures) following explantation is the recommended limit. For many species, new isolations are recommended annually (Skirvin et al., 1994; Rana and Raina, 2000).

6.4. Clonal Fidelity in Adventitious Multiplication Systems

Somaclonal variation is a term introduced by Larkin and Scowcroft (1981) to describe genetically novel shoots or plantlets derived from tissue culture systems. It is not always known if these shoots arise from genetically variant cells that are present prior to culture or if variant cells are induced by the culture process due to environmental stress and/or chemical mutation from exposure to growth medium ingredients (Skirvin et al., 1994). In vitro stresses of environment or chemistry could cause mistakes during nuclear and cell division processes. It is usually unknown if individual changes are heritable or not – for clonally propagated species this is rarely of interest.

6.4.1. Pre-existing chimeral variation

Vegetatively propagated clones are known to accumulate mutations over time. This comes about through microenvironment effects on plant apical and lateral shoot meristems. When more than one genotype is present within a plant, the plant is known as a chimera. Probably all plants are chimeral to some extent, since during normal organ

formation, mistakes in nuclear and cell division may lead to chromosomal changes, both small (point mutations) or large (aneuploidy, polyploidy). In some cases, variant cells within the shoot apical meristem may occur in discrete sectors (sectorial chimera), portions of the tunic (outer histogenic layer) (mericlinal chimera) or an entire tunic layer (periclinal chimera) (see Fig. 2.2). While the sectorial and mericlinal chimeras are transient, the periclinal chimera is a stable arrangement, also known as a hand-in-glove chimera, involving a mutation in the outer histogenic layer(s) or tunic surrounding a wild-type (non-mutated) core or corpus. There are many well known examples of chimeras, of various complexity, such as cv. Russet Burbank potato (Davis, 1992; Tilney-Bassett, 1986), cvs. Bartlett pear, Delicious apple, and thornless *Rubus* species (Loganberry or Thornless blackberry) (Skirvin, 1977).

If a chimeral cultivar, such as Loganberry or Thornless blackberry is propagated through callus and adventitious shoot or embryoid formation, then chimeral disassembly can occur. The individual cells or small groups of cells that contribute to shoot initiation may have only one genotype - in which case the shoot is no longer chimeral. The same is true when single cells develop into somatic embryoids. If an established chimeral cultivar is disassembled, then cultivar status is irrevocably altered in some adventitious propagules. Reversal to chimeral status can only occur if the original mutation is repeated, the likelihood of this is unknown. In the case of Thornless blackberry, 100% of the regenerants were thornless; some were chimeral like the source tissue and some were genetically thornless derived entirely from the mutated LI histogenic tissue layer (Skirvin et al., 1994; 2000; Fig. 6.2). Following field-selection among a population of thornless plants, a commercially interesting genetically thornless (non-chimeral) plant was selected and named, cv. Everthornless.

6.4.2. Reducing genetic risk in micropropagation of chimeral species

If the chimeral status of a tissue cultured plant is unknown, a process of "uncovering" of the chimeral genotypes may occur (van Harten, 1998). When adventitious culture systems are used for putative chimeral species, there may be ways to minimize genetic variation through a better understanding of chimeral structure. For example, most Angiosperm dicotyledonous plants have shoot meristems composed of

two tunic layers surrounding the corpus (Fig. 6.3). The outer (LI) and inner (LII) tunic layers generally develop into the stem epidermis and cortex (or outer cortex), respectively (Lineberger, 2005). The pith or medulla of the stem (sometimes also the inner cortex) develops from the corpus (LIII) of the meristem. Mutations are more likely to accumulate towards the outer periphery of the meristems, within the tunic layers (Bäurle and Laux, 2003). This occurs due to the relatively small number of divisions that occur in the cells in the central zone of the corpus and the greater number of divisions within their derivative cells. For this reason, pith sections may hold fewer variant cells than epidermal or cortical tissues or whole stem sections. However, somaclones derived from the pith are by definition non-chimeral, so 100% somaclonal variation results from this chimeral disassembly.

Does it matter, if a chimeral cultivar is separated into its component genotypes, in terms of plant growth and productivity? For thornless *Rubus*, a mixed population of chimeral shoots and somaclones from LI tunic tissue were compared, and the best non-chimeral clone selected was just as good as the original chimeral cultivar for commercial fruiting attributes. So the answer is that tissue selection is important, non-chimeral clones can be just as good as chimeral clones, but field evaluation is the only guaranteed way at the moment to test the commercial acceptability of these non-chimeral somaclones.

6.4.3. Culture-induced chimeral variation

Somaclonal variation is associated with callus or wound-tissue proliferation and adventitious shoot regeneration systems. The process of accumulation of mutations in this system is said to result from asynchrony between nuclear and cell division that occurs in callus. Contributing to this could be mutation events that result from in vitro selection pressures. If meristems that are initiated in callus accumulate mutations in vitro in the same way as in the field, adventitious chimeral shoot tips could arise. These could have transient sectorial or mericlinal chimeral arrangements or the stable periclinal arrangement. These shoots may appear identical to the source plant tissue, unless the genes involved affect some obvious phenotypic trait. The genetic risk associated with adventitious culture systems varies with the species involved. The risk is estimated to be relatively low (1-3%) for adventitiously regenerated plants (Skirvin et al., 2000).

However, off-types are usually visually assessed and real numbers of clonal variants may be far greater.

Many types of mutations are seen in clones derived adventitiously with or without callus. Are these variants from pre-existing variant cells or culture-induced variants? One way to distinguish the relative frequency of pre-existing and culture-induce variant cells would be through comparison of the incidence of somaclonal variants from indirect and direct shoot regeneration systems of the same explant. However, these studies are not easily controlled, as different regeneration systems require the use of different growth regulators and growing conditions. Some combinations may favour growth of variant cells or adventitious shoots differentiation from them. Furthermore, genetic analysis may not readily distinguish between them.

Molecular techniques are not yet capable of fully characterizing adventitious shoots or embryoids to establish the degree of clonal fidelity. When somaclones present to growers as phenotypically identical or similar to the source plant and to each other, it is difficult to know what the actual genetic picture really is. Clearly, some plant species, pre-culture and culture protocols, and some explants have the potential to yield much greater frequencies of somaclonal variants. For example, somaclonal variation reported in different bananas and plantains ranged from 0-69%, with 6-38% among Cavendish cultivars (Martinez et al., 1998 and Hwang and Tang, 2000 cited in Sahijram et al. 2003). Additional confusion may arise when chromosome or gene mutations occur, but are not stable (Karp, 1995). Plants may outgrow some types of mutations, for example sectorial and mericlinal arrangements where reversion to the stable periclinal or to the wild-type occurs (Hartmann et al., 2002). The incidence of this is unknown and may differ among species. To determine the incidence of reversion to wild-type, genetic analysis of adventitious propagules may have to be repeated at intervals.

6.4.4. Epigenetic variation

Confounding pre-existing and culture-induced somatic variation, is a complex of epigenetic characteristics associated with the culture-induced phenotype. This is developmental variation that has been well characterized in temperate fruit species. It includes a suite of environmentally-dependent anatomical and physiological changes

characteristic of in vitro-grown plants (Donnelly and Tisdall, 1993). These result from exposure to the culture environment, which imposes: saturated atmosphere, low medium water potential, low light level, low rate of gas exchange, high and constant temperature, presence of sugars and exogenous growth regulators in the medium. Some of the many features of the culture-induced phenotype include: miniaturization, mixotrophic nutrition, reduced epicuticular and cuticular wax deposition, reduced and altered trichome population, and altered stomatal function. All of these features affect acclimatization of ex vitro transplants. However, the new tissues formed ex vitro exhibit the control phenotype in response to the climate outside of the culture containers. The culture-induced phenotype is quickly outgrown.

6.5. Conclusion

In summary, single-node cuttings and axillary shoot proliferation techniques have been extensively used for micropropagation of potato and temperature fruit species, respectively. These are believed to be "safe" means of micropropagation, with little opportunity for introduction of genetic variation due to plant derivation from preexisting organized meristems. Nevertheless, at the germplasm repositories, field selection during cultivar audit followed by thermotherapy and in vitro selection of a representative meristem or shoot tip source clone may impose a series of selection pressures on cultivars, and have resulted in the emergence of suspected intraclonal strains or geographic clones. Axillary shoot multiplication can occasionally be confounded by adventitious shoot proliferation and this is more prevalent for specific cultivars of some fruit species and in some commercial situations. In addition, overuse of growth regulators may interfere with normal meristematic growth. Reducing the amount of growth regulators used, and the number of subculture cycles from the time of explantation, may reduce the risk of variation in these cultures.

In adventitious culture systems, the risk of somaclonal variation is much greater than in single-node or axillary shoot multiplication systems. It is not known how much preexisting variation occurs in plant tissues and how much is introduced by adventitious culture prractices. All plants probably are chimeral to some extent. Older cultivars may

have accumulated significant numbers of mutations over the years. Some of these mutations may be distributed in stable periclinal arrangements. When adventitious culture systems are used for putative chimeral species, there may be ways to minimize genetic variation through a better understanding of chimeral structure. In the case of thornless *Rubus* species, an LI-derived genetically thornless somaclonal variant had satisfactory yield characteristics and was commercialized. However, selection of tissue derived from the corpus may be inherently less genetically variable than tissues derived from the tunic, especially the outer tunic layer (LI). The relative somatic variation derived from tissues of different histogenic layers should be evaluated, especially for plant species where somatic variation has been particularly troubling. At the present time, only field-evaluation can determine whether disassembled, non-chimeral clones can perform satisfactorily; a lengthy and costly activity for perennial species. Nevertheless, it is possible that new non-chimeral cultivars may propagate adventitiously with a reduced incidence of somaclonal variation. Molecular techniques cannot yet fully characterize adventitious shoots or embryoids to determine their clonal status but this era is approaching rapidly. It will take a collaborative approach among plant anatomists, tissue culturists and molecular geneticists to solve clonal fidelity issues.

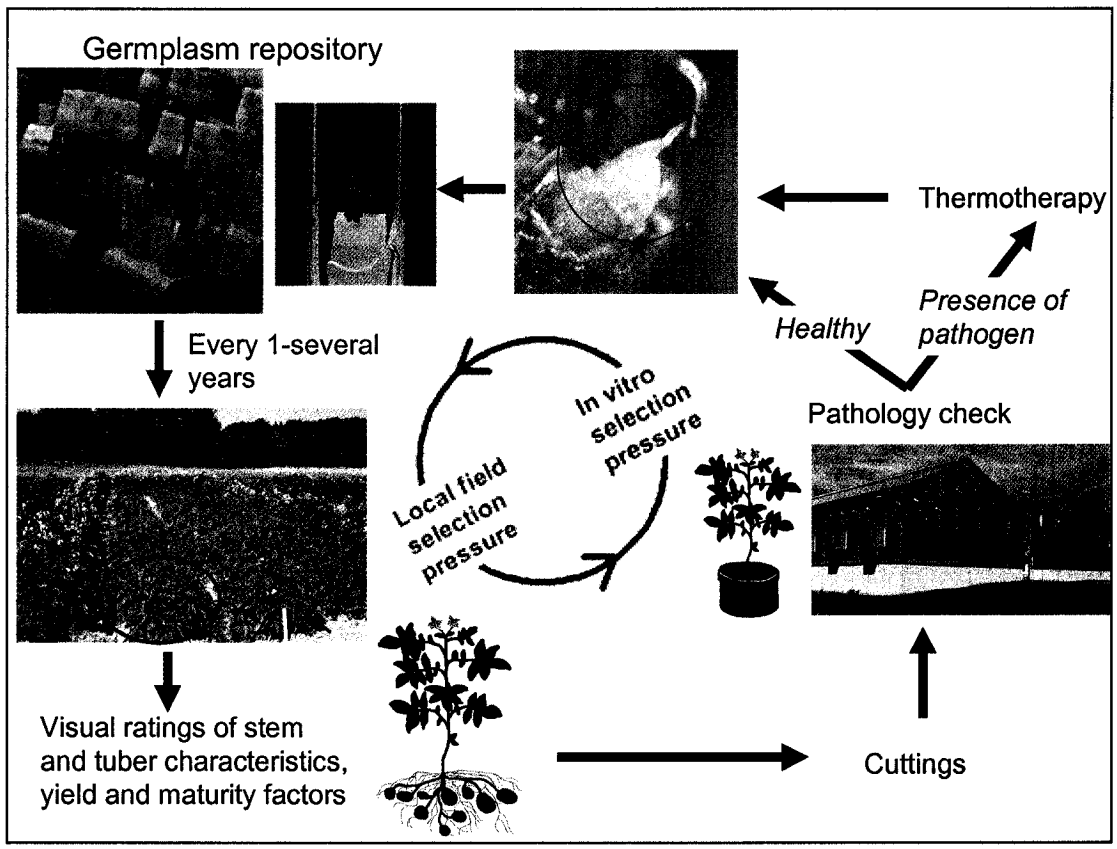

Figure design: Béatrice Riché, 2005.

