

STUDIES OF ANDROGENIC PROCESSES IN DIPLOID POTATO

by

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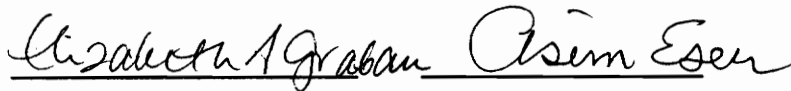
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Horticulture

APPROVED



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(ABSTRACT)

Androgenic processes in diploid potato ($2n=2x=24$) were studied in three interspecific hybrids. Five incubation temperature treatments were examined. Temperature shock (35°C) during the first 12 h of anther culture and elevated incubation temperature (30°C 16h/ 20°C 8h) (hereafter 35°C - 30°C / 20°C) enhanced androgenic embryo production. Variation among experiment dates was highly significant. Temperature treatment (35°C - 30°C / 20°C) during anther culture did not influence the subsequent conversion rate of androgenic embryos, thus providing a simple and effective way to enhance androgenic embryo yield.

Repeated experiments were conducted to study extended anther culture by replacing anthers into medium following the usual harvest 6 weeks after culture initiation. Embryos continued to be produced after the first harvest. Embryo yield at the first harvest was significantly correlated with that at the second harvest ($P < 0.01$). Significantly more embryos were produced when anthers were put back into the same medium compared to fresh medium in extended anther culture. Although relatively high embryo yield was produced in extended anther culture, high contamination and low regeneration rate eliminate any practical use unless a better regeneration protocol is

developed.

Randomly amplified polymorphic DNA (RAPD) techniques were applied to analyze the genetic composition of anther-derived plants, whose ploidy level was pre-determined by flow cytometry. The RAPD fragments amplified from various anther-derived diploid plants ($2n=2x=24$) were compared with those from anther donor ($2n=2x=24$) and anther-derived monoploids ($2n=1x=12$). Anther donor and anther-derived monoploids were distinguished by scoring segregating bands as well as total number of scorable bands that exhibited polymorphism. Thus RAPD has the potential to separate homozygous from heterozygous diploids, since the frequency of RAPDs present in homozygous diploids is expected to be the same as in a group of known monoploids, whereas heterozygous ones will be similar to the anther donor.

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TABLE OF CONTENTS

ABSTRACT.	ii
ACKNOWLEDGEMENTS.	iv
LIST OF TABLES	viii
LIST OF FIGURES	xii
CHAPTER 1. INTRODUCTION	1
Use of Anther Culture in Potato Breeding	2
Molecular Biology As a Tool in Potato Breeding	4
Gamete Selection As a Tool in Breeding Programs	5
Objectives	10
References	11
CHAPTER 2. EFFECT OF TEMPERATURE SHOCK AND ELEVATED INCUBATION TEMPERATURE ON ANDROGENIC EMBRYO YIELD IN DIPLOID POTATO	16
Abstract	16
Introduction	17
Materials and Methods	19
Results	23

Discussion28
References33
Tables36
CHAPTER 3. STUDY OF EXTENDED ANTHOR CULTURE41
Abstract41
Introduction42
Materials and Methods43
Results47
Discussion51
References54
Tables and Figures56
CHAPTER 4. ANALYSIS OF THE GENETIC COMPOSITION OF ANTHOR-DERIVED PLANTS BY RANDOM AMPLIFIED POLYMORPHIC DNA65
Abstract65
Introduction66
Materials and Methods68
Results and Discussion71
References75
Tables and Figures77

APPENDIX. GAMETE SELECTION FOR HEAT TOLERANCE IN DIPLOID
POTATO85

 Abstract85

 Introduction86

 Materials and Methods88

 Results91

 Discussion95

 References98

 Tables101

VITA106

LIST OF TABLES

CHAPTER 2

- Table 1. Temperature treatments applied during anther culture of diploid potato. . . 36
- Table 2. Combined analysis of variance of two clones (APB3 and APM1) for embryo yield in anther cultures under five different temperature treatments cultured on three days.37
- Table 3. Mean embryos per flask (30 anthers/flask) of two diploid potato clones APB3 and APM1, cultured on three days, under five temperature treatments. (Means for treatment and date are combined for the two clones.)37
- Table 4. Analysis of variance for embryo yield at the first harvest in anther cultures of potato clones APB3 and APC3 under three different temperature treatments. Each clone was cultured on three separate days. 38
- Table 5. Mean embryos per flask (30 anthers/flask) at the first harvest for anther cultures of APB3 and APC3 conducted under three temperature treatments.38
- Table 6. Mean embryos per flask (30 anthers/flask) of two diploid potato clones (APB3 and APM1) cultured under five temperature treatments on three days in experiment 1. (Means are presented separately for each clone.) . . . 39

Table 7. Mean embryos per flask (30 anthers/flask) of two diploid potato clones (APB3 and APC3) cultured under three temperature treatments on three days in experiment 2. (Means are presented separately for each clone.) . . .	39
Table 8. Frequency of converted androgenic embryos harvested from various anther culture treatments and ploidy analysis of regenerated plants.	40

CHAPTER 3

Table 1. Analysis of variance for embryo yield at the first and second harvests. . .	56
Table 2. Mean embryos per flask in anther culture of diploid potato. Each experiment was conducted on two genotypes, on three or six different dates, and embryos were harvested twice.	57
Table 3. Regression analysis revealing a positive relationship between the embryo yield of the first and second harvests.	58
Table 4. Analysis of variance for androgenic embryo yield of APB3 and APC3 on extended anther culture using old or fresh medium.	59
Table 5. Medium effect on extended anther culture of APB3 and APC3.	59
Table 6. Comparison of contamination occurring in the first six weeks culture and extended anther culture; first harvest and second harvest embryo culture . .	60
Table 7. Comparison of regeneration potential of embryos from first and second harvests.	61
Table 8. Ploidy analysis of regenerated plants from the first and second harvests .	62

CHAPTER 4

Table 1. The sequences (5'-3') of the oligonucleotide primers used in RAPD analysis	77
Table 2. Segregating RAPD bands amplified from a set of primers in anther-derived plants of CP2 family.	78
Table 3. Segregating RAPD bands amplified from a set of primers in anther-derived plants of APB3 family.	79
Table 4. Total number of scorable RAPD bands that exhibited polymorphism amplified from a set of primers in anther-derived plants of CP2 family.	80
Table 5. Total number of scorable RAPD bands that exhibited polymorphism amplified from a set of primers in anther-derived plants of APB3 family	81

APPENDIX

Table 1. The effect of temperature on pollen viability (%).	101
Table 2. Number of pollinations, fruit set and seed set for four different crosses using either heat-treated or fresh pollen of 2 pollinators on 2 stylar parents. ...	102
Table 3. The seed pairs that were sent to Israel.	103
Table 4. Temperature treatments applied on anther culture selection.	104

Table 5. Plants regenerated from anther culture selection under various temperature treatments. 104

Table 6. Agronomic performance of selected (S) and non-selected (NS) progenies from 3 paired crosses resulting from pollen selection. 105

LIST OF FIGURES

CHAPTER 3

- Fig. 1. Embryonic mass occasionally obtained from extended anther culture of diploid potato clone APB3 after 12 weeks of culture. 63
- Fig. 2. Frequency of regenerated plants after serial subculture. The embryos were harvested either 6 weeks after anthers were placed in culture (1st harvest) or from extended anther culture (2nd harvest). They were subcultured at 3 week intervals. S1, S2, ..., S5 represented 3, 6, ..., 15 weeks after embryos were cultured on regeneration medium. 64
- Fig. 3. Number of regenerated plants after serial subculture. The embryos were harvested either 6 weeks after anthers were placed in culture (1st harvest) or from extended anther culture (2nd harvest). They were subcultured at 3 week intervals. S1, S2, ..., S5 represented 3, 6, ..., 15 weeks after embryos were cultured on regeneration medium. 64

CHAPTER 4

- Fig. 1. Examples of RAPD reactions that produced different band patterns. 82
- Fig. 2. Examples of RAPD polymorphism detected in CP2 family. 83
- Fig. 3. Examples of RAPD polymorphism detected in APB3 family 84

CHAPTER 1: INTRODUCTION

Cultivated potato is an autotetraploid (*Solanum tuberosum* ssp. *tuberosum*) belonging to the family *Solanaceae* of South American origin. Following its introduction into Europe in the 16th century, the crop has now become one of the world's major food crops. Potato is superior to all other crops in protein production per unit area and time, and to most others in terms of energy production (Ross 1986).

The objectives of potato breeders are to improve yield and resistance to diseases, pests, and environmental factors. Conventional breeding involves making crosses among different parental lines, selecting desirable progeny, and evaluating the advanced breeding lines. Because potato is vegetatively propagated through tubers, any desirable genotypes can be maintained, multiplied and released as new varieties. On the other hand, the tetraploid level and high heterozygosity of the crop make selection and evaluation more difficult.

Current research in potato breeding explores unconventional methods to complement traditional approaches. Anther culture techniques, for instance, have been used to produce dihaploids and monohaploids as a way of constructing tetra-allelic genotypes systematically. Recent developments in molecular biology add a potentially important tool to breeding programs. Gamete selection may provide an alternative approach to potato breeding.

Use of Anther Culture in Potato Breeding

Immature pollen grains can be stimulated into an embryogenic pathway through anther culture. Flower buds with microspores at the uninucleate stage are sterilized, the anthers are aseptically removed and placed in liquid anther culture medium (Uhrig 1985). After 4 to 6 weeks, embryos may be produced and subsequently regenerated into whole plants. This procedure provides a rapid technique for producing homozygous breeding lines. Because of the high degree of heterozygosity in potato cultivars, selection at tetraploid level is extremely difficult in the presence of dominance, segregation, recombination, epistasis, etc. Because monoploids have only one set of chromosomes, they are free of all lethal genes due to the effects of the "monoploid sieve" (Wenzel et al. 1979). Such monoploids can be doubled to produce homozygous diploids, which may then be intercrossed with other diploid species. They could also be crossed with tetraploids via sexual polyploidization (Veilleux 1985), thus allowing advances made by breeding at the diploid level to be introgressed into *S. tuberosum*. Monoploids could also be combined to produce somatic hybrids via protoplast fusion. In addition to their breeding application, doubled haploids are also useful in genetic mapping and studies of quantitative traits (Snape 1988; Choo 1981; Singsit and Ozias-Akins 1993).

One of the problems in anther culture is the low yield of embryos. Anther culture response of potato varies among genotypes (Wenzel and Uhrig 1981; Singsit and Veilleux 1989). Incorporation of genes controlling high responsiveness to anther culture into non or low-responsive genotypes can enhance androgenesis (Singsit and Veilleux 1989).

However, it requires many years to accomplish. Therefore, studies on further improvement of the cultural procedures for potato anther culture are needed.

Other factors, such as the microspore development stage, the composition of the medium, and the culture temperature also influence androgenesis (Johansson 1986; Sopory 1978; Uhrig 1985; Calleberg and Johansson 1993). Most research has been focused on medium composition, growth regulators, and developmental stage of microspores. Cool temperature treatment of buds before anther culture has been reported to increase androgenic embryo yield. However, the environmental conditions during anther culture have not received much attention. In other crops, studies have shown that high temperatures may have a beneficial effect on embryo yield (Feng and Wolyn 1991) and subsequent embryo conversion (Afele et al. 1992).

Another problem associated with anther culture is high variation for responsiveness among replicate cultures (Snider and Veilleux 1994). Different embryo yields have been obtained under identical culture conditions, with variation among replicates estimated to contribute 6.94% of total variation (Powell & Uhrig 1987). Buds harvested at different seasons, days, or even at different times of the same day may give different responses. It is generally believed that the developmental stage of microspores, the physical condition of donor plants, even the environmental conditions of donor plant before bud excision all contribute to the variation in responsiveness.

Even under the same culture conditions, embryo yield varies from flask to flask with buds that were excised at the same time. Anthers are usually discarded when embryos are harvested. Whether poor responsiveness of anthers is due to inability to

produce embryos or to the requirement for a longer duration in anther culture remains to be studied.

Molecular Biology As a Tool in Potato Breeding

As molecular biology studies advance, more and more potato breeders are exploring these tools in breeding programs (Flavell 1987). Potato is one of the few major crop species that is infected by *Agrobacterium tumefaciens*, which provides a way to transfer foreign genes into potato. Plants can also be regenerated from single cells that have received new genes. Knapp et al. (1988) transformed the *Ac* element from maize into the potato genome using leaf disc transformation mediated by *A. tumefaciens*. This may allow the cloning of interesting genes by transposon tagging.

Molecular breeding techniques can have an advantage over conventional breeding in that they allow the addition of specific genes encoding desirable traits to a cultivar or advanced breeding line, while preserving its intrinsic features (Huisman et al. 1992). By genetic engineering of potato virus X (PVX), potato virus Y (PVY), and potato leafroll virus (PLRV) coat protein genes, transgenic potato plants resistant to these viruses have been obtained (Huisman et al. 1992).

Various techniques have been used to facilitate selection in breeding programs. Marker based selection is a powerful tool to speed breeding programs. Another potential application of molecular techniques is to analyze the genetic composition of anther-derived plants (Rivard et al. 1989).

The anther culture process results in the regeneration of plants varying in ploidy (Bajaj and Sopory 1986). Regenerants from a diploid donor parent may be monoploid, diploid, tetraploid or mixoploid. The diploids may arise from unreduced microspores or somatic tissue of anther, or by chromosome doubling of monoploids during the culture phase. Those produced by chromosome doubling are expected to be homozygous whereas those produced by embryogenesis of $2n$ microspores or somatic tissue of anthers would be heterozygous. If a heterozygous diploid originated from somatic tissue of an anther, it will be genetically identical to the anther donor plant. On the other hand, if it originated from a $2n$ (unreduced) microspore, the mechanism of $2n$ pollen formation will determine the level of heterozygosity. Microspores derived by FDR (first division restitution) are more heterozygous than those derived by SDR (second division restitution) (Veilleux 1985). The source of tetraploids is even more ambiguous. In order to use spontaneously doubled monoploids in breeding programs, homozygous diploid regenerants must be separated from heterozygous ones.

Gamete selection as a tool in breeding programs

Plant breeding has generally been limited to selection during the sporophytic phase, during which we can observe the phenotypes in angiosperms. In the 1970s, gametophytic selection became a plausible supplemental method of plant breeding. Gamete selection is theoretically more efficient, allowing millions of genotypes to be screened in a short time. In addition, it provides a homogeneous and controlled

environment which greatly simplifies selection procedures compared to those used with plants grown in an open field. Gametic selection is considered as a complementary tool to field techniques (Landi et al. 1989).

The basis of gametophytic selection is that selection of gametophytes for traits such as pathogen or environmental tolerance is positively correlated with expression of the same traits in the following sporophytic generation (Mulcahy 1979; Ottaviano et al. 1988, 1990). Such correlation is evident from the overlapping relationships in gene expression between the diploid and haploid phases, and in behavior of the haploid and diploid phases in response to different external agents.

An overlapping relationship in genetic expression between the sporophyte and gametophyte has been found in isozymes and messenger RNAs. Tanksley et al. (1981) studied nine isozymes in tomato (*Lycopersicon esculentum*), and found that 58% of the isozymes were expressed in both phases. The overlap rates seem to be similar in other enzymatic groups and different plant species. Based on the hybridization between mRNAs synthesized by the gametophyte and cDNAs from the sporophyte in *Tradescantia paludosa*, Willing and Mascarenhas (1984) reported that about 60% of the sequences analyzed were expressed in both gametophytic and sporophytic tissues.

Another type of overlap exists in the response to different environmental stresses between the sporophyte and the gametophyte. There are numerous examples of positive correlation between both phases to temperature, salinity and osmotic pressure, metal tolerance, herbicides and other toxic compounds (Hormaza & Herrero 1992).

Gametic selection may be achieved based on either the variation due to meiotic recombination in a heterozygous anther donor (Veilleux 1985) or gametoclonal variation induced by the anther culture system (Evans et al. 1984). Most gametic selection has involved pollen selection while only a few successful examples involve selection via *in vitro* culture systems.

In vivo gamete selection via cross pollination

Such gamete selection includes both pollination and fertilization conducted under stressful conditions or pollination with pollen that had been pre-incubated under a stressful condition. The progeny from such crosses can then be more resistant to the stress.

There are many successful examples of pollen selection for resistance to environmental stresses including salinity (Sari-Gorla et al. 1988), metals (Searcy & Mulcahy 1985), herbicides (Gorla et al. 1992; Sari-Gorla et al. 1989), disease resistance, and other toxic compounds (Mercuri et al. 1992). For example, by conducting crosses in rapeseed with pollen that had been incubated in toxic compounds, resistance to these toxic compounds from *Alternaria brassicicola* was enhanced in pollen derived from such progenies (Hodgkin 1990). Likewise, Shivanna and Sawhney (1993) found that the responses of pollen grains as well as leaves of various *Brassica* species to the toxin of *A. brassicae* were similar to the degree of susceptibility/resistance of these species reported in the literature, indicating that the genes imparting susceptibility/ resistance are

expressed in the pollen. Therefore, pollen selection offers a simple and effective method for application of selection pressure to eliminate pollen grains susceptible to the toxin. In addition, pollen selection has been used in selection for high photosynthetic efficiency (Medrano & Primo-Millo 1985) and earliness (Pollacsek and Caenen 1979).