Figure 6.1. Cycle of activities involved in auditing cultivars held at a germplasm repository for trueness-to-cultivar. Some pre-micropropagaton activities, such as thermotherapy and meristem tip culture for virus elimination, and in vitro germplasm storage, may serve to decrease the amount of genetic diversity present within a clonal cultivar. Local field selection pressure is followed by selection for growth in culture. The method of maintenance of clonal germplasm has changed a great deal over the years. The older the clonal cultivar the greater the range of genetic mutation that has accumulated within the clone. If a clonal cultivar is represented by one meristem tip-source clone, inherent variation is reduced.

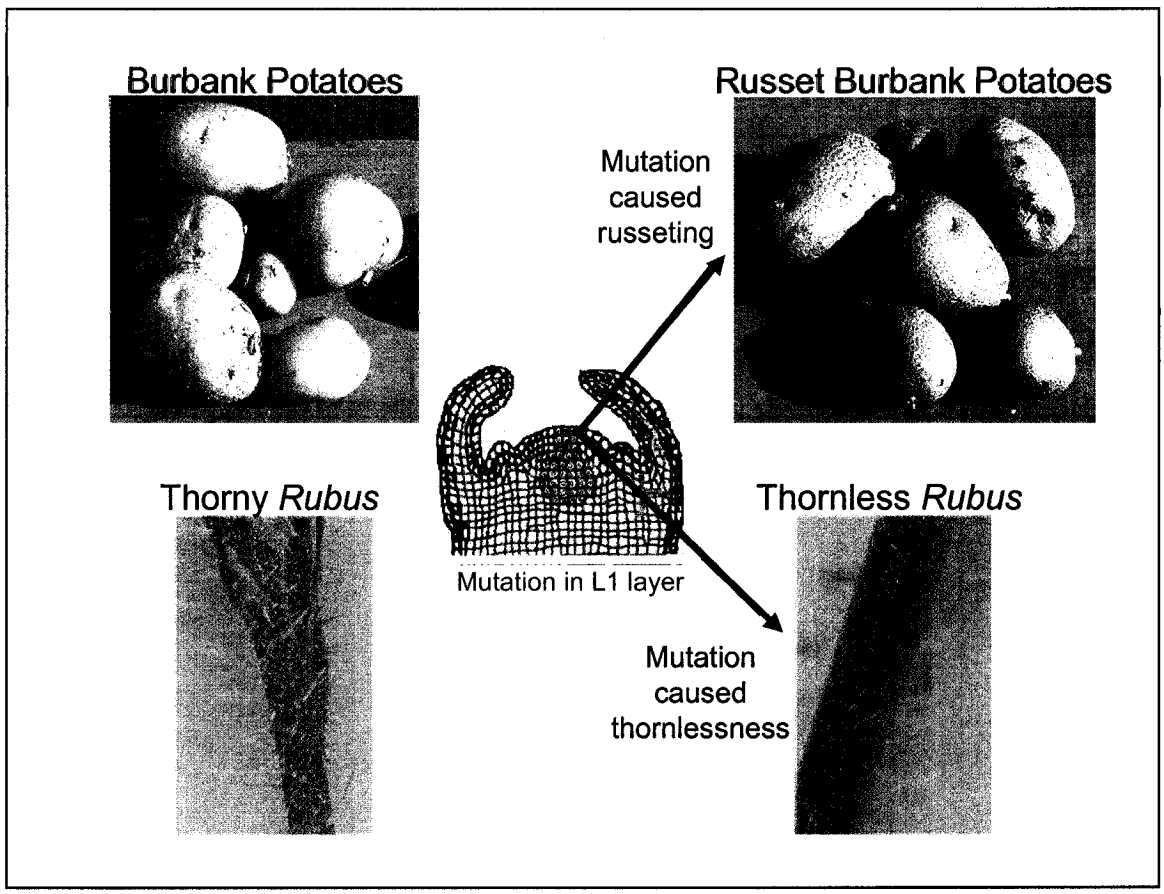

Photo credit cvs. Burbank and Russet Burbank: John Bamberg & Max W. Martin, 2004. US Potato Genebank. Sturgeon Bay, WI. USA.

Figure 6.2. Two examples are illustrated where periclinal mutation of the LI tunic layer has lead to improved cultivars. The potato cv. Russet Burbank is a sport of cv. Burbank. Russet Burbank is a periclinal chimera in which the LI tunic layer has a mutation that causes the russeted periderm phenotype. Thornless *Rubus* species are periclinal chimeras with a mutated gene for thorniness in the LI tunic layer. Through tissue culture, a non-chimeral, genetically thornless *Rubus* cultivar was produced by Skirvin's group in the 1980s.

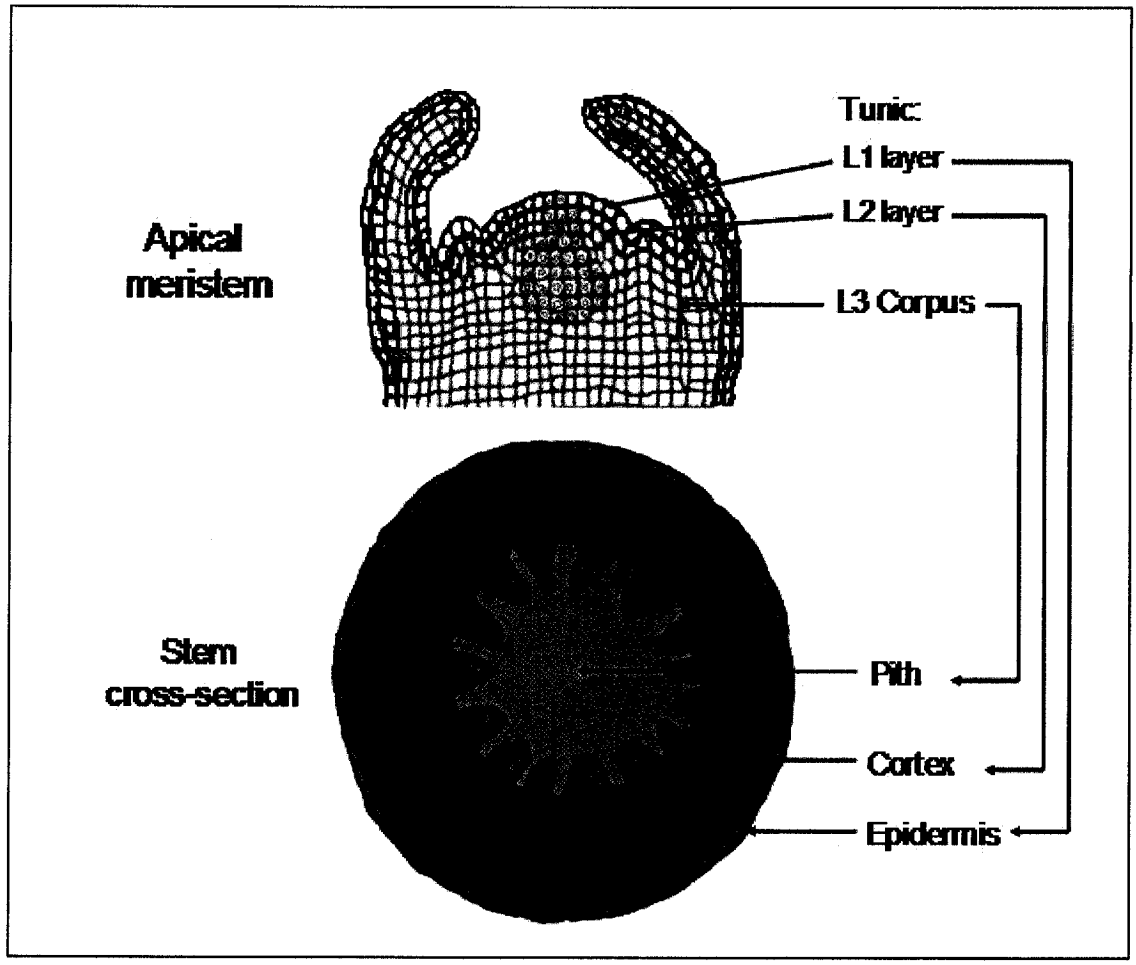

Figure design: Béatrice Riché, 2005.

Figure 6.3. Shoot tip organization, in Angiosperm dicotyledons, involves two tunic layers, designated LI (outer layer) and LII (inner layer) and the corpus, designated LIII. As stem development occurs, the LI layer differentiates into the epidermis, the LII layer grows into the cortex (outer cortex in some species) and the LIII layer becomes the pith (and inner cortex in some species). In the central corpus area is a group of cells that divide infrequently, while their derivatives divide many times. In this way, the genetic integrity of these central corpus cells (stem cells) is conserved.

CONNECTING STATEMENT FOR CHAPTER VII

Chapter VII consists of the manuscript entitled "Testing periclinal chimerism in potato somatic regenerants using tuber characteristics" prepared by E. Ortiz-Medina and D.J. Donnelly. This manuscript will be submitted for publication to Plant Cell, Tissue and Organ Culture.

The characteristic tissue-distribution pattern of total soluble proteins in potato tubers was reported in Chapters III and IV; relatively greater concentration in periderm compared with lesser concentration in cortex and pith tissues. This distribution suggests the hypothesis that protein content may be distributed in a periclinal chimeral way. This chapter reports a test of the periclinal chimeral hypothesis through the disassembly of chimeral and putative chimeral potato cultivars into their component genotypes. The total soluble protein pattern was used as a biochemical marker and the russeting trait as a phenotypic marker. Somatic embryogenesis from tissue-specific explants from source tissue with relatively greater or lesser protein level was used to regenerate non-chimeral plants. These were tuberized and the tubers examined for protein content and distribution. This chapter considered the potential advantages of screening tissue-specific intraclonal variants (discussed in Chapter VI), as a method of nutritionally improving the potato crop.

Chapter VII

TESTING PERICLINAL CHIMERISM IN POTATO SOMATIC REGENERANTS USING TUBER CHARACTERISTICS

E. Ortiz-Medina and D.J. Donnelly[2]

7.1. Abstract

Potato periclinal chimerism was investigated through the disassembly of tuber tissue of potato cultivars Alpha, Bintje, Red Gold, and Russet Burbank. Tissue-specific explants from the periderm, cortex, and pith (derived from histogenic layers LI, LII, and LIII, respectively) were used to produce non-chimeral somatic regenerant (SR_1) plants. Cortex- and pith-derived SR_1 plants were obtained for all cultivars but periderm-derived SR_1 plants were only obtained for Bintje. The russeting trait was used as a phenotypic marker for Russet Burbank (classic example of a periclinal chimera) and total soluble protein (TSP) distribution pattern as a putative biochemical marker for all cultivars. Russet Burbank cortex- and some pith-derived SR_1 plants had non-russeted minitubers similar to the original cultivar Burbank but other pith-derived SR_1 plants produced minitubers with a russeted periderm like Russet Burbank. We conclude that Russet Burbank is a LI periclinal chimera, but chimeral instability is evident. There was no consistent evidence that TSP was distributed in a periclinal chimeral way. Red Gold, a hybrid seedling-derived cultivar, was "uncovered" as an LII periclinal chimera for periderm colour (Red-Gold-Red). Periclinal chimeral disassembly into component genotypes is discussed and potential advantage of screening tissue-specific intraclonal variants is considered.

7.2. Introduction

Most dicots have shoot meristems with three distinct histogenic cell layers that develop independently from each other (Schmidt, 1924; Esau, 1965). The outermost

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layer, the tunica (tunic), consists of an outer layer (LI) that differentiates into the epidermis, and a second layer (LII) that forms subepidermal tissue including cortex (or outer cortex, depending on the species) and gametes. The inner layer, the corpus (LIII), differentiates into the central tissues, the pith (or inner cortex and pith, depending on the species) (Dermen, 1960; Norris et al., 1983; Tilney-Bassett, 1986).

Despite the genetic stability usually associated with vegetative propagation, spontaneous mutations occur in plants that are continuously clonally propagated. These mutations may affect organellar (chloroplastic, mitochondrial) or cellular (nuclear) genomes. These mutated plants, designated "genetic mosaics" are composed of two or more genetically different tissues (Marcotrigiano, 1990; Hartmann et al., 2002). The best-known genetic mosaics are those affecting chloroplasts, resulting in variegated foliage (Norris et al., 1983; Marcotrigiano, 1997). Less understood are the nuclear mutations that occur within the histogenic layers of shoot meristems, leading to genetic mosaics called *chimeras*. Some nuclear mutations may lead to visible phenotypic changes but many more are "silent". There are three known kinds of chimeras, based on their spatial arrangement within histogenic cell layers; periclinal, mericlinal, and sectorial (Marcotrigiano, 1997; Burge et al., 2000; Hartmann et al., 2002). Periclinal chimeras, in which the mutation usually occurs in the LI layer at an early developmental stage of the meristem, are stable to vegetative propagation through conventional cuttage (Tilney-Basset, 1986; Marcotrigiano, 1990; Hartmann et al., 2002).

Genetic mosaics, in the form of periclinal chimeras, distinguish many new cultivars of potato; "sports" of the original cultivars (Crane, 1936; Miller, 1954; Howard, 1959; Tilney-Basset, 1986). Altered tuber characteristics, especially skin (periderm) colour and texture result from periclinal chimerism and usually refer to mutations of the LI with respect to the wild-type internal tissues derived from the LII and LIII (Crane, 1936; Rieman et al., 1951; Howard, 1959).

The classic, often cited, example of a periclinal chimera is Russet Burbank (thick, russeted brown skin, elongate-round shape), a sport of Burbank (thin, smooth white skin, elongate-round shape) (Fig. 7.1). Russet Burbank (originally called Netted Gem) was selected as a russeted mutant from Burbank, by a Colorado potato grower (L.D. Sweet), in 1914 (Davis, 1992). The original Burbank was a seedling selection from a chance fruit

of Early Rose (thin, smooth pink skin, round-oval shape) (Burbank, 1914; Davis, 1992). Improved characteristics seen in Russet Burbank compared with Burbank were attributed to russeted skin and included considerable resistance to potato scab (*Streptomyces scabies*) and late blight (*Phytophthora infestans*) (Davis, 1992). Two russeted cultivars, recognized as periclinal chimeras (Russet Burbank and Russet Norkotah) are among the most widely grown cultivars in North America (Davis, 1992; Hale et al., 2005). To the best of our knowledge, their periclinal chimeral status has never been tested or challenged.

In a survey of the total soluble protein (TSP) content in tubers of 20 important North American cultivars, a pronounced cultivar-specific pattern was observed in TSP distribution between the three tissue layers (periderm, cortex, and pith) (Chapter III and IV; Ortiz-Medina and Donnelly, 2003; 2007a). Russet Burbank and most other cultivars had greater TSP concentration in the periderm compared with the cortex and pith. In Alpha, the pattern was inconsistent; lower or similar TSP concentration in the periderm compared with the internal tissue.

We hypothesized that cultivars with altered TSP concentrations in the periderm were periclinal chimeras (putative periclinal chimeras) that resulted from LI mutation(s) affecting protein deposition pattern, in the same way that LI mutation(s) have affected periderm colour or thickening (russeting) characteristics. This hypothesis suggests that the separation of the periclinal chimeral potato tuber into component genotypes would give non-chimeral plants with non-chimeral tubers (Fig. 7.2). For Russet Burbank, it was expected that periderm-derived plants would produce russeted tubers. We could not determine from the literature whether LII was affected by the same mutation for russeting in Russet Burbank. If LI and LIII are both wild-type for the russeting trait, it was expected that cortex- and pith-derived plants would produce tubers similar in appearance to one another and without russeted periderm (like the original cultivar Burbank). In all four cultivars, it was expected that non-chimeral somatic regenerants, first generation (SR_1) plants would produce tubers with a new protein distribution pattern (greater or lesser) in all tuber tissues that was similar to the explant-source tissue genotype.

The objective of this study was to investigate potato periclinal chimerism through the disassembly of four cultivars (Alpha, Bintje, Red Gold, and Russet Burbank) into

their component genotypes. This was done by explanting tissue derived from each histogenic layer; periderm (derived from LI), cortex (derived from LII) and perimedullary tissue and pith (pith) (derived from LIII) followed by regeneration from these non-chimeral tissues through somatic embryogenesis, a technique in which individual potato cells can be induced to form plantlets (Seabrook and Douglass, 2001; Gray, 2005). Tuberization of regenerated plantlets to produce microtubers and minitubers was followed by evaluation of tubers from non-chimeral SR_1 plants using the russeting trait as a phenotypic marker (for Russet Burbank) and TSP pattern as a putative biochemical marker (for all four cultivars).

7.3. Materials and Methods

7.3.1. Plant material

Field-grown potato tubers of four cvs. Alpha, Bintje, Red Gold, and Russet Burbank were used in this study. These were provided by the Bon Accord Elite Seed Potato Centre (Bon Accord, NB, Canada). The Russet Burbank field tubers we received had thick brown russeted periderm. The TSP distribution for the Bintje, Red Gold, and Russet Burbank tubers was high, low, low (HLL) and for Alpha, low, low, low (LLL) in the periderm, cortex and pith respectively.

7.3.2. Somatic embryogenesis

Plant regeneration through somatic embryogenesis was carried out using a two-step procedure modified from Seabrook and Douglass (2001) that used microtuber slices as explants. We used field-grown tubers and aseptically removed explants from specific tissues derived from each of the three histogenic layers. Individual cells or small clusters of cells within each explant differentiated into new plants ensuring that our SR_1 regenerants were non-chimeral.

Tubers were surface-disinfested in 10% sodium hypochlorite solution for 15 min. and rinsed with sterile distilled water several times. Tuber explants (height x length x width) were removed from the periderm (1 x 5 x 5 mm), cortex (5^3 mm) and pith (5^3 mm) using a dissecting microscope (50X; Wild Heerbrugg SC, USA) located inside a laminar

airflow cabinet (Canadian Cabinets, H4MW-97-BJ, Canada). Any visible buds from tuber eyes on the periderm were excluded from periderm explants. For each cultivar, three petri dishes with five tuber explants from each tissue layer were established in a replicated trial.

The explants were established in petri dishes with MS (Murashige and Skoog, 1962) callus induction medium containing basal salts and organic components, sucrose (30 g l^{-1}), agar (7 g l^{-1}) (Anachemia, Montreal, QC) as well as the plant growth regulators indoleacetic acid (IAA) (19 μM), and thidiazuron (0.15 μM). Cultures were grown under controlled environmental conditions in a walk-in growth room maintained at 23 ± 2°C under a 16/8 h (light/dark) photoperiod with cool-white fluorescent light (GE Pro-Line, Watt-Miser F40) at a flux density of 100 μmol $m^{-2}s^{-1}$. Once callus developed (2-3 weeks), these were transferred into Magenta containers with 40 ml of MS medium containing zeatin (12 μM), IAA (0.05 μM) and gibberellic acid (0.55 μM) for the induction of somatic embryos. After 3-4 weeks of culture under the same environmental conditions, the somatic embryos started to grow. Cultures were observed at 7-day intervals for the regeneration of somatic embryos. Somatic embryos developed through the globular, heart-shaped, torpedo, and cotyledonary stages to become SR_1 plantlets.

7.3.3. Micropropagation

SR_1 plantlets were collected at 1-week intervals for 6 weeks. By limiting the callus and induction phases there were relatively few SR_1 plantlets in total. However, we lessened the chances of somatic changes that might result from the tissue culture process (exogenous variation) and maximized the opportunity to see variation inherent in the source tissue (endogenous variation). SR_1 plantlets were transferred to MS medium without growth regulators (micropropagation medium) when they reached 1-1.5 cm in height, and maintained under the same environmental conditions. Single-node cuttings from in vitro potato plantlets of the four cultivars were provided by the Plant Propagation Centre (Fredericton, NB, Canada) and were used as controls for intact periclinal chimeral (or putative periclinal chimeral) plantlets. After 4 weeks of growth on micropropagation medium, control and SR_1 plantlet lines were used for microtuberization or minituberization.

7.3.4. Microtuberization and minituberization

Microtuberization occurred in a grown-chamber using a two-step layering procedure (Leclerc et al., 1994). Microtubers of approx. 1.0 cm diameter were harvested after 4 weeks in microtuber induction medium.

For minituberization, plantlets were transferred to ProMix (Premier Horticulture, Ltée. QC, Canada) in plug trays and placed into the mist chamber for 1 week before transfer to a greenhouse bench. After 2 weeks the transplants were repotted into larger containers (Nursery Products Inc., pots #12, ON, Canada) and grown under ambient greenhouse conditions. Minitubers were harvested after 16 weeks in the greenhouse and each weighed approx 10 g.

7.3.5. Sample preparation

Samples of field-grown source tubers, and SR_1 plant microtubers and minitubers were randomly taken from three tissue layers (periderm, cortex, and pith) for the quantification of TSP. The periderm was removed in strips using a scalpel for microtubers and a potato peeler for field-grown tubers and minitubers. The cortex and pith were separated with a scalpel and cut into small pieces of 0.5-1.0 g FW per sample. Samples were then immediately frozen under liquid nitrogen. Frozen samples were lyophilized in a freeze-dryer (SNL216V, Savant Instruments Inc. NY, USA) at -50°C, ground-up and stored at -20°C until analysis.

7.3.6. Total soluble protein (TSP) determination

TSP was extracted from 10 mg dry weight (DW) of each freeze-dried stored sample with 2 ml of 0.1 N NaOH, pH 12.8 (Jones et al., 1989). Protein concentration was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA; Bio-Rad Laboratories, ON, Canada) as a standard. The microassay procedure for microtiter plates (Bio-Rad protein assay) was used and TSP was determined at 595 nm in a microplate reader (Synergy HT, Bio-Tek, VT, USA). Results were reported in mg g^{-1} DW of tuber tissue.