Pollen selection has also been used in high (Petolino et al. 1990; Rodriguez-Garay and Barrow 1988) and low temperature adaption (Zamir and Gadish 1987; Maisonneuve et al. 1986; Jones 1982). For example, Zamir et al. (1982) reported preferential transmission of cold tolerant *Lycopersicon hirsutum* alleles in backcrosses of *L. esculentum* \times (*L. esculentum* \times *L. hirsutum*) under low temperature and concluded that, as a result of gametophytic gene expression, pollen grains carrying alleles from the cold tolerant *L. hirsutum* parent were more successful in fertilization. On the other hand, Petolino et al. (1990) demonstrated that exposing maize gametes to high temperature (38°C) during pollination and fertilization resulted in progenies more tolerant to heat. Similarly, in a study of pollen selection for heat tolerance in cotton, Rodriguez-Garay et al. (1988) found that pollen from heat tolerant cultivars in the field generally expressed higher fertility after heat treatment than pollen from heat sensitive cultivars. Pollen was heat treated to eliminate all but those grains with genetic heat tolerance. An increase of fertile pollen after heat treatment in the following generation indicated that genes for heat tolerance were selected in the pollen and effectively transferred by hybridization.

In vitro gamete selection via anther culture

Anther culture diverts the normal development of the male gametophyte to a sporophytic pathway resulting in callus or embryo formation. Anther culture can allow the use of both *in vitro* induced variability and the natural variability following meiotic recombination. Genetic variability resulting from recombination and segregation appears to be the predominant source of variation in anther culture. The amount of variability is determined by the level of heterozygosity of the donor plant. F₁ hybrids that combine desirable characters via interspecific crosses could be an attractive source of heterogeneous gametophytic cell populations (Lashermes 1991). Additionally, haploid embryos express both dominant and recessive traits during the selection process; therefore, they are free of lethal alleles due to the "monoploid sieve" (Wenzel et al. 1979). The embryo as a selection unit may be more likely to respond to selection pressure in a manner similar to a whole plant in contrast to cells in suspension or callus culture. Moreover, in association with doubled haploid breeding methodology in self-pollinated crops, rapid development of new cultivars is possible (Lashermes 1991).

Few attempts have been made to select resistance to environmental stress using microspore culture systems. Ultraviolet (UV) irradiation of cultured microspores of *Brassica napus* resulted in heritable resistance to the pathogen, *A. brassicicola*, and the herbicide, chlorsulfuron, in progenies (Ahmad et al. 1991). Also, in *Brassica napus*, rapid and clear separation of herbicide (chlorsulfuron) tolerant genotypes has been reported from segregating microspore populations isolated from hybrid plants (Swanson et al.

1988). In barley, screening for salt tolerant genotypes was achieved via anther culture of F₁ in salt stress media induced by a high concentration of Na₂SO₄ (Ya et al. 1987). Using microspore culture of oilseed rape, Kenyon et al. (1987) obtained sulfonyleurea herbicide tolerant plants. Similarly, Fadel and Wenzel (1993) obtained *Fusarium* tolerant plants in F₁ microspore populations of wheat. On the other hand, instead of selection via microspore culture, Zelitch (1989) used dihaploid plantlets regenerated from anther culture to select O₂-resistant plants.

Objectives

The overall objectives of the present study were focused on the androgenetic process in complex potato hybrids for better understanding its potential uses in potato breeding programs. Improvement in anther culture techniques as well as effectiveness of gamete selection were studied. Additionally, randomly amplified polymorphic DNA (RAPD) techniques were applied to analyze the genetic make-up of anther-derived plants. Three interspecific diploid hybrids, APB3, APM1 and APC3, were used. They were produced between dihaploid *S. andigena* as female, and one of F₁ (*S. phureja* x *S. berthaultii*), F₁ (*S. phureja* x *S. microdontum*), and F₁ (*S. phureja* x *S. chacoense*) as male, respectively. These clones were used because of their anther culture competence and high 2n pollen frequencies, which facilitates use of anther techniques and their hybridization with *S. tuberosum* cultivars. Additionally, *S. phureja*, *S. microdontum*, and *S. chacoense* accessions used to develop these complex hybrids had been previously selected for heat

tolerance (Reynolds and Ewing 1989; Simmonds 1971). Intercrossing these diploid clones with tetraploid cultivars via production of 2n gametes could add heat tolerant genes into current cultivars to develop heat tolerant breeding lines. The specific objectives include:

- (1) to examine the effect of the incubation temperature during anther culture on androgenic embryo production and subsequent plant regeneration in diploid potato;
- (2) To study the cause of flask to flask variation in a normally responsive clone; to determine whether poor responsiveness of some anthers is due to a lack of embryo initiation or a delay in embryo production beyond the time of normal harvest;
- (3) To analyze the genetic composition of anther-derived plants by RAPD markers in order to use anther-derived plants effectively in breeding programs;
- (4) To assess the effectiveness of gamete selection via both pollen selection and anther culture selection in breeding for heat tolerance.

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CHAPTER 2: EFFECT OF TEMPERATURE SHOCK AND ELEVATED INCUBATION TEMPERATURE ON ANDROGENIC EMBRYO YIELD OF DIPLOID POTATO

Key words: incubation temperature, anther culture, extended anther culture, diploid potato

Abstract: Using three diploid *Solanum* clones as anther donors, experiments demonstrated that temperature shock during the first 12 h after anthers were placed in medium may increase androgenic embryo production. These clones were: APB3 [*S. andigena* x (*S. phureja* x *S. berthaultii*)], APM1 [*S. andigena* x (*S. phureja* x *S. microdontum*)] and APC3 [*S. andigena* x (*S. phureja* x *S. chacoense*)]. Five incubation temperature treatments were tested on APB3 and APM1, and three treatments were repeated on APB3 and APC3. Using APB3 and APM1 as anther donors, temperature treatment, genotype, dates of culture initiation, and their interactions were all significant sources of variation. A treatment combining a high temperature shock (35°C for 12h) with elevated incubation temperature (30°C/20°C) (hereafter 35°C 12h-30°C/20°C) yielded 11 times as many embryos (44 per flask) as the control 20°C (4 per flask). APB3 yielded significantly more embryos than APM1 (26 vs. 7 per flask). Variation among experiment dates was highly significant; thus using date as a blocking factor can increase the sensitivity of the test. When embryos were harvested after 6 weeks in culture, the anthers were put back to medium and cultured for another 6 weeks. The beneficial effect of temperature shock (35°C 12h) during first 6 weeks of culture continued on the extended

culture, although it was less than that on the first embryo harvest. Anthers cultured at 30°C/20°C produced more embryos (23 per flask) than the control (13 per flask) while the 35°C 12h-30°C/20°C treatment produced an intermediate number (18 per flask). The embryo yield for control at the second harvest was increased compared with that at the first harvest (13 vs. 4 per flask). Temperature shock (35°C 12h) during anther culture did not appear to influence the subsequent conversion rate of androgenic embryos.

Introduction

Temperature shocks, high or low, are believed to improve androgenesis by diverting normal gametophytic development onto a sporophytic pathway, thus leading to the formation of haploid embryo-like structures (Nitsch et al. 1982). A cold treatment of buds at 4°C and 10°C before plating has routinely been used for many species. In addition to this pre-treatment, postplating cold treatment (i.e., cold treatment of detached anthers already in culture) was found to increase embryo yield in maize (Nitsch et al. 1982; Pescitelli et al. 1990), and tobacco (Duncan & Heberle 1976). Beneficial effects of elevated temperature during the anther culture phase have been reported on a number of species, such as asparagus where culture at 32°C for 3-4 weeks was critical for the induction of embryogenic callus (Feng and Wolyn 1991). In maize, heat treatments (30°C-35°C) of inoculated anthers and microspores have been used to induce androgenesis (Coumans et al. 1989; Genovesi 1990; Buter et al. 1991; Afele et al. 1992). Beneficial

effects of elevated incubation temperature were reported on pepper (Dumas de Vault et al. 1982), *Brassica* (Keller et al. 1979) and some wheat species (Huang 1987).

The effect of temperature treatments on *Solanum* genotypes has been reported, mostly by placing excised buds at low temperatures (6°C) for two to three days before anther culture (pre-treatment). Johansson (1986) showed that embryogenesis was stimulated by pretreatment of the buds at 6°C. A beneficial effect of high temperature (30°C) pre-treatment has also been found on *Solanum* genotypes. In anther culture of diploid potato originating from complex crosses of 2x *S. tuberosum* clones with several wild 2x species, Powell and Uhrig (1987) found differential genotypic response to bud pretreatment (6°C or 30°C) for 2 days, but both high and low temperature shocks were superior to the control (without pre-treatment). However, no positive effect of 30°C pretreatment was found in anther culture of *S. tuberosum* cvs. Pito and Danva (Tiainen, 1992).

The effect of elevated temperature during culture phase on potato anther culture is not well studied. In anther culture of *S. chacoense* Bitt. and an interspecific diploid hybrid, *S. tuberosum* x *S. chacoense*, Cappadocia et al. (1984) first cultured anthers at 30°C for 2 days in darkness and then transferred them to light at 27°C. However, they did not test whether this 30°C pre-incubation had a beneficial effect on anther culture. Later, Batty and Dunwell (1986) confirmed the beneficial effect of high temperature pre-incubation on the androgenic response of a dihaploid *S. tuberosum* clone. Recently, Calleberg and Johansson (1993) studied the effect of incubation temperature (10, 15, 20, 25, 30°C) on anther culture of potato. They found embryo production of individual

genotypes, tetraploid as well as dihaploids, was dependent on the incubation temperature; 30°C was beneficial for some genotypes, but others preferred 20-25°C.

Successful regeneration of haploid embryos is essential to the development of breeding systems utilizing anther culture. Afele et al. (1992) reported that maize microspores cultured at 32°C for 10 days had more embryo production, higher embryo quality (more compact), and higher frequency of embryos that survived on transfer to regeneration medium than control (25°C). However, the regeneration rate was still low. The objectives of the present study were to examine the effect of incubation temperature on androgenic embryo yield and the subsequent conversion of androgenic embryos of diploid potato.

Materials and Methods

Plant materials

Plant materials included asexually-produced diploid potato clones APB3, APM1, and APC3 selected from APB, APM and APC populations, which were produced between dihaploid *S. andigena* as female and one of F₁ (*S. phureja* x *S. berthaultii*), F₁ (*S. phureja* x *S. microdontum*) or F₁ (*S. phureja* x *S. chacoense*) as male, respectively. *S. berthaultii*, *S. microdontum*, and *S. chacoense* accessions used to develop these complex hybrids had been previously selected for heat tolerance (Reynolds and Ewing 1989; Simmonds 1971).

APB3, APM1 and APC3 were selected for anther culture competence and high 2n pollen frequencies.

Ten plants each of APB3, APM1 and APC3 clones were planted in 7.6 l pots containing 1 : 1 : 1 of sand : weblite (Weblite Co., P.O. Box 12887, Roanoke, Va.) : Sunshine mix (Fisons Horticulture Inc., Vancouver, BC, Canada) in the greenhouse. APB3 and APM1 were planted from Sept. 1992 to Feb. 1993, and APB3 and APC3 were planted from Jan. 1992 to Sept. 1993. During the winter months, a 16 h day length was provided by high pressure sodium vapor lamps to promote flowering. Plants were fertilized weekly with soluble fertilizer (Peter's Fertilizer Products, W.R. Grace & Co., Fogelsville, Pa) containing 20:19:18 (N: P₂O₅: K₂O) .

Anther culture and temperature treatment

Buds with anthers approximately 3 to 4 mm in length were picked from each clone on each given day and placed in cold storage (4°C) for 3 days. For each clone, the anther culture was conducted on 3 different days, 3 replications per day. The buds were surface-sterilized for 30 sec in 70% ethanol and 5 min in full strength commercial bleach [Wonder Chemical Corp.; 5.24% (W/V) sodium hypochlorite] with a drop of 'Tween 20' followed by two rinses in sterile distilled water. The anther culture medium was prepared according to Uhrig (1985) with some modifications: MS basal medium (Murashige and Skoog, 1962) supplemented with 100 mg/l myo-inositol, 0.4 mg/l thiamine, 60 g/l sucrose, 2.5 g/l activated charcoal, 2.5 mg/l N₆-benzylaminopurine (BA), and 0.1 mg/l indole-3-

acetic acid (IAA). The medium pH was adjusted to 5.8 before adding activated charcoal. The culture medium was sterilized by autoclaving at 121°C and 1.1 kg/cm² for 20 min. Anthers from each bud were distributed to 5 Delong culture flasks (Bellco Glass Co., Vineland, NJ) with 15 ml medium until 30 anthers were contained in each flask. The five flasks were covered with a Magenta 2-way cap (Magenta Plastics, Chicago, IL) and sealed with parafilm, then incubated separately at each of the five temperature treatments according to Table 1. Cultures were maintained in the dark on a shaker (120 rpm). After 6 weeks, the number of embryos was counted.

Once the embryos were harvested, the anthers were put back into the same anther culture medium and incubated for another 6 weeks. The same incubation treatments were maintained (Table 1). The number of embryos was again recorded at the second harvest.

Embryo regeneration

The embryos from anther culture were transferred to 100 x 15 mm Petri dishes with 15 ml regeneration medium: 3.2 g/l Gamborg's B5 salts (Gamborg et al. 1968) with minimal organic compounds (Sigma G 5893), 50 mg/l CaHPO₄, 748 mg/l CaCl₂, 250 mg/l NH₄NO₃, 10 g/l sucrose, 6 g/l agarose (type III-A), 0.1 mg/l gibberellic acid (GA₃, filter-sterilized), pH 5.6, and incubated at 20°C under high intensity light (175 μmol sec⁻¹ m⁻²). At 3 week intervals, the embryos that were able to convert into plants were counted and transferred to a 20 x 150 mm glass tube containing 20 ml MS basal media (Murashige and Skoog 1962) while the unconverted embryos were transferred to fresh regeneration

medium. The regenerants were kept at 20°C under a 16h photoperiod and 175 $\mu\text{mol sec m}^{-2}$ provided by cool white fluorescent tubes.

Ploidy analysis

The ploidy level of regenerated plants was determined by flow cytometry (Owen et al. 1988). One gram of leaves and stems of *in vitro* plantlets was chopped on ice for 3 min with a razor blade in a 6 cm diam glass Petri plate containing 3 ml chopping buffer (12 mM sodium citrate, 8 mM MOPS, 38.4 mM MgCl_2 , and 0.04% Triton X-100). The mixture was poured through a 300 μm filter and then through a 60 μm filter to remove debris. One ml filtrate was placed in a microcentrifuge tube and incubated in 0.5 ml RNAase solution (Sigma R 5503) (0.8 mg/ml chopping buffer) at room temperature for 30 min. A 0.25 ml propidium iodide (PI) solution (0.4 mg PI/ml chopping buffer) was added to each sample, incubated on ice for at least an additional 30 min and analyzed within 3 h. Stained samples were then filtered through a 37 μm nylon mesh. Ploidy analysis of the prepared samples was provided by Virginia-Maryland Regional College of Veterinary Medicine using Epics V, Model 752 laser flow cytometer and cell sorter (Coulter Electronics, Hialeah FL).

Statistical analysis

All statistical analyses were conducted after square root transformation of total embryos per flask, using SAS GLM procedure (SAS 1985). The only exception is in the analysis of genotypic response to temperature treatment and experiment dates.

Results

Temperature effect on anther culture

Two experiments were conducted between 1992-1994. In the first experiment, two diploid clones, APB3 and APM1, were used as anther donors. For each replication, 5 flasks of cultures were subjected to 5 different temperature treatments. The combined analysis of APB3 and APM1 is shown in Tables 2 and 3. Treatment, genotype, dates of culture initiation, and all their interactions were highly significant sources of variation (Table 2). At the first embryo harvest, 35°C 12h-30°C/20°C yielded 11 times as many embryos (44 per flask) as the control, 20°C (4 per flask). When 35°C pre-incubation was extended from 12 to 24 h, a severe decrease of embryo yield was found. APB3 yielded significantly more embryos than APM1 (26 vs 7 per flask). Also, there was high variation in anther culture response among the three dates when anthers were cultured (Table 3). Genotypic response to temperature treatments and experiment dates is shown in Table 6. The best temperature treatment for embryo production of APB3 was 35°C

12h-30°C/20°C whereas no significant difference between 35°C 12h-30°C/20°C and 30°C/20°C for APM1 clone was found. This may explain the significant G x T interaction in Table 2. The embryo yield also varied between experiment dates. For APB3, the highest embryo yield was on 11/26/92, whereas for APM1, 11/26/92 produced the same amount of embryos as date 11/24/92. For both APB3 and APM1, 11/23/92 was the day on which the lowest embryo yield was produced. As noticed in Table 2, the G x D interaction was highly significant. Difference in date is mainly due to difference in growth conditions of donor plants as well as developmental stage of microspores. A significant T X D interaction may reflect different response in application of temperature treatments on the different dates.

In the second experiment using APB3 and APC3 as anther donors, the anther culture procedure was the same as in the first experiment, except for each replication, two flasks were treated with 20°C, two flasks with 35°C 12h-30°C/20°C and one flask with 30°C/20°C. The mean yield of the two flasks receiving 20°C or 35°C 12h-30°C/20°C was used in the analysis. The 24 h heat shock treatments were discontinued due to the poor performance of anthers receiving these treatments. The combined analysis is shown in Table 4. Genotype, date, and G x T interaction were not significant sources of variation this time. Only temperature treatment was significant (Table 4). Anthers cultured at 35°C 12h-30°C/20°C yielded significantly more embryos than those at either 20°C or 30°C/20°C (39, 23, and 11 per flask, respectively) (Table 5). Both genotypes responded similarly to temperature treatments allowing sufficient statistical separation of treatment effects in the combined analysis. If genotypes were analyzed separately, however,

treatment effects were not significant (see lack of mean separation for treatment in Table 7). Because dates were nested within genotypes, there was insufficient statistical power to observe significant differences due to date of bud collection although APB3 buds picked on 4/14/93 appear to have been more responsive (Table 7).

In conclusion, both experiments of temperature effect on anther culture revealed that 35°C for 12 h followed by 30°C 16 h/20°C 8 h for the rest of the incubation time yielded the greatest number of embryos (Tables 3 and 5), although genotypic interaction with temperature treatment was observed in the first experiment (Table 6).

Temperature effect on extended anther culture

The combined analysis of APB3 and APM1 in extended anther culture is shown in Tables 2 and 3. The effect of temperature treatments in the first 6 weeks of culture was reduced in extended anther culture, but still remained a significant source of variation (Table 2). Both treatments receiving 24 h temperature shock were considerably inhibited in embryo production (Table 3). The two treatments 30°C/20°C and 35°C 12h-30°C/20°C that had increased embryo yield over control at the first harvest were not significantly different from control at the second harvest. An increased embryo yield for the control at the second harvest (13 vs 4 per flask at the first harvest) was apparent. Variation among the culture initiation dates continued in the same trend as for the first harvest whereas no significant difference between the two genotypes was found (Table 3). T x D interaction was still significant, whereas G x T, G x D, G x T x D interactions were

not significant (Table 2). The genotypic response of APB3 and APM1 to temperature treatment and experiment dates is shown in Table 6. At the second harvest, temperature treatments were not significant for both APB3 and APM1; no significant difference of genotypic response to temperature treatment was found (Table 6). Differential response to experiment dates continued as in the first harvest.

The combined analysis of APB3 and APM1 from both first and second harvests on the effect of temperature treatments on embryo yield is shown in Tables 2 and 3. 35°C 12h-30°C/20°C yielded the greatest number of embryos (62/per flask). No significant difference was found between treatments 35°C 12h-30°C/20°C and 30°C/20°C. However, a longer (24h) pre-incubation at 35°C inhibited embryo yields. Significant difference between genotypes and dates were found. APB3 yielded significantly more embryos than APM1 (41 vs. 18 per flask). In addition, the anthers cultured on one date (11/26/93) yield 58 times as many embryos as those cultured on another date (11/23/93), just 3 days earlier. G x T, G x D, T x D interactions were all significant.

The genotypic response of APB3 and APM1 in the combined analysis from both first and second harvests is shown in Table 6. For APM1, no significant difference in the total embryo yield between treatment 35°C 12h-30°C/20°C and 30°C/20°C was found whereas 35°C 12h-30°C/20°C yielded significantly more embryos than 30°C/20°C for APB3. Difference in experiment dates continued as before for both APB3 and APM1.

Temperature effect on embryo conversion and ploidy analysis

Only plants regenerated from the first anther culture experiment were analyzed. The conversion rate of androgenic embryos into anther-derived plants differed for clones APB3 and APM1. For APB3, at the first harvest, 1153 embryos were cultured, from which 206 plants were regenerated (18% conversion). Ploidy analysis revealed 4 monoploid, 185 diploid and 4 tetraploid plants from a total of 206 plants regenerated; 13 plants were lost to contamination. For APM1, 8 shoots were regenerated from 291 cultured embryos, but only 2 diploids were regenerated into complete plants (0.7% conversion rate) (Table 8).

The irregular number of embryos obtained in the various treatments over a prolonged period of time prevented a meaningful statistical analysis. However, for APB3 at the first harvest, 711 embryos from 35°C 12h-30°C/20°C treatment were cultured, from which 140 plants were regenerated; the embryo conversion rate was similar to the control (Table 8). Due to the poor regeneration of APM1, the effect of temperature treatment during anther culture on embryo conversion could not be examined. The percentage of converted embryos is not statistically reliable because only 2 plants were regenerated.

Embryo conversion was much less frequent for the second harvest embryos. Only 42 APB3 plants regenerated from 556 embryos cultured (7.6% conversion rate). Of these plants, 35 plants were diploid; the other 7 plants died before ploidy analysis could be made. Of 352 APM1 embryos cultured, none regenerated (Table 8).

For APB3 clone, the 35°C 12h-30°C/20°C temperature treatment continued to have beneficial effect on conversion of the second harvest embryos. The 35°C 12h-30°C/20°C treatment held its regeneration rate at 24.2% whereas the 30°C/20°C treatment dropped to 2.1%. Most obviously, embryos produced in culture from continuous 20°C incubation temperature had very loose structure and usually remained too small to be successfully transferred onto regeneration medium. Of 129 control embryos cultured, none regenerated.

Discussion

Androgenesis involves a shift in the developmental fate of the microspores or pollen from a gametophytic pathway toward mature pollen formation to a sporophytic pathway toward embryo formation. The mechanism behind temperature shocks may be to arrest the existing metabolism and shift it toward the new pathway of embryogenesis instead of the formation of mature pollen (Nitsch et al. 1982). Duncan & Heberle (1976) examined the microspore stage of anthers immediately after harvest from donor plants, after 3 days of cold-incubation (5°C) of the cultured anthers, and after 4 more days of culture at 28°C. Fewer binucleate stage and a much lower percentage of dead grains were found in the treated anthers (3 days incubation) compared with the control anthers. A high proportion of control microspores possessed highly condensed vegetative-type nuclei whereas those subjected to cold shock exhibited diffuse vegetative nuclei in most cases. The same trend was observed among anthers after 7 days incubation (3 days 5°C and 4

days 28°C). After 8 weeks of culture, more anther derived plants were obtained from treated anthers. They concluded that cold-treatment delays the degradation process in the pollen-grains and probably allows more grains to start a new cell cycle, leading to androgenesis. In our study, a short temperature shock (35°C 12h) followed by elevated incubation temperature (30°C/20°C) enhanced the production of androgenic embryos. This was demonstrated in repeated experiments for both APB3 and APC3 clones. However, when 35°C pre-incubation was extended from 12h to 24h, a severe decrease in embryo yield was found. Elevated incubation temperature (30°C/20°C) also appeared to be generally beneficial. This can be observed in both 35°C 12 h-30°C/20°C and 30°C/20°C treatments which used 30°C/20°C as incubation temperature. These two treatments produced the greatest embryo yield at both the first and second harvests for all clones. However, the inhibitory effect of 24 h shock obscured any potentially beneficial effect of 30°C/20°C incubation temperature in the 35°C 24h-30°C/20°C treatment. Genotypic response to temperature treatment was also observed. For APM1, the best temperature treatment appeared to be 30°C/20°C, although no significant difference was found between 35°C 12 h-30°C/20°C and 30°C/20°C. The reduced effect of temperature shock on extended anther culture of APB3 suggests that temperature shock increases androgenic embryo yield, probably by reducing the time for producing embryos. The control anthers cultured at 20°C were the only ones to show an increase in embryo production between the first and second harvests (Table 3).

Genotype was again found to be an important factor determining the responsiveness to anther culture. APB3 produced more embryos than APM1 but no

significant difference between APB3 and APC3 was found. Genotypic differences have been consistently found in previous reports (Cappadocia et al. 1984; Uhrig 1985; Sonnino et al. 1989; Singsit and Veilleux 1989).

There was considerable variation in anther culture response among different dates (Nov. 23, 24, and 26, 1992) when anthers were cultured in the first experiment. However, no difference was found in the second experiment (April 11, 14, 18, June 25, 30, and July 2, 1993). The variation among different culture dates may reflect different growth conditions of donor parent which are important to androgenic capacity. The influence of temperature surrounding the donor plant to the androgenic embryo yield has been reported in rapeseed (Lo and Pauls, 1992), maize (Afele et al. 1992), and potato (Snider and Veilleux, 1994). Cheng and Veilleux also found significant influence of time of bud harvest on embryo production in *S. phureja*. In their studies, although all of the anthers were cultured within a ten day period in April, 1992, approximately twice as many embryos were produced on one day than on the other three. With regard to the time at which flower buds were harvested, those picked at midnight yielded significantly fewer embryos than those picked at 6:00 am, 12:00 noon, or 6:00 pm (Cheng and Veilleux, unpublished data).

To control day effect on embryo yield, two or three replications may be carried out in a single day; therefore, variance due to day effects on embryo yield can be partitioned thereby reducing experimental error.

In our study, anthers from the same bud were distributed to five flasks with different treatments to prevent confounding of bud-to-bud variation with treatment effects

(Snider and Veilleux 1994). The variation among buds collected on the same day may be largely due to differences in the developmental stage of microspores, which contributes androgenic ability (Telmer et al 1992).

Highly significant G x T, G x D, T x D, and G x T x D interactions in first harvest of Exp. 1 complicate the analysis and interpretation of the results. Uncontrollable environmental factors surrounding the donor plants preceding bud harvest interact with temperature treatments and genotypes. Such interactions appear to have been less important at the second harvest (Table 2). Our results are consistent with previous reports. For example, Keller and Armstrong (1983) found that initial high incubation temperature had a promotional effect on androgenesis in some species but this effect interacted with the genotype of the donor plant and the growth conditions (donor plant age, vigor and growth environment, Ku et al. 1978). Afele et al. (1992) found that microspores isolated from plants in a growth chamber (18°C/15°C : day/night) produced more than double the embryos after a temperature treatment of 32°C for 10 days compared to the culture incubated at 25°C continuously. On the other hand, no significant differences for incubation treatments were found if microspores were isolated from greenhouse plants (28°C/23°C : day/night).

In our study, temperature treatment (35°C 12h-30°C/20°C) during anther culture did not seem to influence the regeneration rate (Table 8). Calleberg et al. (1993) pointed out that anther culture conditions of potato were important to regeneration. Although the direct regeneration on the anther culture medium after incubation at elevated temperature during anther culture was not as high as control (20°C), the final regeneration rates were

independent of the incubation temperature of the anther culture. On the other hand, Afele et al. (1992) found high culture temperature treatment on maize microspore resulted in a high quality embryo and a two-fold increase in the percentage of embryos that survived on transfer to regeneration medium compared to the control, although the regeneration rate was still low. We have noticed that embryos from 35°C 12 h-30°C/20°C treatment generally were larger, most had cotyledons and roots whereas embryos from control usually were small and mostly globular. However, the final regeneration percentages were equivalent among the first harvest embryos, although plants regenerated from control generally were weaker and grew slower than those plants regenerated from 35°C 12 h-30°C/20°C treatment. In contrast to the first harvest embryos, the second harvest embryos resulted from 35°C 12 h-30°C/20°C treatment had much higher regeneration percentage than embryos from control.

Ploidy analysis of anther derived plants revealed a high percentage of diploids. This is because anther donors APB3, APM1 and APC3 have been selected for high 2n pollen frequency. Meyer et al. (1993) found that anther-donor clones that generate primarily monohaploid plants also have low 2n pollen production.

Our results showed that a short temperature shock (35°C 12h) followed by elevated incubation temperature increase androgenic embryo yield. This provides a simple and effective way to enhance androgenic embryo yield. More studies are needed to examine if temperature shock treatment has generally beneficial effects in other genotypes and species. If the mechanism behind the temperature shock treatment is to block development of those grains that had not yet entered mitosis, it would be worthwhile to

test whether cold temperature shock or chemical reagents that block mitosis have the same beneficial effect on androgenesis. More cytological investigation is also needed to understand the mechanism behind these treatments. The length of temperature shock treatment and the composition of culture medium need to be further examined, since Dumas et al. (1982) found significant interactions between length of temperature shock treatment and concentration of the growth substances in the culture medium.

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Table 1. Temperature treatments applied during anther culture of diploid potato.

Treatment abbreviation	Pre-incubation temperature	Subsequent incubation (6 weeks)	Extended culture (6 weeks)
35°C 12h-30°C/20°C	35°C 12h	30°C 16h/20°C 8h	30°C 16h/20°C 8h
30°C/20°C	30°C 16h/20°C 8h	30°C 16h/20°C 8h	30°C 16h/20°C 8h
35°C 24h-30°C/20°C	35°C 24h	30°C 16h/20°C 8h	30°C 16h/20°C 8h
20°C (control)	20°C	20°C	20°C
35°C 24h-20°C	35°C 24h	20°C	20°C

Before anther culture, buds were treated at 4°C for 3 days. After anthers were placed in the medium, they were exposed to one of the above five different combinations of pre-incubation and incubation temperature treatments.

Table 2. Combined analysis of variance of two clones (APB3 and APM1) for embryo yield in anther cultures under five different temperature treatments cultured on three days.

Source	1st harvest			2nd harvest			Total embryos		
	df	MS	p	df	MS	p	df	MS	p
Genotype(G)	1	80.2	0.0001	1	8.2	0.1852	1	73.2	0.0001
Temperature(T)	4	48.2	0.0001	4	18.1	0.0072	4	62.1	0.0001
Date(D)	2	112.4	0.0001	2	79.4	0.0001	2	225.9	0.0001
Rep(Date)	6	2.8	0.2420	6	11.1	0.0386	6	9.5	0.0495
G x T	4	15.4	0.0001	4	4.2	0.4613	2	12.2	0.0294
G x D	2	39.5	0.0001	2	14.1	0.0534	2	52.6	0.0001
T x D	8	18.2	0.0001	81	12.3	0.0152	8	27.1	0.0001
G x T x D	8	6.7	0.0044	8	4.1	0.5159	8	7.7	0.0906

Table 3. Mean embryos per flask (30 anthers/flask) of two diploid potato clones APB3 and APM1, cultured on three days, under five temperature treatments. (Means for treatment and date are combined for the two clones.)

Treatment		1st harvest		2nd harvest		Total embryos	
		n	mean	n	mean	n	mean
Temperature	35°C 12h-30°C/20°C	18	44 ^a	18	18 ^{ab}	18	62 ^a
	30°C/20°C	17	21 ^b	17	23 ^a	17	45 ^a
	35°C 24h-30°C/20°C	18	10 ^c	16	3 ^b	16	14 ^b
	20°C (control)	18	4 ^c	16	13 ^{ab}	16	16 ^b
	35°C 24h-20°C	18	2 ^c	17	4 ^b	17	5 ^b
Genotype	APB3	45	26 ^a	43	14 ^a	43	41 ^a
	APM1	44	7 ^b	41	11 ^a	41	18 ^b
Date	11/26/92	30	33 ^a	28	23 ^a	28	58 ^a
	11/24/92	30	15 ^b	30	12 ^b	30	27 ^b
	11/23/92	29	1 ^c	26	<1 ^c	26	1 ^c

Mean separation by SNK, 5% level. All analyses were conducted on the square root transformation of embryos per flask. Comparisons were made within each column of each source of variation, e.g., temperature, genotype, or date.

Table 4. Analysis of variance for embryo yield at the first harvest in anther cultures of potato clones APB3 and APC3 under three different temperature treatments. Each clone was cultured on three separate days.

Source	df	MS	p
Genotype	1	27.3	0.0892
Temperature	2	32.6	0.0359
Date (Genotype)	4	1.6	0.9464
Genotype x Temperature	2	0.1	0.9870

Table 5. Mean embryos per flask (30 anthers/flask) at the first harvest for anther cultures of APB3 and APC3 conducted under three temperature treatments.

Temperature treatment	N	Mean embryos/flask
35°C 12h-30°C/20°C	16	39 ^a
30°C/20°C	18	23 ^b
20°C	16	11 ^b

Mean separation by SNK, 5% level. Analysis was conducted on the square root transformation of embryos per flask.

Table 6. Mean embryos per flask (30 anthers/flask) of two diploid potato clones (APB3 and APM1) cultured under five temperature treatments on three days in experiment 1. (Means are presented separately for each clone.)