7.3.7. Statistical analysis

Analysis of variance (ANOVA) and means comparisons by the Least Significant Differences (LSD) method were carried out on TSP concentration data from tissue-derived SR_1 microtubers and minitubers, using the statistical program SAS 9.1 (SAS, 2003) at 0.05 level of significance.

7.4. Results

Disassembly of potato tubers of four cultivars was achieved through explantation of tissues derived from each histogenic layer followed by somatic embryogenesis and regeneration of non-chimeral plants (Table 7.1). Only explants from the cortex and pith tissues consistently regenerated somatic embryos. Approx. 10% of periderm explants from Alpha, Red Gold, and Russet Burbank callused but these subsequently deteriorated. Periderm explants from Bintje survived (53%) and produced somatic embryos. Five-seven periderm- (for Bintje), cortex- and pith-derived SR_1 lines of each cultivar were microtuberized or minituberized along with the respective control tubers. Microtubers and minitubers from SR_1 plants were used for TSP analysis. Due to their similarity, the results of one repetition are presented.

7.4.1. Tuber periderm characteristics

Periderm features were not definitive on the tiny microtubers (data not shown) but readily observed on minitubers. Russet Burbank control minitubers had russeted periderm and elongate-round shape (Fig. 7.3A) and looked like control Russet Burbank field tubers (Fig. 7.1A). All cortex-derived and some pith-derived SR_1 plants from Russet Burbank produced minitubers with smooth white skin that looked like Burbank (compare Fig. 7.3B, D, E with Fig. 7.1B), which conformed to the expected. However, some pith-derived SR_1 plants produced russeted minitubers that looked like Russet Burbank (compare Fig. 7.3C, F, G with Fig. 7.3A, 7.1A).

Of particular interest, all minitubers from Red Gold cortex-derived SR_1 plants had gold (yellow) periderm compared with the pinkish-red colour of all control and pith-derived SR_1 plant minitubers (Fig. 7.4).

7.4.2. TSP in control field-grown tubers, microtubers and minitubers

TSP pattern in control microtuber and minituber tissues were the same as in the field-grown source tubers; HLL in the periderm, cortex and pith, respectively for Russet Burbank and Red Gold (Fig. 7.5A, D), and LHH for Alpha (Fig. 7.5B) as reported by Ortiz-Medina and Donnelly (2003). This was not the case for Bintje, where the protein pattern was not consistent with what was observed in field-grown source tubers (Fig. 7.5C).

TSP concentration was greater in all tissue layers of microtubers compared with the source tubers in all four cultivars. This supports previous results where microtubers of seven cultivars, including Russet Burbank, showed the same TSP pattern but with very significantly increased tissue levels compared with field-grown tubers (mean increase was 37, 60, and 29% in periderm, cortex, and pith, respectively) (Ortiz-Medina and Donnelly, 2003).

7.4.3. TSP patterns from microtubers and minitubers of SR_1 plants

The expected TSP concentration pattern (LLL; Fig. 7.2) but not the same concentrations in each tissue occurred in minitubers but not microtubers of cortex- and pith-derived Russet Burbank SR_1 plants (Fig. 7.5A). It is particularly interesting that all tubers from Russet Burbank SR_1 plants had significantly lesser TSP concentrations in their periderm compared with the periderm concentrations in control microtubers and minitubers (Fig. 7.5A). This suggests that among the Russet Burbank intraclones, regeneration from internal tissue (both cortex and pith derivatives) affects ability to synthesize or store TSP in the periderm and may also affect the composition of periderm protein (and possibly other components).

The expected TSP concentration patterns (Fig. 7.2) did not consistently occur in SR_1 plant tubers of Alpha, Bintje, or Red Gold (Fig. 7.5B-D). In Alpha, cortex- and pith-derived SR_1 plant microtubers but not minitubers, had the expected HHH pattern (Fig.

7.5B). In Bintje, the expected TSP patterns for periderm-derived SR_1 plant tubers (HHH) and cortex and pith-derived SR1 plant tubers (LLL) were not observed (Fig. 7.5C). In Red Gold, the expected TSP pattern of cortex- and pith-derived SR_1 tubers (LLL) partially occurred for microtubers (HLL) but not for minitubers (Fig. 7.5D). There was no evidence for these three cultivars that regeneration from internal tissue affected periderm TSP concentrations in the intraclones

7.4.4. Pith-derived minitubers of Russet Burbank SR_1 plantlets

Significant differences in TSP concentrations occurred in minitubers from the five pith-derived SR_1 lines compared with control minitubers (Fig. 7.3). Minitubers from lines 1 and 2 showed increased cortex and pith TSP levels relative to the periderm while in lines 3, 4 and 5, TSP generally decreased in the cortex and pith compared with the control minitubers. The periderm TSP level was similar in minitubers from the five pith-derived SR_1 lines, but significantly less than control minituber periderm levels.

Visual characteristics of minitubers based primarily on periderm russeting suggested that non-russeted cortex- and pith-derived SR_1 lines 2 and 3 constitute one group and russeted lines 1, 4 and 5 constitute another group with the control (Fig. 7.3). Physiological maturity affects tuber shape; in general, minitubers of Russet Burbank and Burbank tended to be rounder than field-grown tubers. The differences in shape may reflect differences in physiological maturity between these plants at the time of harvest.

7.5. Discussion

Tissue specific explantation followed by somatic embryogenesis is a promising technique for disassembly of tubers into component genotypes. Unfortunately, somatic embryos did not readily form from tuber periderm tissue in three of the four cultivars tested. This may be due to the method we used for explanting periderm tissue; only the most superficial tissue layer was removed from source tubers. It is possible that our explants contained only suberized, non-living phellem cells common in older outer periderm (Sabba and Lulai 2002). Probably, only the innermost cells of the periderm can callus and regenerate somatic embryos. In cv. Bintje, where regenerative plants were

obtained from periderm explants, we could speculate that some phellogen cells were present in these explants.

When russeting and colour of periderm were evaluated in Russet Burbank, cortex-derived SR_1 plants (LII origin) and some pith-derived SR_1 plants (LIII origin) looked like the original Burbank, on which the sport Russet Burbank was found. On this basis, we accept the hypothesis that Russet Burbank represents an LI mutation affecting periderm russeting, and is a periclinal chimera of Burbank. The mixed population of pith-derived SR_1 plants with tubers that looked like Russet Burbank or Burbank, clearly suggest chimeral instability. These results corroborate earlier findings with periclinal chimeras using the "eye-excision" method to induce adventitious shoots from pith of Golden Wonder (LI mutation with russeted periderm); these shoots produced plants with non-russeted and others with russeted tubers (Crane, 1936). Using the same eye-excision method, colour anomalies were noted on tubers from pith-derived adventitious shoots of Red King (LI mutation from splashed pink to full pink periderm); some plants produced tubers with periderm of King Edward VII-type (splashed pink), others had Red King-type (full pink) and others produced entirely white tubers (Howard, 1959). The findings were rationalized as possible faulty experimentation produced from incomplete bud removal from the eyes (Howard, 1970). However, it is clear from our results and those of Crane (1936) and Howard (1959) that classic descriptions of periclinal chimerism are not sufficient to explain variations in periderm texture (russeting) or colour (LI mutations) in pith-derived SR_1 plant tubers as this tissue and its derivatives are expected to be homogeneous and conserved (Tilney-Basset, 1986). LI cells appear to have invaded or replaced the LIII (Howard et al., 1963; Stewart and Dermen, 1970; Klekowski et al., 1985). An instability or breakdown in periclinal chimeral structure has occurred sometime during the almost 100-year history of clonal propagation of Russet Burbank. This is difficult to understand as, by definition, a periclinal chimera is a stable entity (Marcotrigiano, 1997; Burge et al., 2002). More extensive examination of tissue-specific SR_1 plant tubers from Russet Burbank would help to determine the extent of LI replacement and cell mixing in LIII- (and possibly LII-) derived tissues.

The gold periderm, observed in Red Gold minitubers on all cortex-derived SR_1 plants, suggests that Red Gold is a LII periclinal chimera, inadvertently "uncovered"

through our disassembly process. It appears that Red Gold has LII "gold periderm" sandwiched between LI and LIII "red periderm" and masked by LI (RGR periclinal chimera). There was no evidence of cell displacement or replacement between histogenic layers in this cultivar. Red Gold is a hybrid seedling selection from G68211 (gold skin) crossed with G6521-4RY (red skin) (Coffin et al., 1988). Periclinal chimerism is usually associated with spontaneous or induced mutation, not sexual hybridization (Tilney-Basset, 1986; Marcotrigiano, 1997; Hartmann et al., 2002).

Evaluation of TSP concentration among control source tubers, microtubers and minitubers revealed a similar TSP pattern in three of four cultivars. However, consistently greater TSP tissue concentration in microtubers was observed. The TSP concentration increase was cultivar-dependent and may also be related to differences in growing conditions in tissue culture and greenhouse systems that allow a greater availability of nutrients compared with those in the field (Chapter III, Ortiz-Medina and Donnelly, 2003). Concentration of TSP may also be influenced by cell size and/or tissue density in microtubers and other factors including environment. As intraclone tubers of all cultivars had TSP patterns that did not consistently conform to the expected, we must reject the hypothesis that TSP is distributed in a periclinal chimeral manner. However, all tubers from Russet Burbank cortex- and pith-derived SR_1 plants had significantly lesser TSP concentrations in the periderm compared with the periderm concentrations in control field-grown source tubers. This cultivar-specific characteristic may involve differential gene expression due to positional effects, which may affect other important periderm features (protein composition, antipathogenic compounds, etc.) and should be explored.

Somaclonal variation resulting from the disassembly of histogenic layers via tissue-specific explants has many and varied implications for horticultural research. Improvement of plants without disturbing or damaging their primary traits can be achieved when chimeras are separated into their component genotypes, resulting in valuable new varieties, as reported in some grapevines (Franks et al, 2002) and pears (Chevreau, 1989; Abu-Qaoud et al., 1990). It is possible that for potato, non-chimeral somatic variants may represent an untapped resource for plant breeding, especially when the traits for which mutations are desired can not easily be selected for, such as pest and disease resistance (Tilney-Basset, 1986). They are also of great potential interest for

studying patterns of endogenous somatic variation as they enable us to visualize some of the mutations that have accumulated over time in the apical meristem and where these cells have migrated to. For example, in Russet Burbank endogenous variants may represent wild-type cells from earlier clones such as Burbank, or progenitors such as Early Rose. Other potential advantages to "somatic mining" for non-chimeral SR_1 potato plants could involve selection for improved nutrient value. For example, greater or lesser protein concentration in some intraclones was seen in this study; the stability of which is untested, Furthermore, superior plants derived from intraclonal selection could have excellent market acceptance unlike genetically transformed plants (van Harten, 1998).

7.6. Conclusions

In this study, we examinated the periclinal chimeral hypothesis. We produced tissue-specific (non-chimeral) SR_1 plants, which we tuberized to evaluate some phenotypic (russeting) and some biochemical (TSP) characters of tissue derived from the three histogenic layers of four cultivars. Disassembly of the histogenic layers of these cultivars enabled closer examination of two periclinal chimeras. Russet Burbank, now almost 100 years in cultivation, exhibited chimeral instability showing replacement of LI tunic cells into the pith (and possibly the cortex although this was not seen). Red Gold was uncovered as a Red-Gold-Red (LII) unique hybrid seedling periclinal chimera. This cultivar showed no apparent LI or LII cell displacement or replacement and can be recommended for periclinal chimeral investigations.