Treatment		1st harvest		2nd harvest		Total embryo	
		APB3	APM1	APB3	APM1	APB3	APM1
Temperature	35°C 12h-30°C/20°C	79 ^a	10 ^{ab}	15 ^a	21 ^a	94 ^a	31 ^a
	30°C/20°C	20 ^b	23 ^a	26 ^a	20 ^a	46 ^b	43 ^a
	35°C 24h-30°C/20°C	20 ^b	<1 ^b	5 ^a	<1 ^a	24 ^b	1 ^a
	20°C	6 ^b	1 ^b	21 ^a	5 ^a	27 ^b	5 ^a
	35°C 24h-20°C	4 ^b	<1 ^b	3 ^a	4 ^a	6 ^b	5 ^a
Date	11/26/92	56 ^a	10 ^a	35 ^a	12 ^a	94 ^a	22 ^a
	11/24/92	21 ^b	10 ^a	7 ^b	17 ^a	28 ^b	27 ^a
	11/23/92	<1 ^c	<1 ^a	<1 ^b	<1 ^a	1 ^b	<1 ^a

Mean embryos per flask were separated by SNK, 5% level. All analyses were based on untransformed data. Comparisons were made within each column of each category, e.g., temperature, date.

Table 7. Mean embryos per flask (30 anthers/flask) of two diploid potato clones (APB3 and APC3) cultured under three temperature treatments on three days in experiment 2. (Means are presented separately for each clone.)

Treatment		APB3	APC3
Temperature	35°C 12h-30°C/20°C	49 ^a	25 ^a
	30°C/20°C	29 ^a	16 ^a
	20°C	16 ^a	15 ^a
Date	4/11/93	27 ^a	**
	4/14/93	41 ^a	**
	4/18/93	27 ^a	**
	6/25/93	**	11 ^a
	6/30/93	**	16 ^a
	7/02/93	**	21 ^a

Mean embryos per flask were separated by SNK, 5% level. All analyses were based on untransformed data. Comparisons were made within each column of each category, e.g., temperature, date.

Table 8. Frequency of converted androgenic embryos harvested from various anther culture treatments and ploidy analysis of regenerated plants.

Harvest	Clone	Treatment	Embryos cultured	Converted embryos	%converted embryos	No of plants*		
						1x	2x	4x
1	APB3	35°C 12h-30°C/20°C	711	140	20	2	126	3
		30°C/20°C	180	20	11	0	19	0
		35°C 24h-30°C/20°C	177	33	19	2	29	0
		20°C	53	10	19	0	8	1
		35°C 24h-20°C	32	3	9	0	3	0
		total	1153	206	18	4	185	4
	APM1	35°C 12h-30°C/20°C	87	0	0	0	0	0
		30°C/20°C	183	1	0.5	0	1	0
		35°C 24h-30°C/20°C	4	0	0	0	0	0
		20°C	10	1	10	0	1	0
		35°C 24h-20°C	7	0	0	0	0	0
		total	291	2	0.7	0	2	0
2	APB3	35°C 12h-30°C/20°C	132	32	24.2	0	27	0
		30°C/20°C	235	5	2.1	0	5	0
		35°C 24h-30°C/20°C	42	5	11.9	0	3	0
		20°C	129	0	0	0	0	0
		35°C 24h-20°C	18	0	0	0	0	0
		total	556	42	7.6	0	35	0
	APM1	35°C 12h-30°C/20°C	189	0	0	0	0	0
		30°C/20°C	163	0	0	0	0	0
		35°C 24h-30°C/20°C	0	0	0	0	0	0
		20°C	0	0	0	0	0	0
		35°C 24h-20°C	0	0	0	0	0	0
		total	352	0	0	0	0	0

* 13 plants regenerated from the first harvest and 7 plants regenerated from the second harvest were lost before ploidy analysis could be made.

CHAPTER 3: STUDY OF EXTENDED ANTHER CULTURE

Key words: androgenesis, extended anther culture, medium condition, regeneration, diploid potato

Abstract: Repeated experiments were conducted to study extended anther culture by returning anthers into medium following the usual harvest 6 weeks after culture initiation. Three diploid potato clones APB3 [*adg* x (*phu* x *ber*)], APM1 [*adg* x (*phu* x *mcd*)], and APC3 [*adg* x (*phu* x *cha*)] were used. The results demonstrate that additional embryos were produced after the first harvest, although the embryo quality and the regeneration percentages were generally poorer. In addition, embryos from the second harvest required more time to convert into plants than those from the first harvest. More contamination occurred in extended anther cultures. Significantly more embryos were produced when anthers were returned to the same medium (16 per flask) compared to fresh medium (4 per flask) in extended anther culture. Regression analysis showed that embryo yield at the first harvest was significantly correlated with embryo yield at the second harvest ($P < 0.01$). Hence, poor anther culture responsiveness at the first harvest was not caused by a longer lag period but was due rather to some predetermined factor, such as the microspore developmental stage or growth conditions of the donor plants. Highly responsive cultures are likely to continue yielding embryos even 12 weeks after culture initiation.

Introduction

Anther culture is an important technique for producing homozygous lines. Considerable research has been done to study factors that affect androgenic embryo yield, such as growth conditions of donor plants, developmental stage of microspores, medium, growth regulators, culture conditions, etc. Genotype is one of the most important factors that contributes to response of anther culture. However, even for the same genotype and culture conditions, embryo yield may vary from flask to flask. In my previous experiments, APB3 yielded embryos from 0 to 214 per flask (30 anthers/flask) under identical conditions. For Brussels sprouts, embryo yield varied between 0 and 800 embryos per 100 anthers under similar conditions (Biddington & Robinson 1991). In these studies, anthers have usually been discarded after embryos were harvested. No observation has been available on extended anther culture following the first harvest. It is not yet clear whether poor responsiveness of anthers is due to the incapacity for producing embryos, or by a longer lag period for some anthers. If it is the latter case, anthers that respond poorly at the first harvest should produce more embryos in extended culture than those that respond well at the first harvest. The purpose of this study was to determine if embryos will continue to be produced from anthers after the first harvest and if so, the relationship between the embryo yields of the first and second harvests. The effect of medium condition (fresh or old) in extended anther culture on embryo yield at the second harvest, and the regeneration potential of the second harvest embryos were also determined.

Materials and Methods

Plant materials

Diploid potato clones APB3, APM1, and APC3 were used for this experiment. They were selected from APB, APM, and APC populations which were derived from crosses between dihaploid *S. andigena* as female and F₁ (*S. phureja* x *S. berthaultii*), F₁ (*S. phureja* x *S. microdontum*) and F₁ (*S. phureja* x *S. chacoense*) as males, respectively. APB3, APM1 and APC3 were selected for anther culture competence and high 2n pollen frequencies.

Ten plants each of APB3 and APM1 were planted from tubers in the greenhouse between Sept. 1992 to Feb. 1993, and ten plants each of APB3 and APC3 were planted between Jan. 1992 to Sept. 1993. Sixteen hour day length was provided by high pressure sodium vapor lamps to promote flowering. Plants were fertilized weekly with soluble fertilizer containing 20:19:18 (N:P₂O₅:K₂O).

Extended anther culture

Two separate anther culture experiments were conducted. In the first experiment, APB3 and APM1 clones were used, a total of 2,700 anthers (9 replicates x 2 clones x 5 flasks/rep/clone x 30 anthers/flask) was cultured. In the second experiment, APB3 and APC3 clones were used, a total of 2,160 anthers (9 replicates x 2 clones x 4

flasks/rep/clone x 30 anthers/flask) was cultured. Buds with anthers approximately 3 to 4 mm in length were picked, wrapped in moist paper towels, and placed in cold storage (4°C) for 3 days. For each clone, the anther culture was conducted on 3 different days, 3 replications per day. The buds were surface sterilized for 30 sec in 70% ethanol and 5 min in full strength commercial bleach [Wonder Chemical Corp.; 5.24% (W/V) sodium hypochlorite] with a drop of 'Tween 20', followed by two rinses in sterile distilled water. The anther culture medium was prepared according to Uhrig (1985): MS basal medium (Murashige & Skoog 1962) supplemented with 100 mg/l myo-inositol, 0.4 mg/l thiamine, 60 g/l sucrose, 2.5 g/l activated charcoal, 2.5 mg/l N₆-benzylaminopurine (BA), and 0.1 mg/l indole-3-acetic acid (IAA). The medium pH was adjusted to 5.8 before adding activated charcoal. The culture medium was sterilized by autoclaving at 121°C and 1.1 kg/cm² for 20 min. To distribute bud-to-bud variation across treatments, anthers from each bud were distributed to five 125 ml Delong culture flasks (Bellco Glass Co., Vineland, NJ), each with 15 ml medium, until 30 anthers were contained in each flask. The five flasks were covered with a Magenta 2-way cap and sealed with parafilm. Cultures were maintained in the dark on a shaker (120 rpm).

After 6 weeks, embryos were harvested by pouring the contents of the flask through a sterilized wire mesh sieve, recovering the medium in a sterile beaker, and transferring the embryos and anthers to a sterile petri plate. The number of embryos per flask was recorded (first harvest) and embryos were transferred to regeneration medium. For the extended anther culture, after the embryos were harvested, the anthers were put back either into the same medium or into fresh medium for 4 or 6 weeks. Embryo yield

per flask was again recorded (second harvest). The relationship between the embryo yield of the first harvest and that of the second harvest was analyzed by regression analysis.

Medium effect on extended anther culture

Nine replicates each of APB3 and APC3 consisting of a total of 2,160 anthers (9 replicates x 2 clones x 4 flasks/clone/rep x 30 anthers/flask) were cultured. For each clone, the cultures were initiated on 3 different days, 3 replications per day. Anthers from each bud were distributed to the 4 flasks. After 6 weeks in culture, the embryos were harvested as above. For extended anther culture, two flasks of anthers from each replication were carefully put back to the same medium, whereas the other two flasks of anthers were transferred to fresh anther culture medium of the same composition. The embryos were harvested again after another 4 weeks.

Regeneration of anther derived embryos

The embryos from anther culture were transferred to regeneration medium: 3.2 g/l Gamborg's B5 salts (Gamborg et al. 1968) with minimal organic compounds (Sigma G 5893), 50 mg/l CaHPO₄, 748 mg/l CaCl₂, 250 mg/l NH₄NO₃, 10 g/l sucrose, 6 g/l agarose, 0.1 mg/l gibberellic acid (GA₃), pH 5.6, and incubated at 20°C under high intensity light (175 μmol sec⁻¹ m⁻²). At 3 week intervals, the regenerated embryos were counted and transferred to a 20 x 150 mm glass tube containing 20 ml MS basal media (Murashige

and Skoog 1962) while the unregenerated embryos were transferred to fresh regeneration medium. The regenerants were kept at 20°C under a 16 h photoperiod and 175 $\mu\text{mol sec}^{-1} \text{m}^{-2}$ provided by cool white fluorescent tubes.

Ploidy analysis

The ploidy level of regenerated plants was determined by flow cytometry according to Owen et al. (1988). One gram of leaves and stems of *in vitro* plantlets was chopped on ice for 3 min with a razor blade in a 6 cm diam glass Petri plate containing 3 ml chopping buffer (12 mM sodium citrate, 8 mM MOPS, 38 mM MgCl_2 , 0.04 % Triton X-100). The mixture was poured through a 300 μm filter and then through a 60 μm filter to remove debris. One ml of the filtrate was placed in a microcentrifuge tube and incubated in 0.5 ml RNAase solution (Sigma R 5503) (0.8 mg/ml chopping buffer) at room temperature for 30 min. A 0.25 ml DNA staining reagent propidium iodide (PI) solution (0.4 mg /chopping buffer) was added to each sample, incubated on ice for at least an additional 30 min and analyzed within 3 h. Stained samples were filtered through a 37 μm nylon mesh. Ploidy analysis of the prepared samples was provided by Virginia-Maryland Regional College of Veterinary Medicine using Epics V, Model 752 laser flow cytometer and cell sorter (Coulter Electronics, Hialeah FL).

Statistical analysis

Regression analysis was conducted on the square root transformation of total embryos per flask. All other statistical analyses were based on untransformed data using SAS GLM procedure (SAS 1985).

Results

Recovery of second harvest embryos in extended anther culture

In both experiments, embryos continued to be produced by anthers replaced in culture after the first embryo harvest. Genotype was a significant source of variation (Table 1). In both experiments, APB3 yielded significantly more embryos than APM1 in the first experiment (20 vs 9 per flask) and APC3 in the second experiment (22 vs 10 per flask) (Table 2). A significant difference between harvests was found in the second experiment where embryo yield at the first harvest was significantly higher than at the second harvest (24 vs 8 per flask). However no difference between harvests was found in the first experiment (Table 2). Further analysis indicated that both APB3 and APC3 had higher yield at the first harvest, but APM1 had higher yield at the second harvest (Table 3). Date of culture initiation was a significant source of variation in the first experiment (28, 14, 0.5 embryos per flask at 3 different dates) whereas no difference between dates of culture initiation was found in the second experiment (Table 2).

Embryo yield at the first harvest was significantly correlated with that at the second harvest. In the first experiment, averages of 26 and 7 embryos/flask were produced from APB3 and APM1, respectively, at the first harvest. Similarly, averages of 14 and 11 embryos/flask were produced from the two clones at the second harvest. Regression analysis showed that the embryo yield per flask at the second harvest was significantly positively correlated with the embryo yield at the first harvest ($\text{embryo2} = 0.8 + 0.5 \cdot \text{embryo1}$, $P < 0.001$). In the second experiment, mean embryo yields per flask at the first and second harvests were 33 and 11 for APB3 and 14 and 5 for APC3. Regression analysis revealed the same trend as before ($\text{embryo2} = 0.6 + 0.3 \cdot \text{embryo1}$, $P < 0.01$) (Table 3).

Medium effect on extended anther cultures

Two medium conditions were compared for their effect on the embryo production in extended culture where anthers were either returned to the same used medium or transferred to fresh medium. APB3 and APC3 were used as anther donors. Significantly more embryos were produced at the second harvest in the old medium (16 per flask) than in the fresh medium (4 per flask) (Tables 4 and 5). Genotype, date (Genotype), and genotype x medium interactions were not significant in extended anther culture (Table 4).

Embryo quality and regeneration potential of the second harvest embryos

Embryo quality was estimated by general appearance, fragility on transfer, and regenerability. Generally, the embryo quality of the second harvest embryos was poorer than that of the first harvest embryos. Some of the flasks of both APB3 and APM1 in the first experiment and APB3 in the second experiment produced masses of tiny embryos (Fig 1). These embryos were generally very poor quality, no plants were regenerated from them. Most of these embryos were tiny globular structures, whereas others were loosely compacted into fragile globs that were shattered on handling. These flasks were counted as missing values when data were analyzed. Depending on the health condition of the plants when buds were picked, more contamination was likely to occur in extended anther culture than in the first 6 weeks culture period. However, there was no difference in contamination rate between the regeneration phase of the cultured embryos from first and second harvest (Table 6).

The conversion rate of second harvest embryos was lower than that of first harvest embryos (Table 7). In the first experiment, using APB3 and APM1 as anther donor, for the first harvested embryos, a total of 208 plants was regenerated from 1,444 embryos cultured, yielding an overall regeneration percentage of 14.4%. From 1153 APB3 embryos cultured, 206 plants were regenerated; the regeneration percentage was 17.9%. APM1 had lower regeneration percentage (0.7%) than APB3; only 2 plants were regenerated from 291 embryos cultured. For the second harvested embryos, the overall regeneration percentage was 4.6%. Of 556 embryos cultured, 42 APB3 plants were

regenerated (9.7%). However, from 352 APM1 embryos cultured, none regenerated into plants (Table 7). Six flasks of anthers from APB3 and APM1 were continued in culture for another 6 weeks after second harvest; at the third harvest, only 1 flask of APB3 yielded 193 embryos, 4 of which formed shoots but none regenerated into whole plants (data not shown).

In the second experiment, using APB3 and APC3 as anther donors, a total of 51 plants was regenerated from 810 first harvest embryos; the overall regeneration percentage was 6.3%. Of 600 APB3 embryos cultured, 33 plants were regenerated, yielding a regeneration percentage of 5.5%. Similarly, 18 APC3 plants were regenerated from 210 embryos cultured; regeneration percentage was 8.6%. At the second harvest, the overall regeneration percentage was 2.9%. Only 1 APB3 plant was regenerated from 120 embryos cultured, and 7 APC3 plants were regenerated from 156 embryos cultured (Table 7).

In addition to the difference in regeneration percentage between the first and second harvest embryos, embryos from the second harvest required more time to regenerate into plants than those of the first harvest (Figs. 2). In the culture of first harvest embryos from APB3, 37.4% of total embryos regenerated after 6 weeks in regeneration medium, embryos continued to regenerate even after 5 subcultures, that is, 15 weeks after embryos were cultured on regeneration medium. However, in the culture of the second harvest embryos, no embryos regenerated within first 3 weeks of culture, and only 19% of total embryos regenerated after 6 weeks in regeneration medium. Because of poor quality of the second harvest embryos, they were discarded after 4

subcultures (Fig. 2). The number of embryos regenerated during each transfer is shown in Fig. 3. More embryos from the first harvest regenerated during the first 9 weeks culture compared to that from the second harvest (Fig. 3). The clone APM1 was not included because only 2 plants regenerated from the first harvest embryos and no plants regenerated from the second harvest embryos (see Table 7). Due to frequent contamination and small number of regenerated plants from the second harvest embryos, the data from the second experiment using APB3 and APC3 as anther donor were not reliable; therefore, it was not included in data analysis, but the regeneration data from the first harvest embryos appeared to exhibit the same trend as shown in Figs. 2 and 3.

Ploidy analysis revealed high percentage of diploids (Table 8). This is expected because anther donors APB3, APM1 and APC3 have all been selected for high $2n$ pollen frequency.

Discussion

The results showed that embryos can continue to be produced from cultured anthers after one or even two harvests. Considerably high embryo yields were produced at the second harvest, especially for clone APM1 which produced more embryos in the second harvest than in the first. For clones APB3 and APC3, more embryos were produced in the first harvest than in the second, indicating that androgenic embryo yield in extended anther culture may be genotype dependent. However, the embryo quality and the regeneration percentage were generally poorer for the second harvest embryos

compared to the first harvest embryos. The microspores which are able to convert into androgenic embryos in a shorter period are more likely to regenerate into plants whereas those requiring longer period are more recalcitrant.

Although anther culture has been a routine technique in breeding programs and genetic studies, little understanding has been gained about the induction process involved. Even for a highly responsive clone, the number of embryos produced per cultured anther is generally low, considering the large number of microspores within each anther. In our study, some flasks produced a large mass of embryos at the second harvest. However, none of these tiny embryos was able to regenerate into whole plants under the regeneration procedure generally used for embryo conversion. These flasks were counted as missing values in all data analyses. Because only a few flasks produced masses of embryos, some unknown factors critical to the conversion of microspores to androgenic embryos may have been required.

A significant regression between the embryo yield of the first and second harvests was found. This means that the anthers that produced high embryo yield at the first harvest time were likely to continue to yield embryos in the extended anther culture. On the other hand, if anthers are unable to produce embryos or produce only a few embryos, they are less likely to produce embryos in the extended anther culture. This suggests that some predetermined factors, such as microspore developmental stage or the growth condition of the donor plant, is more important to the high yield of androgenic embryos. Longer culture period did not increase regenerable embryo yields. The importance of the microspore developmental stage and the growth condition of donor plants to the

androgenic embryo yield has been reported in a number of species, such as potato (Snider & Veilleux 1994) and *Brassica napus* (Lo et al. 1992; Telmer et al. 1992).

In both experiments, genotype was a significant source of variation. This observation is consistent with previous reports (Cappadocia et al. 1984; Uhrig 1985; Sonnino et al. 1989; Singsit and Veilleux 1989).

The date of culture initiation was a significant source of variation in the first experiment, but not in the second experiment. The quality of buds and frequency of buds with microspores at the proper developmental stage may vary from time to time. Other factors, such as temperature surrounding the donor plants or growth conditions of the donor plants preceding bud harvest may also contribute to androgenic embryo yield. By conducting two or three replications per day, the error term can be reduced by subtracting day to day variance and thus allowing a more powerful statistical analysis of treatment effects.

The old medium appeared to be better than the fresh medium for the extended anther culture. This may indicate that the old medium acted as a conditioned medium. Alternatively, the old medium may actually contain minute embryos that passed through the sieve and developed in extended anther culture. Embryos from the second harvest had lower regeneration rates and required more time to regenerate into plants compared to those from the first harvest. This may be due to poor quality of the second harvest embryos.

Our results showed that embryos can continue to be produced from cultured anthers after one or even two harvests. However, the source of the embryos produced

after the first or second harvest, which was as late as 18 weeks after culture initiation, remains to be studied. They might have developed from individual microspores released in the medium or still remaining in the replaced anthers. It is also possible that microscopic secondary embryos passed through wire mesh sieve during first harvest, remained in the culture medium and developed in the extended anther culture. Cho and Kasha (1989, 1992) reported that androgenic induction occurs only in early anther culture stage. If the second or third harvest embryos are from individual microspores, further cytological studies should be made to clarify whether they came from early androgenic induction followed by a latent period for induced microspore to develop into embryos, or from a delayed androgenic induction. Studies are also needed to examine the factors that may be required in production of massive numbers of embryos in some flasks. Although a relatively high embryo yield was obtained during extended anther culture, high contamination and low regeneration rate eliminate any practical use of extended anther culture. A better regeneration protocol may be needed to improve regeneration rate of second harvest embryos.

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Table 1. Analysis of variance for embryo yield at the first and second harvests.

Exp no.*	Source	df	MS	p
1	Harvest	1	674.6	0.4043
	Genotype	1	5692.8	0.0162
	Date	2	11056.4	0.0001
2	Harvest	1	7884.4	0.0018
	Genotype	1	5091.2	0.0116
	Date(Genotype)	4	246.5	0.8657

* In experiment 1, clones APB3 and APM1 were used, anthers were cultured on three different dates. In experiment 2, clones APB3 and APC3 were used, anthers were cultured on six different dates.

Table 2. Mean embryos per flask in anther culture of diploid potato. Each experiment was conducted on two genotypes, on three or six different dates, and embryos were harvested twice.

Exp no.	Treatment		N	Mean embryos/flask*	
1	Harvest	1	89	16 ^a	
		2	84	12 ^a	
	Genotype	APB3	88	20 ^a	
		APM1	85	9 ^b	
	Date	11/26/92	58	28 ^a	
		11/24/92	60	14 ^b	
		11/23/92	55	<1 ^c	
	2	Harvest	1	68	24 ^a
			2	64	8 ^b
Genotype		APB3	70	22 ^a	
		APC3	62	10 ^b	
Date		4/11/93	24	19 ^a	
		4/14/93	23	26 ^a	
		4/18/93	23	22 ^a	
		6/25/93	22	8 ^a	
		6/30/94	22	8 ^a	
		7/02/93	18	15 ^a	

* Mean embryos per flask were separated by SNK, 5% level. Comparisons were made within each category, e.g., harvest, genotype, or date for each experiment.

Table 3. Regression analysis revealing a positive relationship between the embryo yield of the first and second harvests.

Exp no.	Clone	Mean embryos/flask*		Regression analysis**		
		1st harvest	2nd harvest	p	R ²	Equation
1	APB3	26±5.5 ^a	14±5.6 ^a	0.0001	0.32	emb2= 0.8+0.5 emb1
	APM1	7±3.2 ^a	11±3.1 ^a			
2	APB3	33±5.6 ^a	11±5.7 ^b	0.0048	0.12	emb2= 0.6+0.3 emb1
	APC3	14±3.4 ^a	5±3.5 ^a			

* Mean embryos per flask were separated by SNK, 5% level. Comparison were made within each row, i.e., between the first and second harvest for each clone.

** Regression analyses were conducted on the square root transformation of embryos per flask. Emb 1 and emb 2 represent embryo yield at the first and second harvests.

Table 4. Analysis of variance for androgenic embryo yield of APB3 and APC3 on extended anther culture using old or fresh medium.

Source	df	MS	p
Genotype	1	300.8	0.2448
Medium	1	1001.6	0.0405
Date(Genotype)	4	50.2	0.9133
Genotype*Medium	1	185.3	0.3583

Table 5. Medium effect on extended anther culture of APB3 and APC3.

Medium	N	Mean embryos/flask
Old	13	16 \pm 4.0 ^a
New	16	4 \pm 3.6 ^b

Mean embryos per flask were separated by SNK, 5% level.

Table 6. Comparison of contamination occurring in the first six weeks culture and extended anther culture; first harvest and second harvest embryo culture.

Exp no.	Clone	Anther culture		Embryo culture	
		first 6 weeks culture	extended culture	1st harvest embryos	2nd harvest embryos
1	APB3	0/45 flask	4/45 flask	0/49 plate	0/20 plate
	APM	10/45 flask	4/45 flask	0/15 plate	0/19 plate
2	APB3	1/36 flask	2/35 flask	0/21 plate	1/5 plate
	APC3*	4/36 flask	2/35 flask	1/8 plate	**

* During the time of picking buds for anther culture, APC3 plants were suffering from insects; therefore, contamination is higher in the first 6 weeks culture.

** No observation.

Table 7. Comparison of regeneration potential of embryos from first and second harvests.

Exp no.	Clone	First harvest			Second harvest		
		Total embryos cultured	No. of plants regen	Regen %	Total embryos cultured	No. of plants regen	Regen %
1	APB3	1153	206	17.9	556	42	7.6
	APM1	291	2	0.7	352	0	0
	Total	1444	208	14.4	908	42	4.6
2	APB3	600	33	5.5	120	1	0.8
	APC3	210	18	8.6	156	7	4.5
	Total	810	51	6.3	276	8	2.9

Table 8. Ploidy analysis of regenerated plants from the first and second harvests.

Exp no.	Clone	First harvest embryos(%)			Second harvest embryos(%)		
		1x	2x	4x	1x	2x	4x
1	APB3	2.0	95.9	2.0	0	100	0
	APM1	0.0	100.0	0.0	**	**	**
	Total	2.0	95.9	2.0	0	100	0
2	APB3	**	**	**	**	**	**
	APC3	0	100	0	0	100	0
	Total	0	100	0	0	100	0

** No observation.

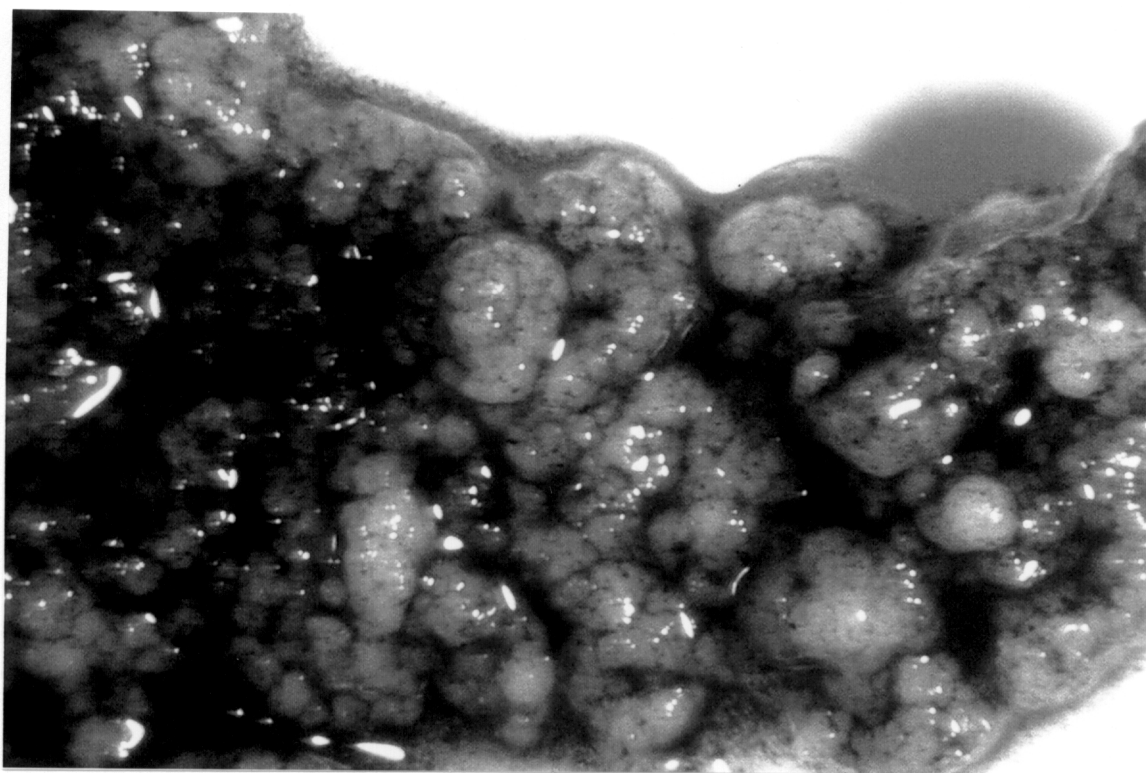


Fig. 1. Embryonic mass occasionally obtained from extended anther culture of diploid potato clone APB3 after 12 weeks of culture.

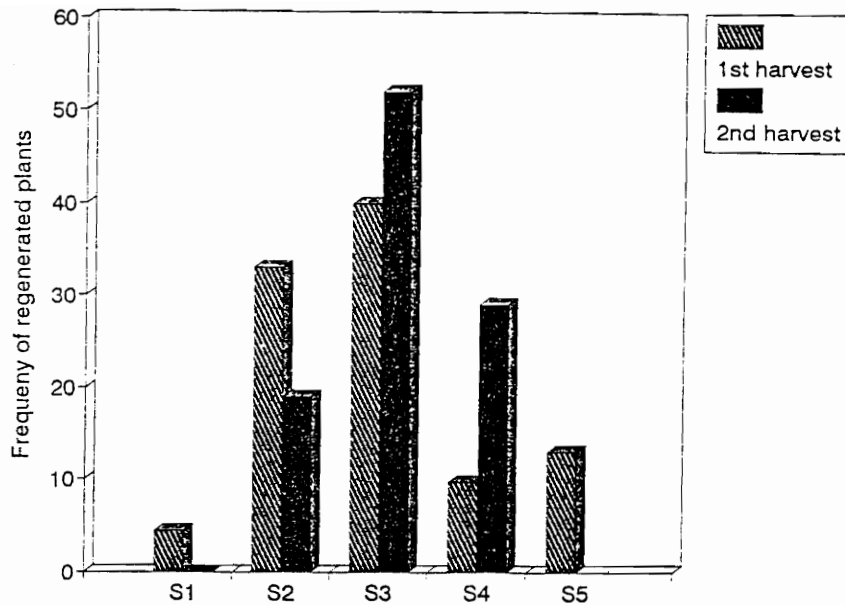


Fig. 2. Frequency of regenerated plants after serial subculture. The embryos were harvested either 6 weeks after anthers were placed in culture (1st harvest) or from extended anther culture (2nd harvest). They were subcultured at 3 week intervals. S1, S2, ..., S5 represented 3, 6, ..., 15 weeks after embryos were cultured on regeneration medium.

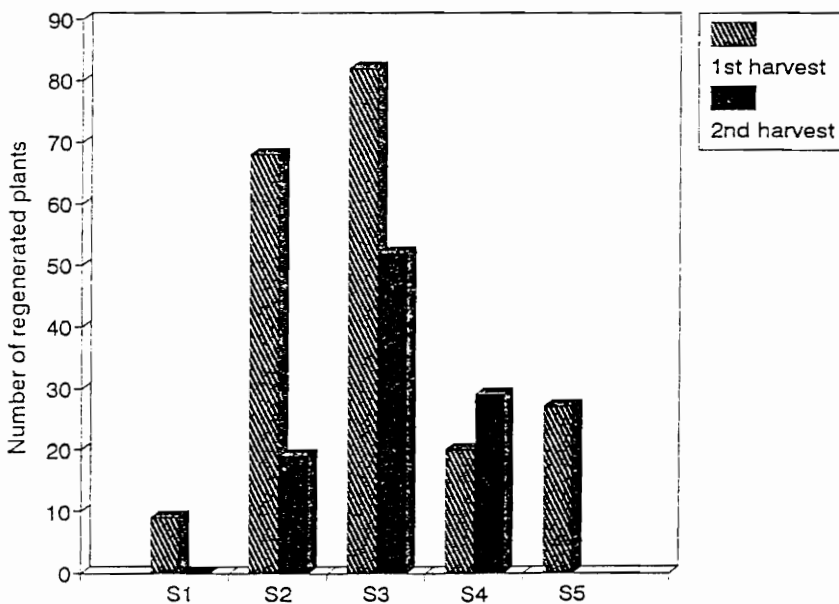


Fig. 3. Number of regenerated plants after serial subculture. The embryos were harvested either 6 weeks after anthers were placed in culture (1st harvest) or from extended anther culture (2nd harvest). They were subcultured at 3 week intervals. S1, S2, . . . , S5 represented 3, 6, . . . , 15 weeks after embryos were cultured on regeneration medium.

**CHAPTER 4: ANALYSES OF THE GENETIC COMPOSITION OF
ANTHER-DERIVED PLANTS BY
RANDOMLY AMPLIFIED POLYMORPHIC DNA**

Key words: RAPD markers, anther culture, *Solanum sp.*

Abstract: As a novel application, RAPD analysis was used to characterize the genetic composition of anther-derived plants. The ploidy level of anther-derived plants was first determined by flow cytometry. Two sets of anther-derived plants from diploid potato clones CP2 and APB3 were analyzed for their genetic compositions. A total of sixty decamer primers was screened for polymorphism within each family. The RAPD fragments amplified from various anther-derived diploid plants were compared with those from anther donor and anther-derived monoploids. The loci which segregated within each family were selected and scored, the anther-derived monoploids have only half as many loci carrying RAPD markers compared to the anther donor. A clear separation between anther donor and anther-derived monoploids was also obtained by scoring the total number of scorable bands that exhibited polymorphism within each family. Six anther-derived diploids in APB3 family were identified as heterozygous diploids since they exhibited band patterns more similar to the anther donor. This provides a rapid and efficient way of indirect genetic analysis of an anther-derived population.