Total soluble protein distribution was inconsistent in tubers from SR_1 plants, and we conclude that it is not distributed in a periclinal chimeral manner. The reduced TSP levels in the periderm of tubers from all internally-derived SR_1 plants of Russet Burbank were not observed in the other three cultivars. Cultivar differences, including periclinal chimerism, may affect the utility of specific source tissues for somatic embryogenesis. Chimeral disassembly provides a unique opportunity to study meristems and many fascinating aspects of plant biology. It may help elucidate some long-standing questions on somaclonal variation including relative incidence of endogenous and exogenously-caused somaclonal variation.

Table 7.1. Regeneration of SR_1 plants from somatic embryogenesis of four potato cultivars. Fifteen explants of each tissue per cultivar were induced to produce somatic embryos. Percentage of callused explants, somatic embryos, and number of regenerated shoots (SR_1 plants) are indicated for each cultivar.

Cultivar	**Callused explants (%)**			**Regeneration of somatic embryos (% explants)**			**Total number of plantlets regenerated (SR_1 plants)**		
	periderm	cortex	pith	periderm	cortex	pith	periderm	cortex	pith
Alpha	7	73	67	-	33	27	-	11	5
Bintje	60	87	93	53	67	73	14	16	15
Red Gold	13	80	87	-	47	60	-	9	12
Russet Burbank	13	93	87	-	73	60	-	21	15

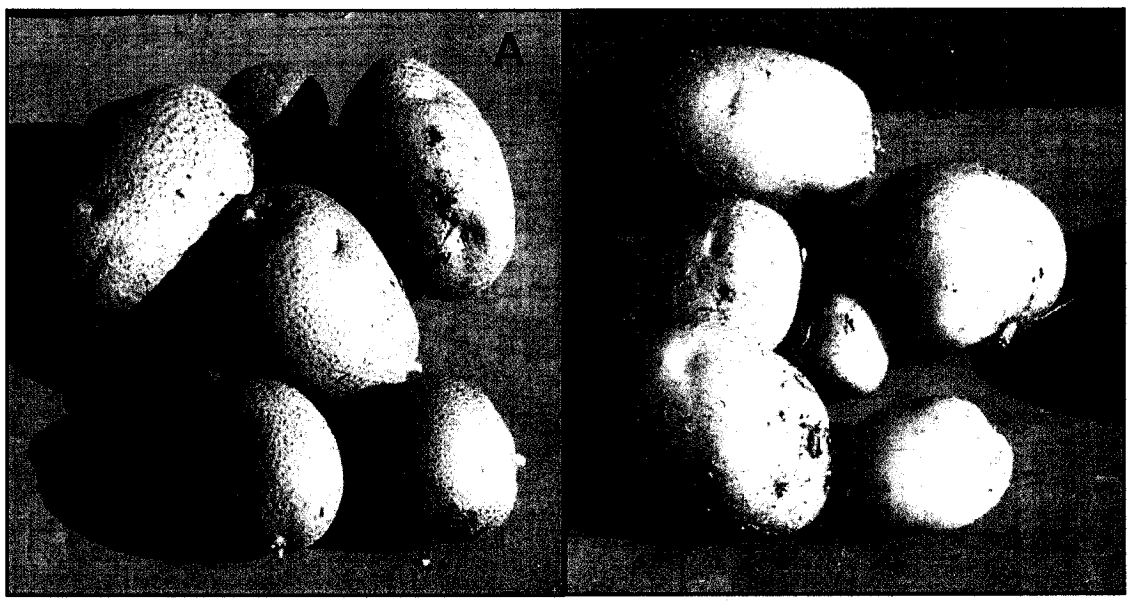


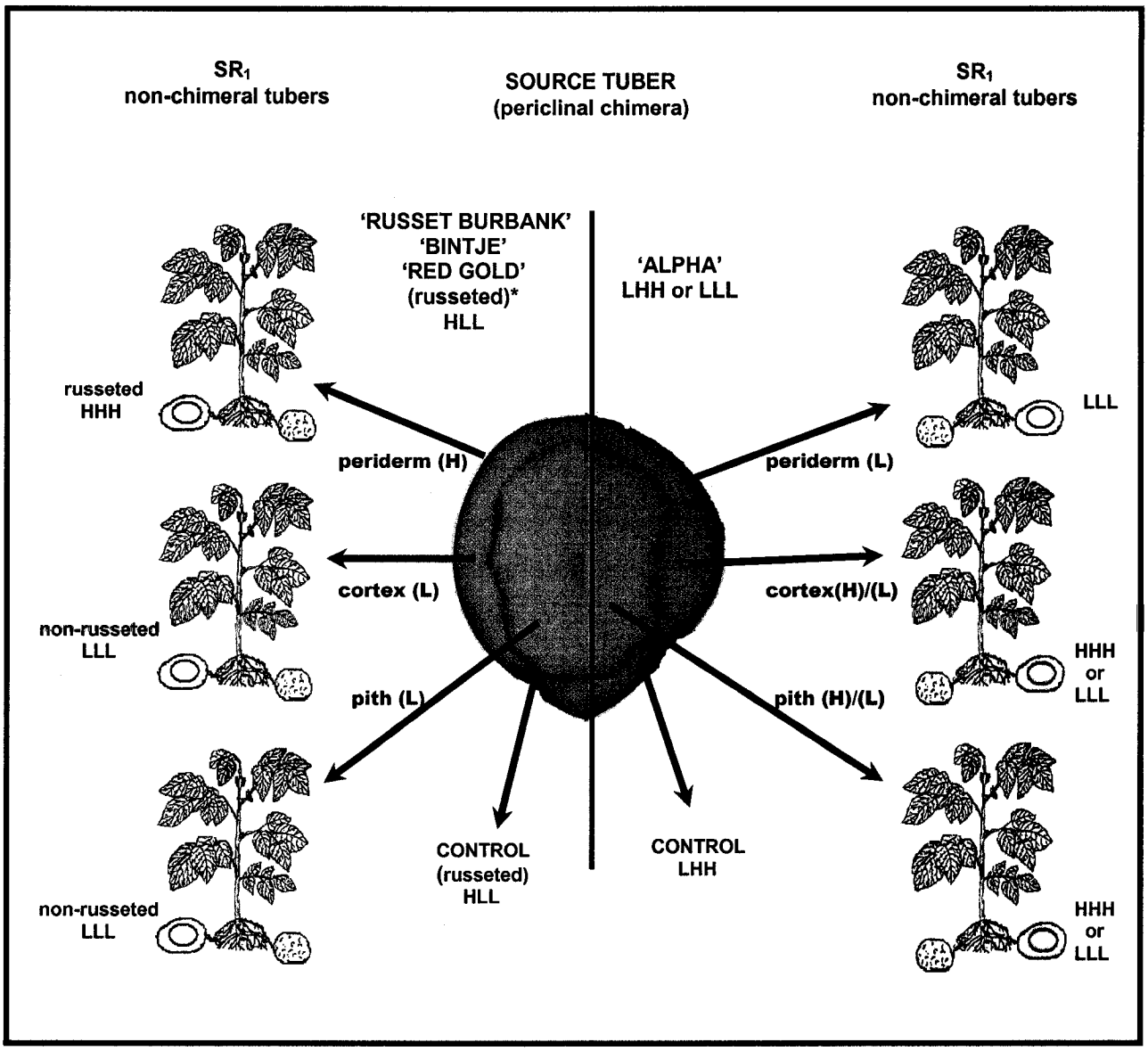
Photo credit: John Bamberg & Max W. Martin, 2004. US Potato Genebank. Sturgeon Bay, WI. USA.

Figure 7.1. Potato tuber characteristics of Burbank and Russet Burbank cultivars.

A. Russet Burbank showing thick, russeted brown periderm and elongate-round shape.

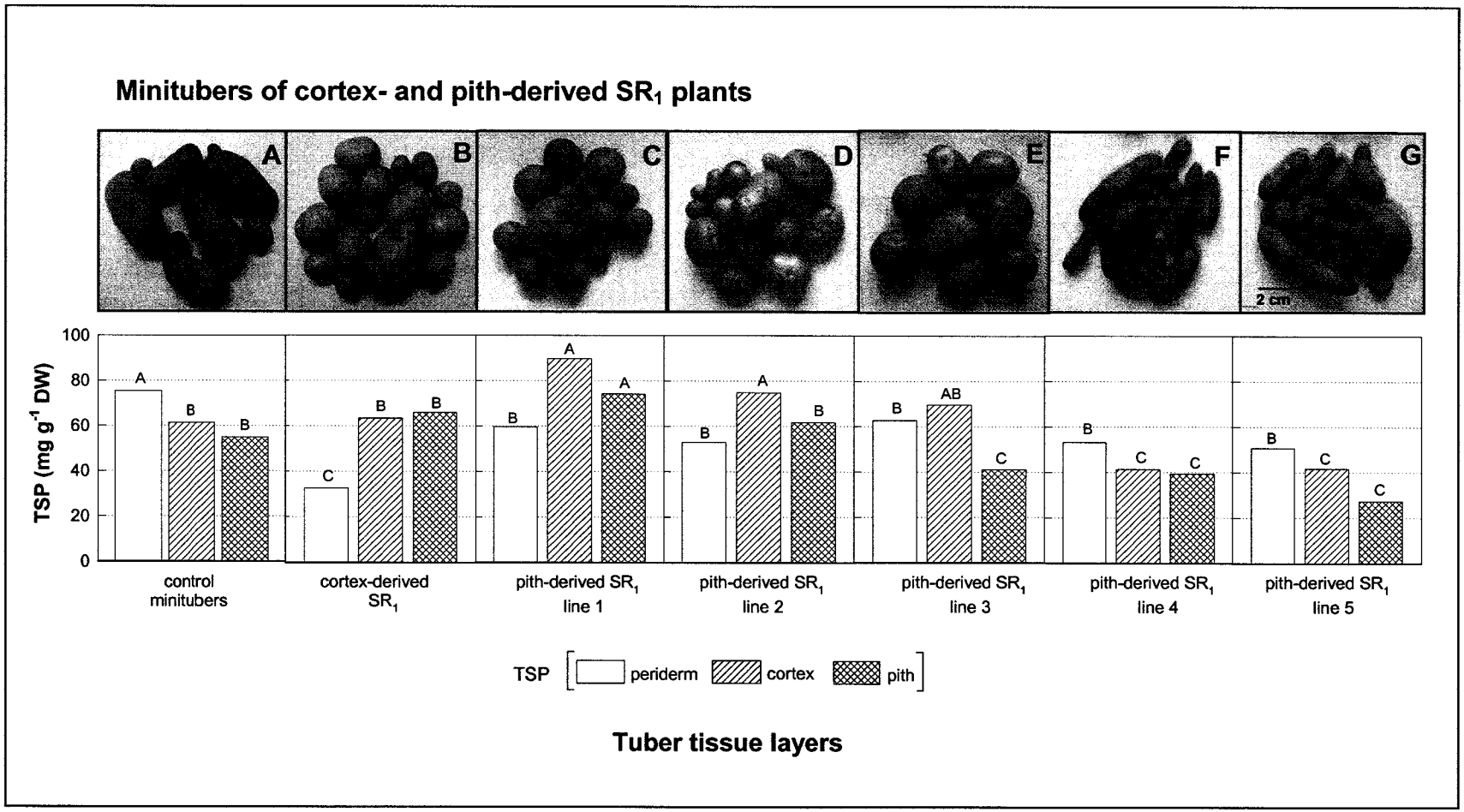
B. Burbank showing thin, non-russeted (smooth) white periderm and elongate-round shape.

Figure 7.2. Schematic representation of the hypothesis of this study. The field-grown source tuber of Russet Burbank is a classic example of a periclinal chimera with L1 russeted periderm. The putative periclinal chimeral TSP pattern is high, low, low (HLL) in the periderm, cortex and pith, respectively. Tissue-specific explants derived from the LI (periderm), LII (cortex) and LIII (pith) are expected to produce SR_1 plantlets with tubers that are non-chimeral. Periderm- explants will lead to SR_1 plants with russeted HHH tubers, while cortex and pith explants will lead to SR_1 plants with non-russeted LLL tubers. Bintje and Red Gold present the same TSP pattern as Russet Burbank (HLL), but not the russeting trait. Alpha has different TSP pattern, it was reported as LHH or similar LLL. Therefore, periderm explants will lead to SR_1 plants with LLL tubers, while cortex and pith explants will form SR_1 plants with HHH or LLL tubers, according with TSP of the source explant.



* The russeted characteristic corresponds only to 'Russet Burbank'.

Figure 7.3. Phenotypic variation (periderm texture and tuber shape) and total soluble protein (TSP) levels (mg g^{-1} DW) of minitubers from one (typical) cortex-derived SR_1 plant and five pith-derived SR_1 plants (lines 1-5) of Russet Burbank. Differences in TSP concentration for the three tissues layers between SR_1 and control minitubers are represented by letters (0.05 level of significance). **A.** Control minitubers: russeted, long; **B.** cortex-derived SR_1: non-russeted, round; **C.** pith-derived SR_1 line 1: russeted, round; **D, E.** pith-derived SR_1 line 2 and line 3: non-russeted, round; **F, G.** pith-derived SR_1 line 4 and line 5: russeted, long.


Minitubers of cortex- and pith-derived SR1 plants
A
B
C
D
E
F
G
2 cm
TSP (mg g-1 DW)
100
80
60
40
20
0
control minitubers
cortex-derived SR1
pith-derived SR1 line 1
pith-derived SR1 line 2
pith-derived SR1 line 3
pith-derived SR1 line 4
pith-derived SR1 line 5
TSP
periderm
cortex
pith
Tuber tissue layers

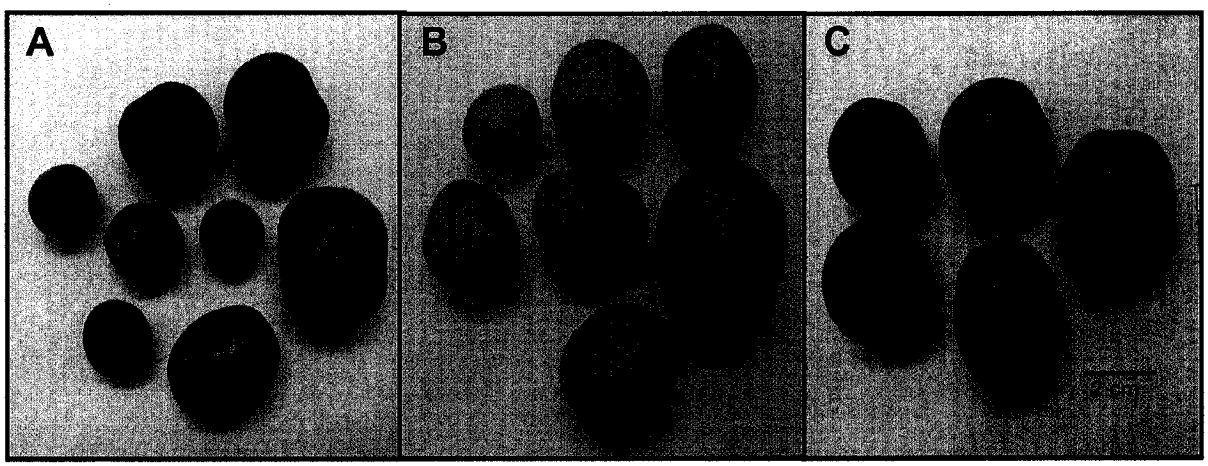


Figure 7.4. Phenotypic variation (periderm colour) of minitubers from cortex- and pith derived SR_1 plants of Red Gold. **A.** Control minitubers: pinkish-red colour, **B.** cortex-derived SR_1 minitubers: gold colour, **C.** pith-derived SR_1 minitubers: pinkish-red colour.

Chapter VIII

GENERAL SUMMARY AND CONCLUSIONS

As the world's fourth most important food crop, potato constitutes a valuable component of the human diet in many countries. It is an important dietary source of carbohydrate, protein, and vitamins (Woolfe, 1987; Juliano, 1999; Buckenhüskes, 2005). Many traits of potato have been improved over the last few years, mostly for pest and disease resistance. However, less attention has been given to improving characteristics with potential nutritional significance (Tarn, 2005). This thesis focused on tuber protein content as an important nutritional component for potential improvement.

This study was divided into two main sections. In the first part, total soluble proteins and patatin tissue distribution were examined in tubers of 20 potato cultivars. Tuber tissues with greater and lesser protein content were identified. In the second part, potato chimeral disassembly through somatic embryogenesis, from specific tuber tissue explants with defined protein levels, was evaluated as a strategy for production of nutritionally improved intraclones of cultivated potato. The major findings and contributions of this thesis are described in this section.

Chapter III and IV described the tissue-specific distribution of total soluble proteins (TSP) in 20 field-grown potato cultivars. TSP was determined in tuber periderm, cortex, and pith, at the time of tuber harvest (fresh) and after 6 months of storage. Protein extraction buffer for TSP determination differed in Chapters III and IV. However, a clear distribution pattern of TSP on a dry weight basis was observed; relatively greater concentration in periderm and lesser in cortex and pith tissues. In some cultivars, periderm TSP concentration was twice that of internal tissues, while TSP concentration of the cortex and pith tissues was similar.

From a nutritional point of view, important practical implications can be derived from this study. The common practice of peeling (removal of the outer layers of potato tubers) substantially decreases the nutritional composition of the food. Caustic peeling is used industrially, and gives losses in the range of 10-20% tuber weight (Huxsoll and Smith, 1975). Even careful domestic peeling can remove 10 to 25% of tuber weight (Burton, 1989) contributing to substantial protein waste.

Similarities in TSP distribution between fresh field-grown tubers and in vitro-grown tubers (microtubers) found in Chapter III, suggest that microtubers are a good model system for tuber protein research. This supports the use of microtuber systems as experimental research tools for different areas of plant metabolism (Coleman et al., 2001; Donnelly et al., 2003)

While Chapter III and part of Chapter IV underlined the tissue-specific distribution of TSP, Chapter IV focused on the tissue-specific distribution of patatin, the major tuber storage protein. To the best of our knowledge, this is the first report of patatin distribution in partitioned tubers (periderm, cortex, and pith) for different potato cultivars. The main finding of this chapter was that patatin concentration shows a consistent distribution pattern, but in the opposite direction to TSP. Patatin concentration was lesser in periderm and greater in cortex and pith tissues. This suggested that tissue-specific expression of patatin is highly regulated in potato tubers. It is clear from this chapter that TSP in periderm tissue is mainly composed of other proteins besides patatin. Identification and nutritional value assessment of those proteins is necessary. SDS-PAGE analysis helped to confirm that patatin protein is distributed in all tuber tissue layers including the periderm, in contrast to the Sonewald et al. (1989) study, where patatin was not found in periderm cells. Findings in this chapter were valuable in the identification of tissues with relatively greater and lesser protein concentration, which were selected as source explants for the tuber chimeral disassembly described in Chapter VII.

As Chapters III and IV reported TSP and patatin concentration measured on a specific tissue basis (mg g^{-1} DW), conversion factors were needed to transform these measurements to a uniform weight whole tuber basis for intercultivar comparisons. In this context, Chapter V described a mean of converting the specific tissue-based nutritional TSP and patatin information (DW) of Chapter IV into typical whole tuber information (FW).

Potato cultivars are characterized by distinctive tuber shape and size, and in consequence have differential internal tissue proportions. Therefore, in Chapter V percent weight proportions of each tuber tissue were determined for 20 cultivars. Weight estimations were based on the volume (calculated through an ellipsoid formula) and density of each component tissue. Percent weight values together with percent dry matter

content for each tissue provided conversion factor values that were used to estimate the TSP and patatin content in each tuber tissue and (by summation) in a typical whole tuber of 100 g FW for all 20 cultivars. Protein estimation obtained with this method facilitated intercultivar comparisons on a whole tuber basis, giving nutritional information more practical than the absolute concentration data of each tissue. Cultivars with greater or lesser TSP and patatin content for each tissue layer, and on a whole tuber basis, were clearly identified. This constitutes useful information for people interested in potato genotypes with enhanced nutritional value, especially for consumers and for potato processing industries (Keijbets, 2005; van Gijssel, 2005). In addition, protein estimation, based on weight tissue proportion, constitutes valuable knowledge as many protein studies were limited to internal tuber sections or whole peeled tubers, but reported on a whole tuber basis (Seibles, 1979; Désiré et al., 1995; Espen et al., 1999a). Based on the information generated in Chapter V, it was suggested that specific-tissue conversion values can be beneficial to estimate the content of other nutritional compounds that are unevenly distributed throughout the tuber tissues in these cultivars.

Chapter VI consisted of a review of the main factors that cause variation in clonally propagated plants derived through tissue culture systems. This chapter emphasized the impact of tissue culture-induced variation on the clonal integrity of cultivars. Intraclonal strains or geographic clones may arise even in crops with strict clonal germplasm certification programs, as seen in some strains of potato cultivars (Love et al., 1992; Leever et al., 1994; Coleman et al., 2003). As vegetatively-propagated clones accumulate mutations over time, probably all clonally-propagated cultivars are chimeral to some extent. However, by a better understanding of plant chimeral structure, the "unpredictable" nature of tissue culture-induced variation may be reduced.

Chapter VII evaluated periclinal chimeral theory through disassembly of chimeral (Russet Burbank) and putatively chimeral (Alpha, Bintje, Red Gold) tubers into their component genotypes. In this chapter chimeral disassembly was assessed as a strategy for production of improved intraclonal variants. Somatic embryogenesis from tissue-specific explants with relatively greater or lesser protein level was used to separate the chimeral tubers into their histogenic component layers (LI, LII, and LIII). Russeting trait was used as a phenotypic marker and TSP distribution pattern as a putative biochemical marker.

The expressed variability of phenotypic characteristics in tubers of non-chimeral plantlets, as a result of chimeral disassembly, was an important finding. Although Russet Burbank was confirmed to be a periclinal chimera, chimeral instability was evident, since some non-chimeral regenerants showed displacement of LI tunic cells with the russeting mutation into the pith (and possibly the cortex). Similar variation was previously observed in potato chimeras disassembled using the "eye-excision" method (Crane, 1936; Howard, 1959). The classic descriptions of periclinal chimerism are not sufficient to explain the variation in periderm texture or colour (characteristic of LI mutations) in non-chimeral regenerants from Russet Burbank pith tissue, which were expected to be homogeneous and conserved (Tilney-Basset, 1986).

The gold periderm, observed in tubers of regenerated plants from cortical explants of Red Gold, and lack of evidence of cell displacement between histogenic layers, suggested that cv. Red Gold is an LII periclinal chimera (RGR) inadvertently "uncovered" through the disassembly process. This cultivar is proposed as a good future model for the study of periclinal potato chimeras. As a model, Red Gold would be even better than Russet Burbank due to the possible chimeral instability of Russet Burbank.

Variation in protein content of non-chimeral SR_1 tubers was also observed. The inconsistent TSP distribution of the regenerants demonstrated that TSP pattern was not distributed in a periclinal chimeral manner, as was hypothesized. However, useful intraclones were selected with increased or decreased protein content in the whole tuber. It is not yet known whether these altered protein levels will remain stable.