Introduction

Potato (*Solanum tuberosum* L.) is a highly heterozygous tetraploid and may harbor deleterious recessive alleles at many loci without any apparent effects on growth and performance (Uijtewaal et al. 1987). Homozygous lines may have various applications in potato breeding schemes because they are free of lethal genes and presumably possess a minimum of deleterious genes. Such lines can be obtained from other diploid *Solanum* species so that they can be incorporated into current cultivars to construct vigorous hybrids with wide genetic components, desirable traits, low genetic load and high level of heterosis.

The anther culture technique allows rapid production of homozygous lines equivalent to those produced after many generations of inbreeding. Nevertheless, regenerants from diploid plants may vary in ploidy level (Bajaj and Sopory 1986). In addition to monoploids, diploids and tetraploids are also obtained. Diploids can either be homozygous if derived by spontaneous chromosome doubling of monoploids during culture, or heterozygous if from unreduced microspores (Veilleux 1985). The source of tetraploids is even more ambiguous. The precise determination of the genetic architecture of these plants is important for using homozygous diploids in potato breeding programs.

Molecular markers are useful tools in assessing genetic variation and determining the genetic composition of anther-derived plants. Restriction fragment length polymorphism (RFLP) can identify DNA variation between individual samples. Since RFLP markers are codominant, heterozygous diploids which have two different alleles at

most loci show two bands whereas homozygous diploids which have two copies of the same allele per locus show only one band. Therefore, heterozygous diploids can be distinguished from homozygous ones with RFLPs. Using this method, Rivard et al. (1989) separated homozygous diploid from heterozygous lines of *S. chacoense* Bitt. produced by anther culture. Later, Meyer et al. (1993) also identified homozygous plants from a diploid anther-derived population by RFLP markers.

RFLP analysis, however, requires considerable technical skill and laboratory equipment. Also, appropriate DNA probes, Southern blotting, and radioisotopic labelling of probes are required. Randomly amplified polymorphic DNA (RAPD) analysis (Williams et al. 1991) uses short (10-mer) oligonucleotide primers to target homologous sites throughout the entire genome to amplify random sequences. Polymorphism among DNA samples is revealed by different amplified fragments due to the differences in the frequency of target DNA sequences. It has been reported that RAPD markers are sufficiently sensitive to detect naturally occurring polymorphism in *Solanum* species. RAPDs were used to select somatic hybrids (Baird et al. 1992) and detect genetic variation in androgenic monoploids of diploid *Solanum* species (Singsit et al. 1993). RAPD markers are suitable, and reportedly reliable, for use as genetic markers to detect segregation in F₂ populations of potato (Hosaka & Hanneman 1994), alfalfa (Echt et al. 1992), and oilseed rape (Tanhuanpaa et al. 1994). However, a major difference between RAPDs and RFLPs is that RAPD markers behave as dominant alleles. Therefore, the difference between a heterozygous anther donor and an anther-derived monoploid would be that the monoploid is expected to carry fewer RAPDs due to segregation of those that

were heterozygous in the anther donor, i.e., monoplasts will carry a number of null alleles. Where a RAPD is homozygous in an anther donor, all monoplasts derived from it should carry the same RAPD marker. If RAPD markers can separate homozygous from heterozygous diploids in anther-derived populations, it would be technically more efficient than RFLP analysis.

The purpose of this study is to distinguish the homozygous and heterozygous diploid plants produced by *in vitro* anther culture by RAPD analysis.

Materials and Methods

Plant materials

Plant materials included anther-derived plants from two diploid potato clones CP2 and APB3. CP2 is a hybrid between *S. chacoense* clone 80-1 as female parent and *S. phureja* clone 1-3 as male parent. APB3 is a clonal selection of APB population [*S. andigena* × (*S. phureja* × *S. berthaultii*)]. Anther culture and plant regeneration followed the procedure described by Taylor and Veilleux (1992). The ploidy level of anther-derived plants was determined by flow cytometric examination (Epics V, Model 752 laser flow cytometer and cell sorter, Coulter Electronics, Hialeah FL) of propidium iodide stained nuclei from *in vitro* leaf and stem tissue (Owen et al. 1988). Laser excitation was 300 mW at 488 nm from a 5 W Innova 90 Argon Laser (Coherent Inc., Palo Alto, CA).

The RAPD patterns of anther donors and anther-derived monoploid plants were compared to those of anther-derived diploids.

DNA isolation

DNA was isolated from *in vitro*-grown anther donor and anther-derived plants according to Singsit et al. (1993) with some modifications. The material was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Extraction buffer (0.1 M Tris-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% hexadecyltrimethylammonium, 1% fresh 2-mercaptoethanol, pH 8.0) was added at 3.3 ml for each 1 g plant material. The slurry was transferred to an autoclaved microcentrifuge tube and incubated in a 60°C water bath for 1-2 h. Then chloroform/isoamyl alcohol (24:1) was added at equal volume to the slurry, the tube was mixed by inverting 6-10 times and centrifuged for 15 min. The aqueous phase was transferred into a new autoclaved microcentrifuge tube containing 200 µl of cold isopropanol and the microcentrifuge tube was gently shaken to precipitate the genomic DNA. The tube was incubated at -20°C overnight, and centrifuged at 1500 rpm for 10 min. The pellet was washed twice with cold 75% ethanol, air-dried, then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and treated with RNase (10 µl/ml). The DNA concentration was measured using a mini-fluorometer (Hoefer Scientific Instruments) at the absorbance of 260 nm.

RAPD analyses

DNA samples included anther donors and anther-derived plants. The 10-mer RAPD primers were obtained from Operon Technologies. Three sets of 20 primers (Kit OPA, OPC, and OPG) were screened. The amplification conditions were as described by Williams et al. (1991) with some modifications: the reaction mixture (25 μ l) contained 50 ng of genomic DNA, 0.6 μ M primer, 200 μ M dATP, dTTP, dCTP, dGTP, 1 unit of *Taq* polymerase (Perkin Elmer Cetus), 1x Cetus buffer (gelatin free) which included 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, overlaid with a drop of mineral oil. The amplification reactions were carried out using a Perkin Elmer Cetus thermal cycler under the following conditions: 94°C for 1 min (denaturation), 37°C for 1 min (annealing), 72°C for 2 min (extension) for 45 cycles, followed by 4 min at 72°C and a 4°C soak until recovery. The amplified products were assayed by electrophoresis in 1.4% agarose-TBE gels in standard conditions (Sambrook et al. 1989). The gels were stained with ethidium bromide and photographed on a UV transilluminator (Sambrook et al. 1989).

Data analysis

Only those RAPD markers that revealed polymorphism within each family were selected for analyses of anther-derived plants. The RAPD fragments amplified from various anther-derived plants were compared with those from anther donor and anther-

derived monoploid plants. Two scoring methods were used in this study: (1) scoring only clearly segregating bands in each family; (2) scoring all scorable bands, that is, both monomorphic and segregating bands. If any band was uncertain for any of the plants within each family, that band was not used.

Results and Discussion

Sixty decamer oligonucleotides were screened in RAPD analysis using genomic DNA of two diploid anther donors (CP2 and APB3) and an anther-derived monoploid from each. The results of PCR reactions with the 60 primers ranged from no amplification, smear, to discrete bands. Examples of several different types of banding patterns that resulted from RAPD reactions are shown in Fig. 1. As a result, 28 decamers which showed clear and meaningful segregation in each family were selected for RAPD analyses of the genetic composition of anther-derived plants. The sequences of these primers are shown in Table 1. Twenty-three primers including 3 from OPC Kit A, 13 from Kit C, and 7 from Kit G, were used in RAPD analysis of the six samples from the CP2 family. For the eight samples from APB3 family, twelve primers, 5 from Kit C and 7 from Kit G, were used in RAPD analysis. Since each primer amplifies DNA throughout the genome during RAPD-PCR reaction, different bands varying in size are presumably amplified from target sequences at different loci. Each amplified RAPD band was thus assigned as a locus. The nomenclature for an individual RAPD locus describes the primer and the position of band detected in the gel, e.g., "A1-1" indicates the first

band from the top, or the largest fragment amplified by the primer OPA-01. The total number of segregating bands for individual samples was scored. Examples of RAPD polymorphism detected in both CP2 and APB3 families are shown in Figs. 2 and 3.

The RAPD bands or loci that segregated in the CP2 and APB3 families are shown in Tables 2 and 3, respectively. RAPD markers segregate in a dominant fashion (Williams et al. 1991), i.e., the presence of an amplified band is dominant to its absence. All genotypes containing the dominant allele or the presence of a RAPD band have been designated as "1", whereas those missing the band at the same site in a gel are designated as "0". Because the absence of a band (allele "0") is recessive to the presence of a band (allele "1"), genotype "0" could be either homozygous "00" or monoploid "0".

In our experiment, 5 known monoploids (C2-C6) and 1 known heterozygous diploid (anther donor C1) were tested (Table 2). The anther donor (C1) has genotype "1" at all of the 41 segregating loci, whereas the monoploids (C2-C6) have genotype "1" at 14 to 18 (34-44%) loci (Table 2). In other words, each monoploid had genotype "0" at more than half of the loci where the anther donor had "1". Loci C5-3, C5-8, C7-2, C15-3, C16-4, C17-1, G9-3, G14-7, G15-2 all had genotype "1" in the diploid anther donor, but had genotype "0" in all five anther-derived monoploids. The chance of such an event occurring randomly is 0.03 (0.5^5), indicating possible lethal alleles at those loci. However, due to small sample size tested, conclusive evidence of the lethality of these alleles will require further investigation. Monoploids C5 and C6 share the same band pattern at all segregating loci. The chance of such an event occurring randomly is 4.55×10^{-13} (0.5^{41}), indicating that both monoploids may have been derived from the same

microspore (Table 2).

In the APB3 family, only a single monoploid (B8) was available. Therefore, the comparison was made between the anther donor, the monoploid, and 6 anther-derived diploids (B2-B7). The anther donor (B1) has genotype "1" at all of the 26 segregating loci, whereas the monoploid (B8) has genotype "1" at only 6 (23%) loci (Table 3). In anther-derived diploids, however, genotype "1" was found at 18 to 24 (69-92%) of the 26 segregating loci. None had the same band pattern as the anther donor, indicating they are partially heterozygous. Therefore, they are not originated from somatic tissue. Instead, they most likely result from 2n microspores that were generated via FDR, since most of loci have genotype "1" (Table 3). The "1" allele at locus C2-6 and C20-4 was absent in all anther-derived APB3 plants, suggesting a possible association with deleterious effects at these loci.

Another way to estimate genetic differences between an anther-derived heterozygous and homozygous diploid is to score the total number of scorable RAPD bands that exhibited polymorphism within each family (Tables 4 & 5). A total of 60 primers was screened and those that showed polymorphism within each family were selected. Twenty-four (40%) primers were selected for analysis of CP2 family, and eighteen (30%) primers for APB3 family. As expected for both CP2 and APB3 families, the total number of RAPD bands amplified from monoploids was less than from the diploid anther donor. In the CP2 family, as shown in Table 4, the number of bands from anther-derived monoploids (C2-C6) ranged from 69 to 71 (74-76%), compared with 93 from the anther donor CP2 (C1). In APB3 family, the single monoploid (B8) had total

of 61 (69%) amplified bands compared with 88 for the anther donor. The six anther-derived diploids had amplified bands ranging from 77 to 84 (88-95%) which was close to the number of bands amplified from anther donor B1 (Table 5). This can be taken as indirect evidence that the anther-derived diploids were heterozygous because homozygous diploids would be expected to exhibit the same frequency of RAPDs as the monoploids, i.e., approximately 69-76% of the anther donor as observed in the two families in the present study. Anther-derived monoploids C5 and C6 again appear to be identical on analysis of the total number of scorable bands (Table 4) providing additional evidence of their origin from the same microspore.

In conclusion, RAPD markers have the potential to separate homozygous doubled monoploids from heterozygous diploids among anther-derived plants. Care should be taken that sufficient markers are used. Since RAPDs are dominant markers, the genotype at an individual locus cannot be ascertained. However, when sufficient markers were used, the frequency of RAPDs present in a group of known monoploids is expected to be the same as in homozygous diploids compared with those in the anther donor.

Heterozygous diploids are expected to exhibit a higher frequency more similar to the anther donor. This provides a rapid and efficient way of indirect genetic analysis of an anther-derived population.

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Table 1. The sequences (5'-3') of the oligonucleotide primers used in RAPD analysis.

Primer	Sequence	Primer	Sequence
OPA-01*	CAGGCCCTTC	OPC-17*	TTCCCCCAG
OPA-02*	TGCCGAGCTG	OPC-18*	TGAGTGGGTG
OPA-04*	AATCGGGCTG	OPC-19*	GTTGCCAGCC
OPC-01*	TTCGAGCCAG	OPC-20**	ACTTCGCCAC
OPC-02 ⁺	GTGAGGCGTC	OPG-02**	GGCACTGAGG
OPC-04 ⁺	CCGCATCTAC	OPG-03**	GAGCCCTCCA
OPC-05*	GATGACCGCC	OPG-05**	CTGAGACGGA
OPC-06*	GAACGGACTC	OPG-08 ⁺	TCACGTCCAC
OPC-07*	GTCCCGACGA	OPG-09*	CTGACGTAC
OPC-09*	CTCACCGTCC	OPG-14*	GGATGAGACC
OPC-10*	TGTCTGGGTG	OPG-15*	ACTGGGACTC
OPC-13**	AAGCCTCGTC	OPG-17 ⁺	ACGACCGACA
OPC-15**	GACGGATCAG	OPG-18**	GGCTCATGTG
OPC-16*	CACACTCCAG	OPG-19 ⁺	GTCAGGGCAA

* Primers used for analysis of CP2 family.

⁺ Primers used for analysis of APB3 family.

Table 2. Segregating RAPD bands amplified from a set of primers in anther-derived plants of CP2 family. C1 is the anther donor CP2, and C2-C6 are monoploid plants derived from CP2. "1" = band present; "0" = band absent.

Locus	C1	C2	C3	C4	C5	C6
A1-1	1	0	0	0	0	0
A1-2	1	1	1	0	1	1
A2-7	1	0	0	1	0	0
A4-1	1	1	1	0	1	1
A4-3	1	1	1	0	1	1
A4-6	1	0	0	1	0	0
A4-8	1	1	1	0	1	1
C1-1	1	0	0	1	0	0
C5-3	1	0	0	0	0	0
C5-5	1	0	1	1	1	1
C5-8	1	0	0	0	0	0
C6-5	1	0	0	1	0	0
C7-2	1	0	0	0	0	0
C9-1	1	1	0	0	0	0
C9-3	1	1	1	0	1	1
C10-2	1	1	1	0	1	1
C13-1	1	1	1	0	1	1
C13-2	1	1	1	0	1	1
C13-3	1	1	1	0	1	1
C13-4	1	0	0	1	0	0
C15-3	1	0	0	0	0	0
C15-4	1	1	1	0	1	1
C16-4	1	0	0	0	0	0
C17-1	1	0	0	0	0	0
C17-2	1	0	0	1	0	0
C18-5	1	0	0	1	0	0
C18-7	1	1	1	0	1	1
C19-1	1	0	0	1	0	0
C19-3	1	1	1	0	1	1
C20-2	1	1	1	0	1	1
G2-3	1	0	0	1	0	0
G3-3	1	0	0	1	0	0
G5-3	1	0	0	1	0	0
G5-5	1	1	1	0	1	1
G9-1	1	1	1	0	1	1
G9-3	1	0	0	0	0	0
G14-4	1	1	0	0	1	1
G14-7	1	0	0	0	0	0
G15-2	1	0	0	0	0	0
G15-3	1	1	1	0	1	1
G18-8	1	0	0	1	0	0
Total	41	18	17	14	18	18
Ploidy	2x	1x	1x	1x	1x	1x

Table 3. Segregating RAPD bands amplified from a set of primers in anther-derived plants of APB3 family. B1 is the anther donor APB3, B2-B7 are anther-derived diploids, and B8 is an anther-derived monoploid from APB3. "1" = band present; "0" = band absent.