In summary, this chapter contributes to knowledge of plant chimerism and its importance as a component of somaclonal variation. Chimeral disassembly through tissue-specific explantation followed by somatic embryogenesis can contribute to the production of intraclonal variants with improved features. Improvement to the protein content of intraclones is possible. These techniques, followed by in vitro screening and field-evaluation can contribute to the production of improved cultivated potato.

8.1. Suggestions for Future Research

1. Tuber protein content data (TSP and patatin) obtained in this study for 20 major cultivars has particular relevance for nutritionists and dietitians. A similar survey should be conducted on the balance of cultivars grown in Canada and all results summarized for a nutrition journal.

2. Microtubers of seven cultivars were shown to have TSP distributed in a similar way to field-grown tubers, but with significantly greater tissue levels. Preliminary studies examined medium nitrogen concentrations in relation to TSP tissue levels but were not conclusive (data not shown). Studies should be designed to explore the relationship between available nitrogen, other medium components, and tuber tissue TSP levels.

3. Information on patatin distribution in potato tubers was gained with this study. It is curious that its concentration was relatively low in the periderm compared with the cortex and pith tissues; a consistent feature in all cultivars. This information may have relevance and could be explored by those studying the role of patatin in plant defense.

4. A more extensive study is necessary to explore the instability or breakdown in periclinal chimeral structure observed in Russet Burbank. The extent of LI displacement and cell mixing into LIII- and possibly LII-derived tissues could be examined more efficiently using molecular marker(s) for the russeting trait to screen populations of SR_1 plantlets and tubers. Possible molecular techniques to distinguish periclinal genotypes could include RAPD, RFLP, AFLP, or SSR.

5. Red Gold, a hybrid seedling-derived cultivar, was found to be as an LII periclinal chimera, with no phenotypic evidence of cell displacement or replacement between histogenic layers. Red Gold is proposed as good model to explore periclinal chimeral separation and its relationship to somatic variation. Studies involving somatic embryogenesis from specific tissues of periderm, cortex, and pith (LI, LII, and LIII) should be repeated, and molecular markers identified for the two coloured phenotypes.

6. Disassembly of periclinal potato chimeras produced non-chimeral somaclonal variants with altered phenotype and distinctive protein characteristics. The stability of these intraclones should be evaluated in successive tuber generations in the field. Those with the greatest protein levels should be retained for further selection and testing.

Chapter IX

CONTRIBUTIONS TO KNOWLEDGE

The following contributions can be considered as original in this thesis:

1) Chapter III. Determination of TSP concentration in specific tissues (periderm, cortex, and pith) from fresh and stored tubers of 20 important cultivars generated new information about protein distribution in potato tubers. It was established that:
   a. TSP concentration (expressed as mg g^{-1} DW) was generally greater in the periderm and less in the cortex and pith tissues. This was more evident when performed with a better extraction buffer (Chapter IV).
   b. After 6 months of storage, TSP content was not consistently affected. However, the cultivar-specific TSP tissue distribution pattern was maintained.
   c. TSP distribution in microtuber tissues followed the same distribution pattern as in field-grown tubers but tissue concentrations were significantly greater. The reason for this was not determined. However, microtubers provide a useful model system for tuber protein studies.

2) Chapter IV. Patatin concentration was determined for the first time in specific tissues (periderm, cortex, and pith) from fresh and stored tubers of 20 important potato cultivars. It was determined that:
   a. Patatin was present in all tuber tissues including periderm, cortex, and pith as detected by ELISA and SDS-PAGE.
   b. Patatin showed a consistent tuber distribution pattern, but in the opposite direction to TSP. Patatin concentration (expressed as mg g^{-1} DW) was generally less in the periderm and greater in cortex and pith tissues.

3) Chapter V. A new method was developed for measuring the percent weight contribution of each specific potato tissue through calculations of its volume and density. The utility of this method was established.

a. Calculated % weight values together with % dry matter content for each tissue provided conversion factor values that were used to estimate the TSP and patatin content in each tuber tissue and (by summation) in a typical whole tuber of 100 g FW for 20 cultivars. These estimates facilitated intercultivar comparisons on a whole tuber basis, giving nutritional information more useful than the absolute concentration data for each tissue.

b. The calculated specific-tissue conversion factors can be use to estimate the content of other nutritional compounds that are unevenly distributed throughout the tuber tissues in these cultivars.

4) Chapter VI. On the basis of a review of the factors implicated in causing variation of clonally propagated plants derived through micropropagation systems, it is suggested that:

a. Under some circumstances, variation may occur in tissue culture-propagated plants, even in those that are propagated through axillary means.

b. As vegetatively-propagated clones accumulate mutations over time, it is probable that all clonally-propagated cultivars are chimeral to some extent.

c. Intraclonal variation may arise in some cases from the disassembly of chimeral plants into their component genotypes; this may be a major unrecognized contributer to somaclonal variation.

5) Chapter VII. This is the first report of disassembly of periclinal (and putatively periclinal) potato chimeras through somatic embryogenesis.

a. There was no consistent evidence that TSP was distributed in a periclinal chimeral way.

b. Russet Burbank was confirmed to be a periclinal chimera, although chimeral instability was evident, since some non-chimeral regenerants showed displacement of LI tunic cells with the russeting mutation into the pith (and possibly the cortex).

c. Red Gold, a hybrid seedling-derived cultivar, was "uncovered" as an LII periclinal chimera (Red-Gold-Red). This cultivar is proposed as a good model for the study

of periclinal potato chimeras. Cv. Red Gold illustrates very clearly the contribution that chimeral disassembly can make towards a better understanding of somatic variation.

d. R_1 plants from disassembled cv. Russet Burbank produced potentially valuable somaclonal variants with altered phenotype and unique protein characteristics.

e. Screening of tissue-specific intraclonal variants may have potential advantages in nutritional and other improvements to cultivated potato.