Locus	B1	B2	B3	B4	B5	B6	B7	B8
C2-4	1	1	0	1	1	1	1	1
C2-5	1	1	0	1	1	0	1	1
C2-6	1	0	0	0	0	0	0	0
C4-1	1	1	1	1	1	1	0	1
C4-3	1	1	1	1	1	1	1	0
C4-4	1	1	1	1	1	1	0	1
C4-6	1	1	1	1	1	1	0	1
C13-2	1	1	1	1	1	1	1	0
C15-3	1	1	0	1	0	0	0	0
C15-5	1	1	0	1	1	0	1	0
C20-2	1	1	1	1	1	1	1	0
C20-3	1	1	1	1	1	1	1	0
C20-4	1	0	0	0	0	0	0	0
G2-1	1	1	1	1	0	1	1	0
G2-2	1	1	1	1	0	1	1	1
G2-3	1	0	1	1	0	1	1	0
G2-4	1	1	1	1	0	1	1	0
G2-5	1	1	1	1	0	1	1	0
G3-1	1	1	1	1	1	1	1	0
G3-9	1	1	1	1	1	1	1	0
G5-6	1	1	1	1	1	1	0	0
G8-6	1	1	1	1	1	1	1	0
G8-7	1	1	1	1	1	1	1	0
G17-4	1	1	1	1	1	1	1	0
G18-2	1	1	1	1	1	1	1	0
G19-5	1	1	1	1	1	1	1	0
Total	26	23	20	24	18	21	19	6
Ploidy	2x	2x	2x	2x	2x	2x	2x	1x

Table 4. Total number of scorable RAPD bands that exhibited polymorphism amplified from a set of primers in anther-derived plants of CP2 family. Plant code as in Table 2.

Primer	C1	C2	C3	C4	C5	C6
A1	5	4	4	4	4	4
A3	3	2	2	3	2	2
C1	1	0	0	1	0	0
C5	5	3	3	3	3	3
C6	5	3	3	4	3	3
C7	3	2	2	2	2	2
C9	4	4	3	2	3	3
C10	3	3	3	1	3	3
C12	3	2	2	2	2	2
C13	3	2	2	2	2	2
C15	2	2	2	1	2	2
C16	4	3	3	3	3	3
C17	3	1	1	2	1	1
C18	5	4	4	5	4	4
C19	6	5	5	6	5	5
C20	2	2	2	1	2	2
G2	4	3	3	4	3	3
G3	2	1	1	2	1	1
G5	5	4	4	4	4	4
G9	3	2	2	1	2	2
G14	6	6	5	5	6	6
G15	3	2	2	1	2	2
G18	5	4	4	4	4	4
G19	8	7	7	7	7	7
Total	93	71	69	70	70	70
Ploidy	2x	1x	1x	1x	1x	1x

Table 5. Total number of scorable RAPD bands that exhibited polymorphism amplified from a set of primers in anther-derived plants of APB3 family. Plant code as in Table 3.

Primer	B1	B2	B3	B4	B5	B6	B7	B8
C2	6	5	3	5	5	4	5	5
C4	7	7	7	7	7	7	5	6
C13	7	7	7	7	7	7	7	6
C15	5	6	2	5	5	2	3	3
C18	2	2	2	2	2	1	2	1
C20	4	4	3	4	4	4	4	2
G3	8	8	8	8	8	8	8	6
G5	7	8	8	8	7	8	7	6
G6	4	4	4	4	4	4	4	3
G7	3	2	2	2	2	2	2	2
G8	6	6	6	6	6	6	6	4
G9	5	4	4	4	4	4	4	4
G10	5	4	4	4	4	4	4	3
G12	5	4	4	4	4	4	4	1
G13	3	3	3	3	3	3	2	2
G17	4	4	4	4	4	4	4	3
G18	2	2	2	2	2	2	2	1
G19	5	4	4	4	4	4	4	3
Total	88	84	77	83	82	78	77	61
Ploidy	2x	2x	2x	2x	2x	2x	2x	1x

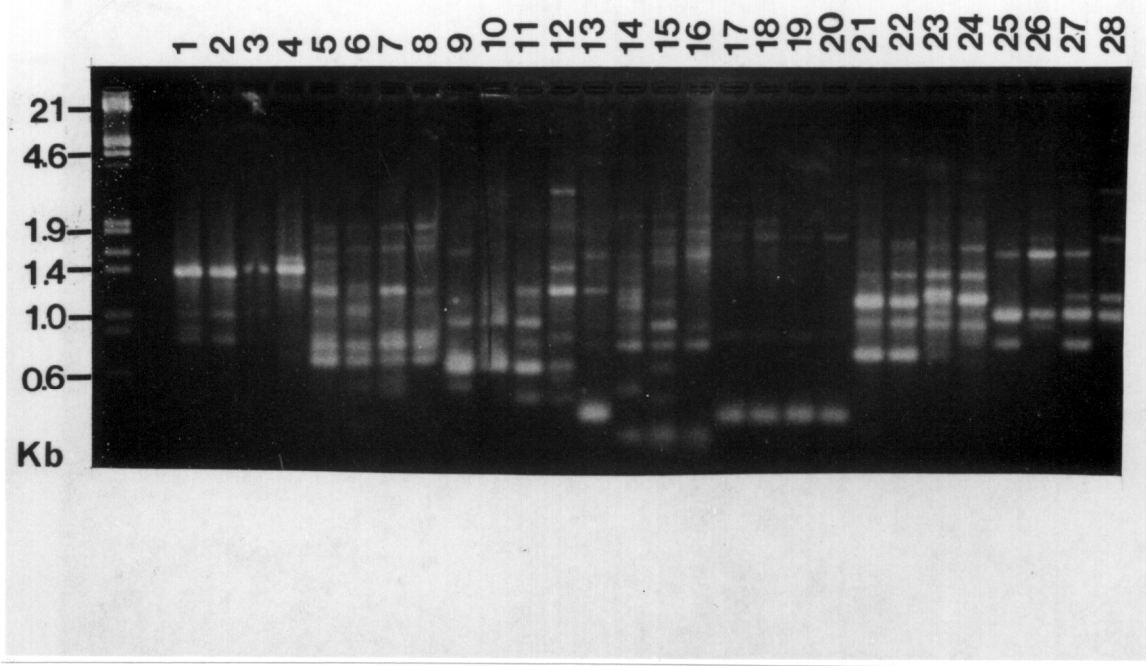


Fig. 1. Examples of RAPD reactions that produced different band patterns. From left to right, primers OPG-15, OPG-14, OPG-13, OPG-12, OPG-11, OPG-10, OPG-09 were used for each four DNA samples. Within each set are contained anther donor C1 (lanes 1, 5, 9, 13, 17, 21, 25), its anther-derived monoploid C2 (lanes 2, 6, 10, 14, 18, 22, 26), anther donor B1 (lanes 3, 7, 11, 15, 19, 23, 27), and its anther-derived monoploid B8 (lanes 4, 8, 12, 16, 20, 24, 28). The left lanes (M) contains *EcoR* I and *Hind* III digested lambda DNA. Some primer and DNA combinations show polymorphism (e.g., OPG-09, lanes 25, 26, 27, 28) whereas others show no segregation (e.g., OPG-11, lanes 17, 18, 19, 20).

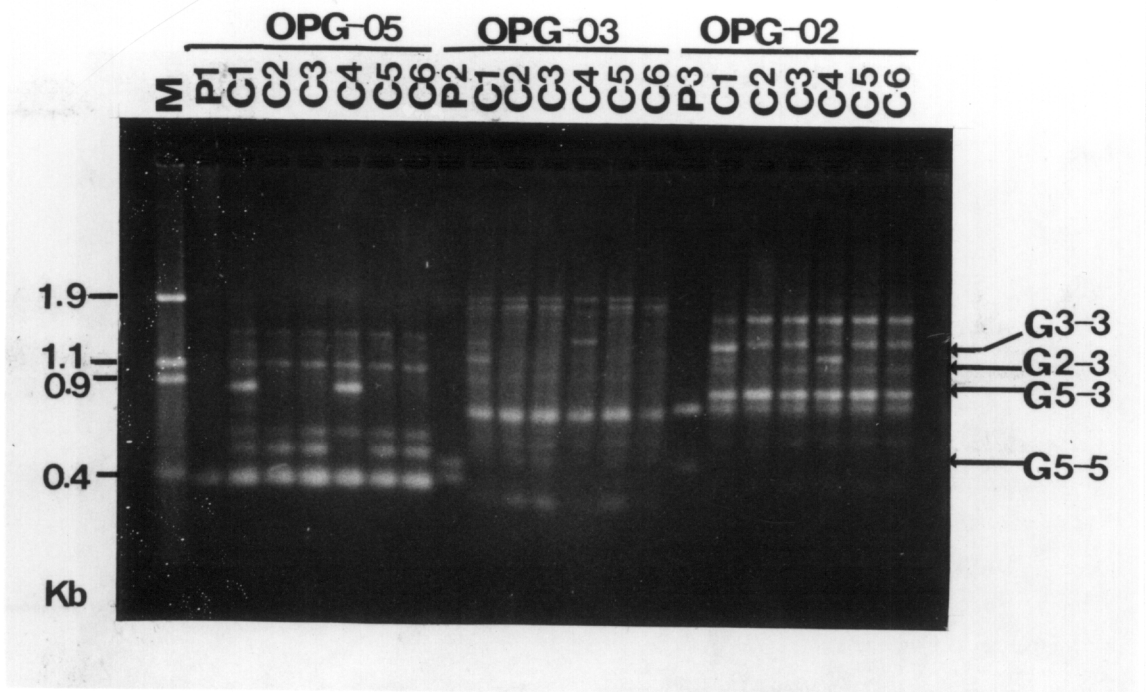


Fig. 2. Examples of RAPD polymorphism detected in CP2 family. Plant code as in Table 2. From left to right, primers OPG-05, OPG-03, OPG-02 were used for each 6 DNA samples. The left lane (M) is *Bst*N 1 digested pBR 322 marker. P1 and P2 are controls containing reaction mixtures with *Taq* polymerase and corresponding primers but no genomic DNA. Note segregating loci G5-3, G5-5 with primer OPG-05, G3-3 with OPG-03, G2-3 with OPG-02.

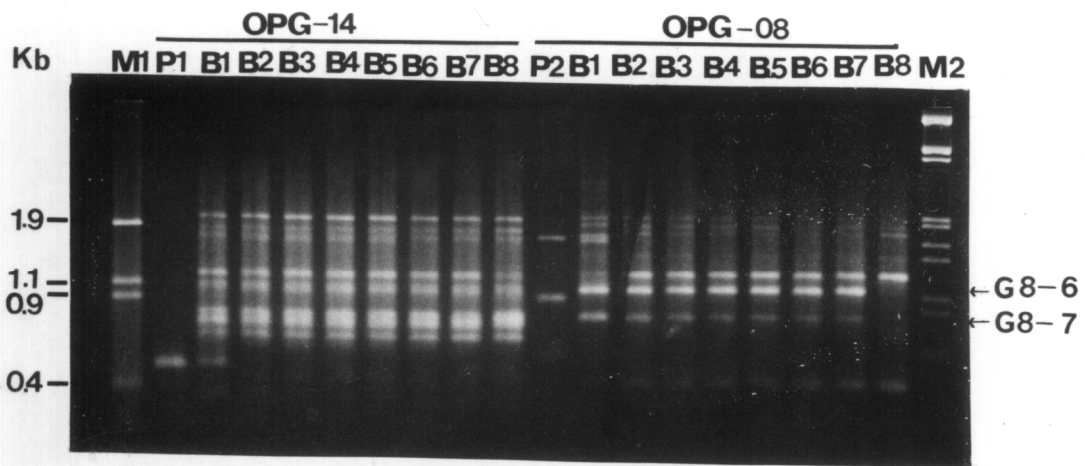


Fig. 3. Examples of RAPD polymorphism detected in APB3 family. Plant code as in Table 3. From left to right, primers OPG-14 and OPG-08 were used for each 8 DNA samples. The left lane (M1) is *Bst*NI digested pBR 322 marker, the right lane (M2) is *Eco*R I and *Hind* III digested Lambda DNA. P1, P2 and P3 are controls containing a reaction mixtures with *Taq* polymerase and corresponding primers but no genomic DNA. Note segregating loci G8-6, G8-7 with primer OPG-08. No polymorphism was detected with primer OPG-14.

APPENDIX: GAMETE SELECTION FOR HEAT TOLERANCE IN DIPLOID POTATO

Key Words: gamete selection, heat tolerance, pollen selection, anther culture selection

Abstract: The present study was designed to assess the feasibility of utilizing gamete selection as a means of developing heat tolerant potato germplasm. Pollen viability tests showed that temperatures higher than 35°C and incubation time longer than 24 h resulted in low pollen viability. Based on this pollen viability test, the temperature for pollen selection was set at 35°C 24h and later reduced to 35°C 4h because no seed set occurred at the harsher treatment. Anther culture selection was set to 35°C 12h followed by incubation at 30°C 16h/20°C 8h. Control treatments in both were at 20°C. Gamete selection was performed by exposing pollen to heat stress before pollination or incubating anthers under high temperatures. The agronomic performance of progenies was evaluated by Dr. D. Levy in Israel in a controlled high-temperature (30°C day/ 20°C night) environment. Although significant differences were occasionally observed between selected (S) and non-selected (NS) seed progenies resulting from pollen selection, e.g., greater haulm weight or stem length for S seedlings, such differences were inconsistent among families. Tuberization under heat stress was minimal. The agronomic performance of progenies resulting from anther culture selection are currently underway.

Introduction

The cultivated potato (*Solanum tuberosum* L.) was originally adapted to cool climates and short days in mountainous equatorial regions of South America. Over the years, potato cultivation in the temperate regions resulted in selection for tuberization under long days. However, in the subtropics of Asia and Africa, potato crops exposed to high day and night temperatures produce a significant lower tuber yield and quality (Ewing 1981; Levy 1986). Various tuber deformities, such as secondary growth, enhanced sprouting, reduced dormancy and poor keeping quality often limit potato production in warm regions (Levy 1986).

Breeding for heat tolerant cultivars has been focused on germplasm developed through interspecific hybridization. Within *S. tuberosum*, heat tolerance has been found to vary among cultivars (Levy et al. 1990, 1991). *S. andigena*, *S. phureja*, *S. berthaultii*, *S. microdontum* and *S. chacoense* have often been suggested as additional gene pools for heat tolerance (Mendoza and Estrada 1979, in Ewing 1985; Reynolds and Ewing 1989; Simmonds 1971). Intercrossing these different species followed by selection of heat tolerant derivatives under high temperature may be a promising route to develop heat tolerant breeding lines. Selection at the gametophytic level has the potential to become an alternative approach.

There has been increasing interest in the possible role of gamete selection in plant breeding (Hormaza & Herrero 1992; Landi et al. 1989). The basis of gametophytic selection is that selection at the gametophyte level could be positively correlated with

changes in the following sporophytic generation. Overlapping gene expression in both gametophytic and sporophytic phases as well as correlation in behavior of both phases in response to different external agents such as temperature, salinity, metals, herbicides, fungal toxin and other factors support this theory (Hormaza & Herrero 1992). Successful examples include selection for heat-stress tolerance in maize (Petolino et al. 1990) and cotton (Rodriguez-Garay & Barrow 1988). By exposing gametes to high temperature during pollination and fertilization (Petolino et al. 1990) or incubating pollen under high temperature before pollination (Rodriguez-Garay & Barrow 1988), the progenies from such crosses were more resistant to the high temperature.

While most gamete selection has involved pollen selection, gamete selection can also be achieved *in vitro*. Selection during anther culture can expose both gametoclonal variation induced by anther culture system (Evans et al. 1984) and the variation due to meiotic recombination in a heterozygous anther donor (Veilleux 1985). The advantage of using an anther culture system over pollen selection is that selection for stress can be combined with passage of androgenic embryos through the "monoploid sieve" (Wenzel et al. 1979) to pursue rapid development of homozygous lines free of lethal alleles (Lashermes 1991). A microspore culture selection system has been used to select herbicide and pathogen resistance *in Brassica napus* (Ahmad et al. 1991; Swanson et al. 1988), wheat (Fadel and Wenzel 1993), and oilseed rape (Kenyon et al. 1987). In barley, an anther culture selection system has also been used to improve salt tolerance (Ya et al. 1987). However, no instance of using anther culture to select for heat-stress tolerance has been reported.

The objective of this study is to assess the feasibility of utilizing both pollen selection and anther culture as a means of developing heat-stress tolerant potato germplasm.

Materials and Methods

Plant materials

Diploid clones APB3 and APM1, tetraploid potato cultivar Atlantic (*Solanum tuberosum* L.), doubled monoploid *S. phureja* Juz. & Buk. clone AD 2-4 were used in this study. APB3 and APM1 were produced between dihaploid *S. andigena* as female, and one of F₁ (*S. phureja* x *S. berthaultii*) and F₁ (*S. phureja* x *S. microdontum*) as male, respectively. *S. berthaultii* and *S. microdontum* accessions (PI 2658958 and PI 320304) used to develop these complex hybrids had been previously selected for heat tolerance (Reynolds and Ewing, 1989). APB3 and APM1 were selected from APB and APM populations, respectively, for their anther culture competence and high 2n pollen frequencies. AD 2-4 was obtained through *in vitro* shoot regeneration of a monoploid that was derived by anther culture of a heterozygous clone of *S. phureja*.

Ten plants each of APB3 and APM1 clones were planted in the greenhouse as pollinators, and ten plants each of Atlantic and AD2-4 were planted as female parents. Tubers were planted in 7.6 l pots containing 1 : 1 : 1 of sand : weblite (Weblite Co., Roanoke, VA.) : Sunshine mix (Fisons Horticulture Inc., Vancouver, BC, Canada).

Sixteen-hour day length was provided by high pressure sodium vapor lamps to promote flowering. Plants were fertilized weekly with soluble fertilizer (Peter's Fertilizer Products, W.R. Grace & Co., Fogelsville, Pa) containing 20:19:18 (N:P₂O₅:K₂O).

Pollen viability assay

Pollen from flowering plants was collected in microcentrifuge tubes, sealed with parafilm, and placed in petri dishes humidified with moist filter paper. Petri dishes were then sealed with parafilm and incubated at different temperatures. After different periods of temperature treatments, pollen was incubated for 30 min at room temperature in a 0.5 M sucrose solution to which fluorescein diacetate (FDA) (2 mg dissolved in 1 ml acetone) had been added. After incubation, the samples were washed by adding drops of water and centrifuged for 1 min, the supernatant was removed with a Pasteur pipet. This procedure was repeated until the solution was clear and colorless (Trognitz 1991).

The samples were then filtered through a 75 µm nylon mesh and analyzed with an Epics V, Model 752 laser flow cytometer and cell sorter (Coulter Electronic, Hialeah FL). Because only viable pollen absorbs FDA stain, it can be distinguished from dead pollen by absorbance when passing through a laser beam. The peaks of fluorescence intensities for viable and dead pollen were selected so that the percentage of viable pollen detected by flow cytometry was equal to that measured microscopically. Ten thousand pollen grains were counted per sample. The percentages of viable pollen of 3 samples of each of the 5 temperature treatments were analyzed using a randomized complete block

design. Analysis of pollen viability was based on square root transformation of percent data using SAS GLM procedure (SAS 1985).

Pollen selection

Pollen samples were collected from flowering plants APB3 and APM1 and treated at 35°C for 24 h, 12 h, or 4h. Treated pollen samples were used to pollinate Atlantic and AD 2-4. Fresh pollen samples were also collected as control. For each clone, pollination was done on the same inflorescence of pistillate parent, half with heat treated pollen and half with control pollen. Fruits were harvested approximately 6-8 weeks after pollination and stored for three weeks before seeds were extracted. The progenies resulted from select and non-selected populations were called S and NS, respectively.

Anther culture selection

Anther culture selection data presented in this study were from Chapter 2. APB3 and APM1 were used as anther donor plants. The anther culture and plant regeneration followed the procedure described by Taylor & Veilleux (1992). Anthers were cultured either continuously in 20°C or treated with different temperature treatments. The plants regenerated from control and temperature treatments were called C and HT populations, respectively. The ploidy of regenerated plants was analyzed by flow cytometry (Owen et al. 1988). Only diploid plants were placed on the microtuberization medium (Espinoza

et al. 1984): MS basal medium (Murashige & Skoog, 1962) supplemented with 8% sucrose, 5 mg/l BAP, 500 mg/l chlorocholine chloride (CCC), 100 mg/l myo-inositol and 3.5 g/l gelrite, pH 5.7-5.8. After 5-6 months, microtubers were harvested and shipped to Israel for agronomic evaluation in controlled greenhouses.

Agronomic evaluation

The selected and non-selected populations via either *in vivo* gametic selection (pollen selection) or *in vitro* gametic selection (anther culture selection) were sent to Israel. Progenies were sent as seeds, whereas anther-derived plants were sent as microtubers. In Israel, the clones were propagated and assessed for heat tolerance according to Levy (1984). The effectiveness of the *in vivo* or *in vitro* selection were assessed by haulm weight, node number, stem length, and the tuberization response under 30°C day/20°C night, in a controlled environment greenhouse.

Results

The effect of high temperature on pollen viability

In vitro pollen viability has been highly correlated with fertility in crosses (Trognitz 1991). Temperature is an important factor determining pollen viability (Egea et al. 1992). In this experiment, we treated pollen with different temperatures and

assessed the pollen viability with fluorescein diacetate (FDA) in order to determine the best selection intensity for pollen selection, i.e., the temperature at which most of the pollen is killed but what remains may carry heat tolerant genes. On the other hand, this temperature also provided a reference of selection temperature for anther culture selection.

Pollen samples were collected from APB3 and APM1 on the same date and incubated at 6 different temperatures: 20°C, 30°C 16h/20°C 8h, 35°C, 37.5°C or 40°C for 24 h, and 35°C for 48 h. There were 3 replications per treatment for each clone. The result is shown in Table 1.

Genotype and temperature treatments were both significant sources of variation ($p < 0.0001$ and 0.0106 , respectively). Control pollen had the highest percent pollen viability. Temperatures higher than 35°C and incubation time longer than 24 h resulted in very low pollen viability. Pollen viability after 30°C 16h/20°C 8h treatment was reduced by more than half compared to the control. Thus 35°C 24h appeared to be the best selection temperature among these 6 different treatments.

In vivo gametic selection via cross pollination (pollen selection)

Sporadic flowering of plants prevented our use of replicates of each clone for pollination on any given day. The differences between crosses and treatments (selected and non-selected populations) are given as fruit and seed set. Based on the pollen viability test, 35°C 24h was used as selection treatment for pollen. Pollen from APB3 and APM1 was incubated at 35°C for 24h before pollinating Atlantic or AD2-4. No fruit

from this treatment, however, was obtained from either cross despite considerable fruit set in the control (Table 2). Therefore, incubation times were reduced to 12h and 4h. Only 1 fruit was obtained from cross ATL x APM1 when pollen was incubated at 35°C for 12h. Reducing incubation time of pollen to 4h at 35°C resulted in more fruits from these crosses using pre-incubated pollen as pollinators; 1, 2, and 4 fruits were obtained from ATL x APM1, AD2-4 x APM1, AD2-4 x APB3, respectively; the number of seeds per fruit ranged from 21 to 76, providing sufficient population for further study. As a result, 3 pairs from cross AD2-4 x APB3 were sent to Israel for agronomic evaluation. The fruit set of these pairs is shown in Table 3.

Numerous fruits were obtained in control crosses. Although pollination resulting in fruit sets varied among different crosses, heat-treated pollen resulted in much less fruit than control, providing evidence that the heat treatments to pollen effectively reduced pollen viability (Table 2).

In vitro gametic selection via anther culture (anther culture selection)

From the study of temperature effect on pollen viability, selection temperatures for anther culture were designed between 35°C 24 h to 30°C 16h/20°C 8h, with 20°C as control. The 35°C 24h treatment was expected to be more lethal whereas 30°C 16h/20°C 8h would be less stringent. Several different temperature treatments were used during anther culture process (Table 4). Neither treatment with 35°C for 24h pre-incubation yielded many embryos for either clone. Interestingly, 35°C 12h-30°C/20°C yielded

considerably more embryos than control (20°C) for both clones. For APB3, a total of 711 embryos was produced from 35°C 12h-30°C/20°C treatment resulting in 140 regenerated plants, whereas only 53 embryos and 10 plants were produced from the control (Table 5). Only 2 plants were regenerated from clone APM1; therefore, they could not be used in further study. Regenerated plants from APB3 clone were tested for ploidy level. Only diploid plants regenerated from both 35°C 12h-30°C/20°C (HT) and control (20°C) (C) were used for further study. Ten clones from C and 50 clones from HT were randomly selected, and 20 copies each for C and 5 copies each for HT were placed on microtuberization medium. After 5-6 months, microtubers were harvested. Some clones produced no microtubers whereas others averaged two per plantlet. They were shipped to Israel for agronomic evaluation.

Agronomic evaluation

The plants resulting from both *in vivo* and *in vitro* gamete selection were evaluated for agronomic performance under high-temperature environments. Seeds from crosses AD2-4 x APB3 that developed from selected (S) and non-selected (NS) pollen were planted in Dept. of Field and Garden Crops, The Volcani Center, Bet Dagan, Israel. Agronomic performance of the resultant seedlings was evaluated pairwise by Dr. D. Levy at three different stages (screens 1, 2, and 3) under heat stress (30°C 16h/20°C 8h) in the greenhouse. Pairwise t-test was made between S and NS for haulm weight, node number and stem length measured at the three stages. Significant differences between the two

populations (S and NS) were found for P2 family in haulm weight, and for P3 family in stem length, both measured during screen 1. No difference, however, was found for these two traits of the same families measured in other stages. All other measurements made at any stages for any families had no significant differences between S and NS (Table 6). Tuberization under heat stress was minimal.

As a result of anther culture selection, 192 microtubers from the two temperature treatments were harvested and shipped to Dr. D. Levy in Israel for field trial. Only 64 of them survived. These microtubers have been planted in a replicated greenhouse trial to determine if the anther culture treatment has had any general effect on the performance of anther derived plants. The trial is currently in progress.

Discussion

Heat treatment of pollen prior to pollination had no consistent effect on the resulting hybrids with regard to heat tolerance. The lack of documented selection may be due to the following factors: the genotype of parent materials, the system used for selection, the evaluation system and the traits chosen to measure heat tolerance. The parent used as a potential source of heat tolerance in this study was a complex three-way interspecific hybrid (APB3); therefore, considerable segregation among pollen grains was expected. The pistillate parent in the cross was a homozygous doubled monoploid so that variation in the progeny should have reflected segregation in the pollen parent exclusively. Petolino et al. (1990) pointed out that the greater the level of tolerance in the original

pollen source the more effective the selection. They found that difference in agronomic performance between selected and non-selected progenies was greater in the single cross (S x H) between a highly heat tolerance source (H) and a heat sensitive cultivar (S) compared to the backcross [S x (S x H)] under heat stress conditions. In one backcross, selection did not result in superior agronomic performance (Petolino et al. 1990). In our study, pollen source APB3 was expected to segregate for heat tolerance, due to the contribution of potential heat tolerant alleles from the *S. berthaultii* genome to individual microspores. Heat tolerance in *S. berthaultii* (PI265858) had been assessed by vine vigor, fresh and dry weight after growing in a hot greenhouse (40°C day/30°C night) (Reynolds and Ewing 1989). Therefore, our evaluation of haulm weight and stem length under heat stress should have allowed us to observe heat tolerant contributions of the *S. berthaultii* genome to the progenies evaluated. The heat stress differed between the two studies. The less severe heat stress conditions used in the present study (30°C day/20°C night) may not have provided sufficient selection pressure for the population difference to be revealed.

Second, the selection system used in this study was to pre-incubate pollen under high temperature before pollination to eliminate heat-sensitive pollen. Hopefully what pollen remained carried heat-tolerant genes. This procedure may not have been as effective as the *in vitro* pollination system used by Petolino et al. (1990) where pollen tubes were required to grow and fertilize cultured ovules under controlled high temperature and humidity. It has been demonstrated that selection pressure may be higher during pollen tube growth than during pollen grain formation (Zamir & Vallejos 1983).

An example of selection pressure applied during pollen formation was reported by Rodriguez-Garay and Barrow (1988). Flowers were picked the day before anthesis and placed in a growth chamber at 35°C for 15h; selection was exerted during the pollen formation process. Increased heat tolerance, as measured by fertile pollen after heat treatment was observed in progenies resulting from a cross made with pollen treated for 15 h at 35°C. No field test at high temperatures was conducted to confirm the ultimate level of heat tolerance by the pollen selection method (Rodriguez-Garay and Barrow 1988). Pollen collected in our study, however, was mature. Selection at this stage may have been less effective than during pollen formation.

Third, the evaluation system for agronomic performance was destructive in order to measure haulm weight. Plants were discarded after each screen. The time from planting to destructive screen was obviously inadequate for tuberization. The traits selected to measure heat tolerance in our study were haulm weight, node number, and stem length. Different responses of various traits to selection pressure were reported by both Landi et al. (1989) and Petolino et al. (1990). Some traits were positively associated with selection, whereas other traits had no correlation or even a negative correlation with selection pressure. Landi et al. (1989) found that leaves per plant, kernel moisture, and plant height were actually higher in a non-selected population compared to a selected population. They postulated the negative correlation of these traits could have been due to higher pollen competitive ability. Observation of more traits in these progenies under a broader set of environments may have revealed population differences due to gametic selection that were undetected in the present study.

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Table 1. The effect of temperature on pollen viability (%).

Treatment		N	Pollen viability (%)
Temperature	20°C 24h	6	24.02 ^a
	30°C 16h/20°C 8h	6	11.05 ^a
	35°C 24h	6	6.48 ^b
	35°C 48h	6	0.30 ^b
	40°C 24h	6	0.21 ^b
	37.5°C 24h	6	0.00 ^b
Genotype	APB3	18	11.00 ^a
	APM1	18	3.02 ^b

Mean separation by SNK, 5% level. All analyses were conducted on the square root transformation of percent data. Comparison were made within each category, e.g., temperature or genotype.

Table 2. Number of pollinations, fruit set and seed set for four different crosses using either heat-treated or fresh pollen of 2 pollinators on 2 stylar parents.

Cross	Treatment	No. of pollinations	Fruit set	Seeds/fruit	
				Mean	Range
ATL x APM1	35°C 24h	68	0	0	0
ATL x APM1	control	68	25	168	25-346
ATL x APM1	35°C 12h	58	0	0	0
ATL x APM1	control	50	1	238	238
ATL x APM1	35°C 4h	26	1	52	52
ATL x APM1	control	23	2	98	69-126
ATL x APB3	35°C 24h	10	0	0	0
ATL x APB3	control	9	6	24	11-37
ATL x APB3	35°C 12h	18	0	0	0
ATL x APB3	control	17	8	21	9-35
ATL x APB3	35°C 4h	34	0	0	0
ATL x APB3	control	31	2	10	10**
AD2-4 x APM1	35°C 24h	8	0	0	0
AD2-4 x APM1	control	7	5	51	43-71**
AD2-4 x APM1	35°C 12 h	156	1	47	47
AD2-4 x APM1	control	149	89	55	24-114**
AD2-4 x APM1	35°C 4h	27	2	23	21-25
AD2-4 x APM1	control	23	2	55	51-59
AD2-4 x APB3	35°C 24h	28	0	0	0
AD2-4 x APB3	control	26	0	0	0
AD2-4 x APB3	35°C 12h	165	0	0	0
AD2-4 x APB3	control	162	74	90	31-136
AD2-4 x APB3+	35°C 4h	61	4	52	32-76
AD2-4 x APB3+	control	62	7	42	8-117**

* ATL: Atlantic.

** Seeds/fruit data were obtained from a part of fruit set.

+ The three seed pairs sent to Israel were selected from this cross.

Table 3. The seed pairs that were sent to Israel.

Family			No. of seeds
Pair 1	AD2-4 x APB3	35°C 4h	32
	AD2-4 x APB3	control	85
Pair 2	AD2-4 x APB3	35°C 4h	127
	AD2-4 x APB3	control	199
Pair 3	AD2-4 x APB3	35°C 4h	49
	AD2-4 x APB3	control	117

Table 4. Temperature treatments applied on anther culture selection.

Treatment abbreviation	Pre-incubation temperature	Subsequent incubation (6 weeks)
35°C 24h-30°C/20°C	35°C 24h	30°C 16h/20°C 8h
35°C 24h-20°C	35°C 24h	20°C
35°C 12h-30°C/20°C	35°C 12h	30°C 16h/20°C 8h
30°C/20°C	30°C 16h/20°C 8h	30°C 16h/20°C 8h
Control (20°C)	20°C	20°C

Table 5. Plants regenerated from anther culture selection under various temperature treatments.

Clone	Treatment	No. of anthers cultured	No. of embryos produced	No. of plants regenerated
APB3	35°C 24h-30°C/20°C	270	177	33
	35°C 24h-20°C	270	32	3
	35°C 12h-30°C/20°C (HT)	270	711	140
	30°C/20°C	270	180	20
	20°C (C)	270	53	10
	total	1350	1153	206
APM1	35°C 24h-30°C/20°C	270	4	0
	35°C 24h-20°C	270	7	0
	35°C 12h-30°C/20°C	270	87	0
	30°C/20°C	270	183	1
	20°C	270	10	1
	total	1350	291	2

Table 6. Agronomic performance of selected (S) and non-selected (NS) progenies from 3 paired crosses resulting from pollen selection.

Screen	Trait	Pair 1		Pair 2		Pair 3	
		NS	S	NS	S	NS	S
1	Haulm wt.	42	71	53*	95*	41	52
	Node no.	31	32	33	33	32	31
	Stem length	89	102	105	108	76*	107*
2	Haulm wt.	64	71	59	54	nt	nt
	Node no.	37	37	36	36	nt	nt
	Stem length	119	117	111	118	nt	nt
3	Haulm wt.	nt	nt	74	49	nt	nt
	Node no.	nt	nt	40	35	nt	nt
	Stem length	nt	nt	136	113	nt	nt

* significant at the 0.05 level.

nt: not tested.

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