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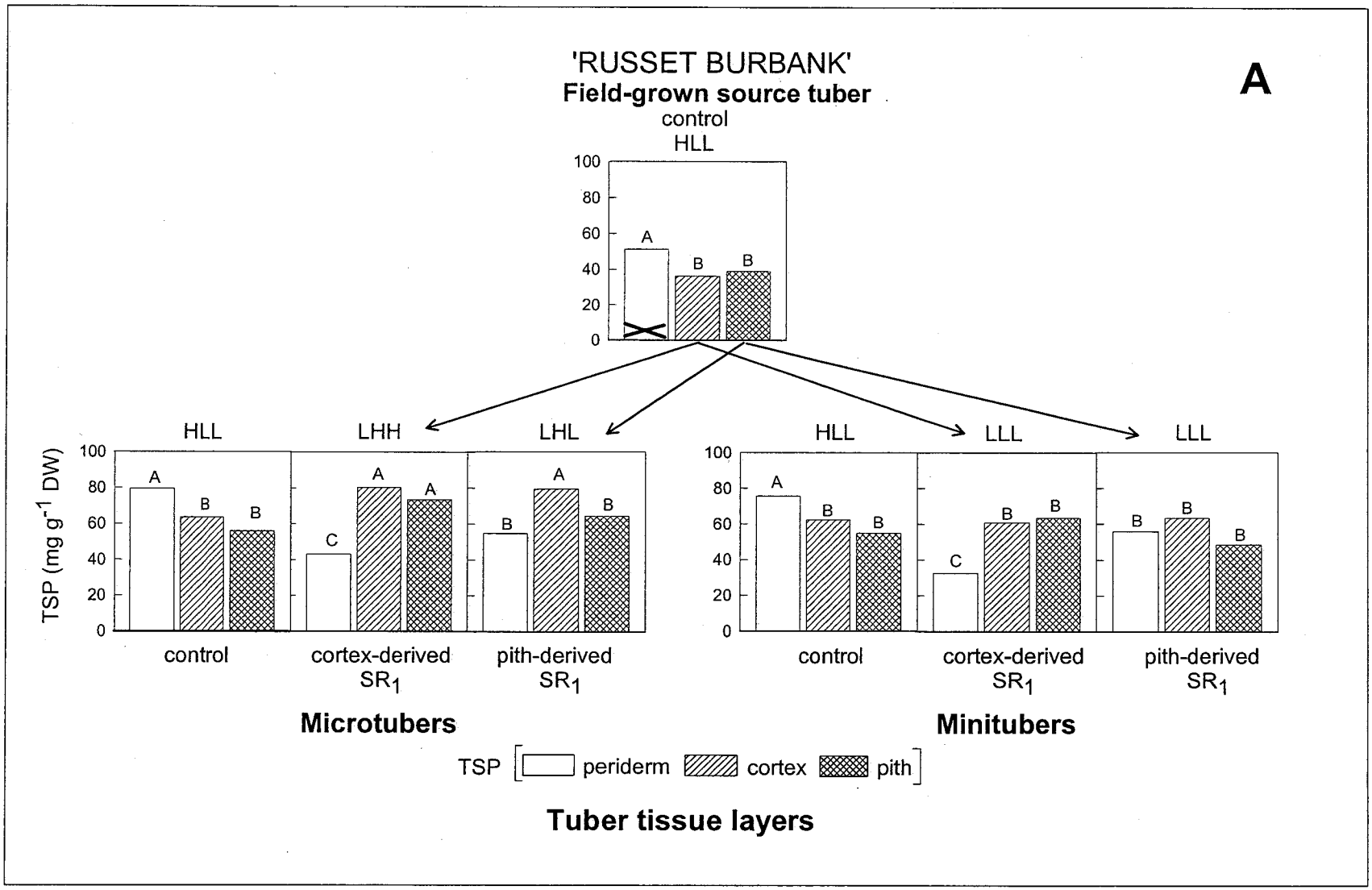
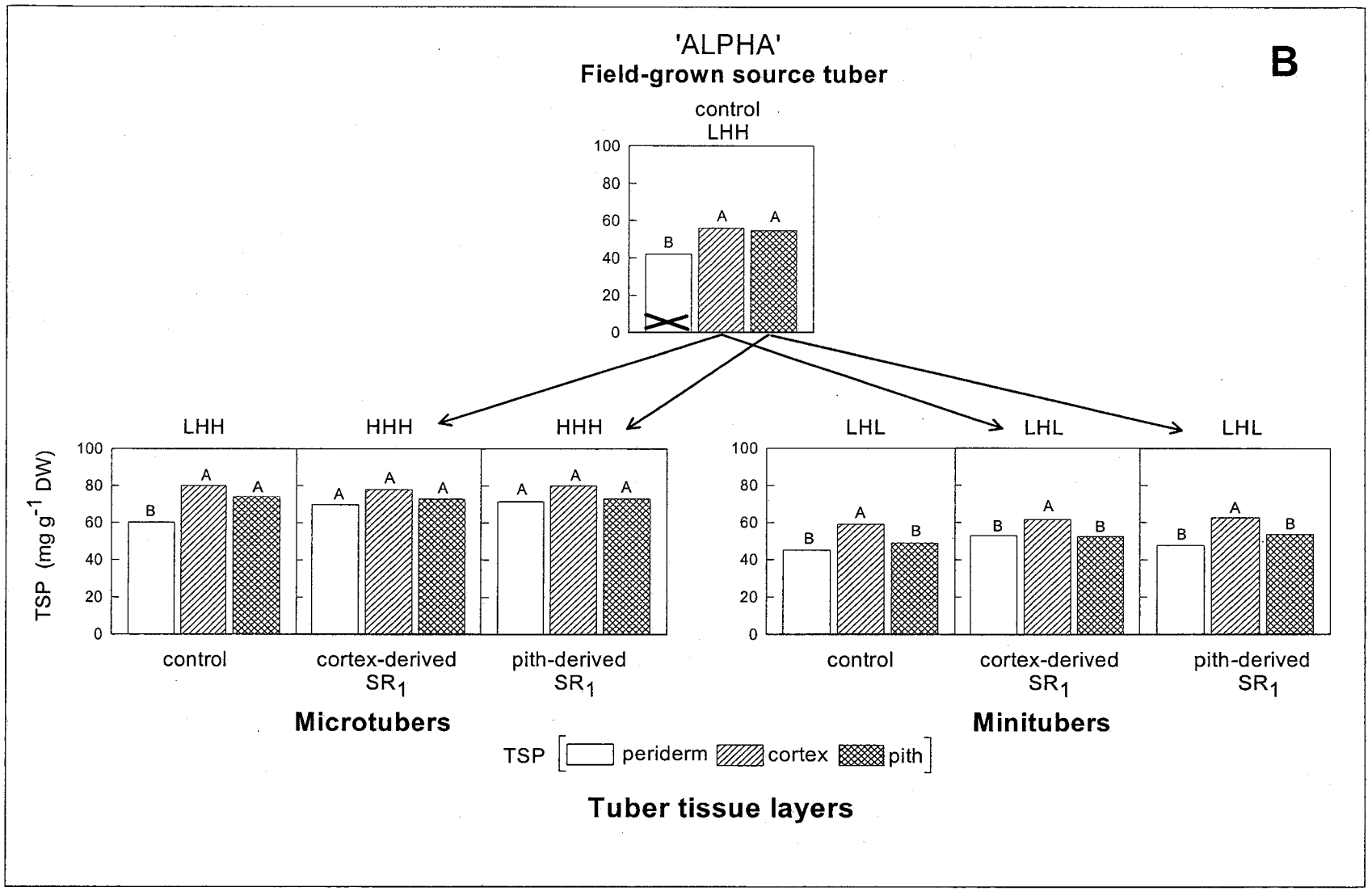
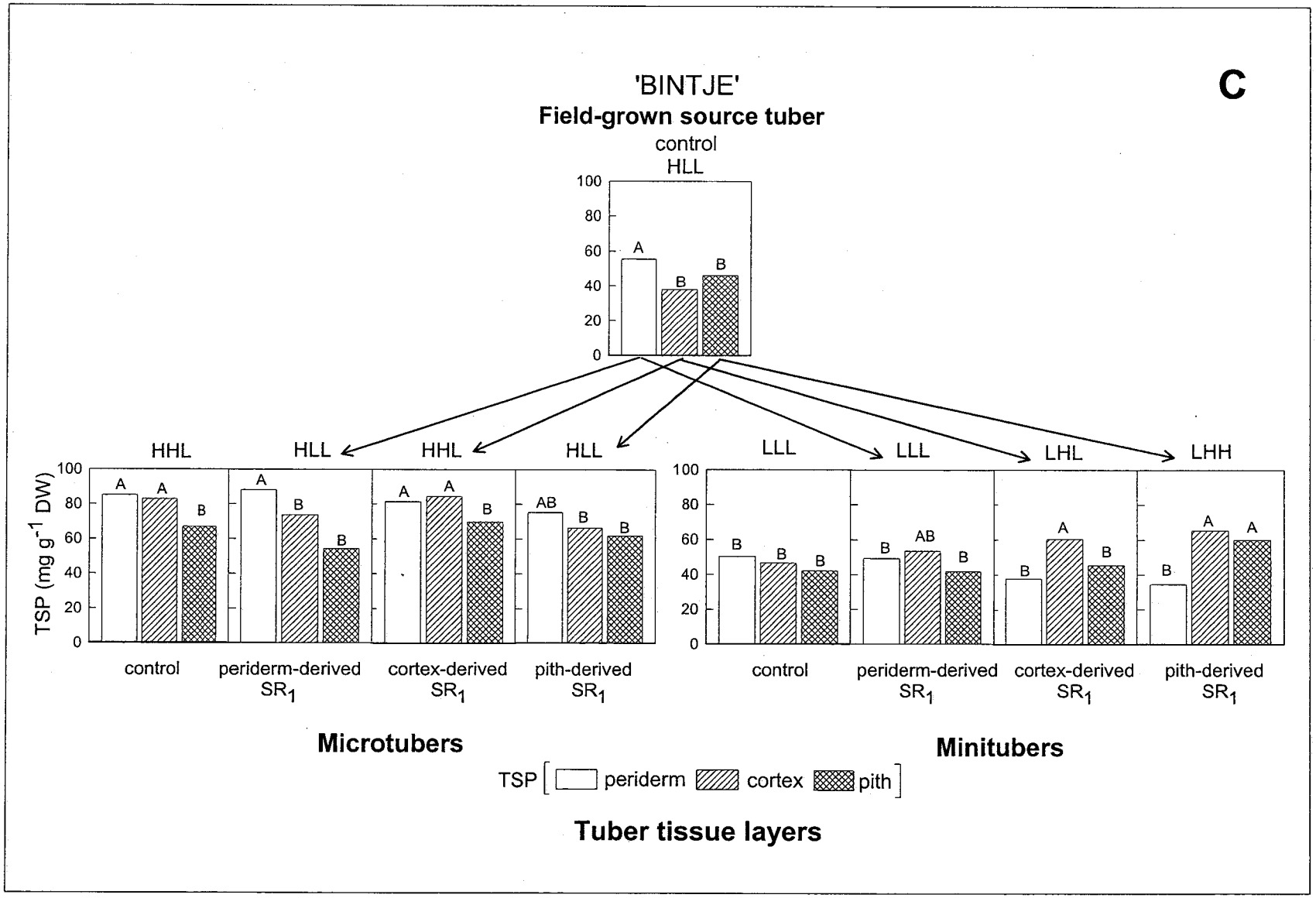
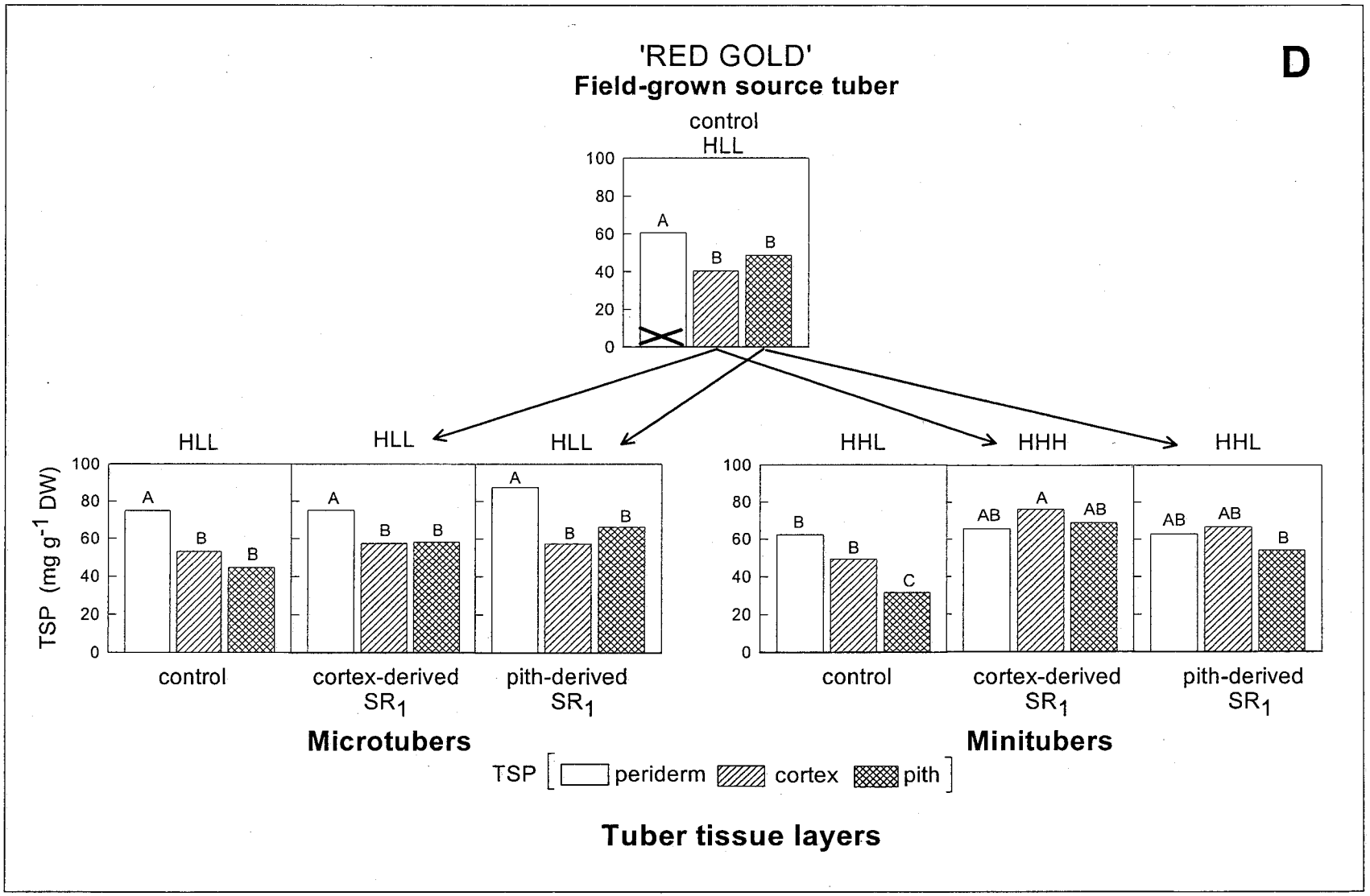
Figure 7.5. Total soluble protein (TSP) (mg g^{-1} DW) in three tissue layers (periderm, cortex and pith) qualitatively rated as H or L from cortex- and pith-derived microtubers and minitubers from somatic regenerant (SR_1, first generation) plants of Russet Burbank, Alpha, Bintje, and Red Gold. The relative TSP concentration patterns, for periderm, cortex and pith are shown for controls (field-grown, microtubers, minitubers) and SR_1 plant microtubers and minitubers. Differences in TSP concentration for the three tissues layers between SR_1 and control microtubers or minitubers are represented by letters (0.05 level of significance). **A.** Russet Burbank, **B.** Alpha, **C.** Bintje, **D.** Red Gold.


'RUSSET BURBANK'
Field-grown source tuber
A
control
HLL
100
80
60
40
20
0
A
B
B
HLL
LHH
LHL
HLL
LLL
LLL
TSP (mg g-1 DW)
control
cortex-derived SR1
pith-derived SR1
control
cortex-derived SR1
pith-derived SR1
Microtubers
Minitubers
TSP
periderm
cortex
pith
Tuber tissue layers


'ALPHA'
Field-grown source tuber
B
control
LHH
100
80
60
40
20
0
B
A
A
LHH
HHH
HHH
LHL
LHL
LHL
TSP (mg g-1 DW)
control
cortex-derived SR1
pith-derived SR1
Microtubers
Minitubers
TSP
periderm
cortex
pith
Tuber tissue layers


'BINTJE'
C
Field-grown source tuber
control
HLL
100
80
60
40
20
0
A
B
B
HHL
HLL
HHL
HLL
LLL
LLL
LHL
LHH
TSP (mg g-1 DW)
control
periderm-derived SR1
cortex-derived SR1
pith-derived SR1
Microtubers
Minitubers
TSP periderm cortex pith
Tuber tissue layers


'RED GOLD'
Field-grown source tuber
D
control
HLL
100
80
60
40
20
0
A
B
B
HLL
HLL
HLL
HHL
HHH
HHL
TSP (mg g-1 DW)
A
B
B
A
B
B
A
B
B
B
A
B
C
AB
A
AB
AB
AB
B
control
cortex-derived SR1
pith-derived SR1
Microtubers
Minitubers
TSP
periderm
cortex
pith
Tuber tissue layers

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