HETEROPLOID GENE TRANSFERS IN *Vaccinium*, SECTION *Cyanococcus*

By

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Heteroploid Gene Transfers in *Vaccinium* Section *Cyanococcus*

By

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Two methods for interspecific gene transfers between 6x rabbiteye (*V. ashei* Reade) and 4x highbush (*V. corymbosum* L.) blueberries were investigated. One method involved an attempt to produce a synthetic 4x by crossing the 6x rabbiteye to the 2x *V. darrowi* Camp. This synthetic 4x could then be crossed to 4x highbush. The other method was an attempt to produce a synthetic 6x highbush by doubling 4x highbush with colchicine to produce an 8x individual and then backcrossing it to 4x highbush.

Two 6x-2x hybrids and one 8x plant were evaluated for fruit set, fruit weight, number of well developed seed/fruit and number of seedlings/pollination. The two 6x-2x hybrids differed significantly in all parameters except seeds/fruit when crossed as males onto 4x highbush

clones. The 8x clone was significantly less fertile than 4x controls in all parameters. Tetrapolid x octoploid crosses produced one 6x hybrid and 124, 4x seedlings from 739 pollinations. Chromosome numbers of seedlings from the 6x-2x hybrids reciprocally crossed to each other and 4x highbush have not been determined.

Meiosis in PMC's of the 8x appeared normal except for the occasional appearance of 2 extra nuclei at Telophase II. The 6x-2x hybrids showed numerous cytogenetic irregularities including 60 somatic chromosomes instead of the expected 48, 2 synezetic knots, 2 nucleolar organizing regions, lagging chromosomes, nonassociating chromosomes, meiotic nonsynchrony, micronuclei at Telophase I and II, nonparallel spindles, extra nucleoli, increased percentage of unreduced gametes, incomplete tetrads, and polyspory.

A technique for producing 8x plants from 4x highbush was developed using tissue culture and colchicine. A 7 day pretreatment of cold (4°C) darkness followed by a growth phase of 48 to 96 hours enhanced the effect of colchicine when 2-node cuttings were treated for 24 hours in liquid media. Modified Anderson's Rhododendron Medium produced highbush cultures with greater vigor than either modified McCown and Lloyd's Woody Plant Medium or modified Knop's Medium. Shoot proliferation of highbush was better with 10mg/l 2iP than 5mg/l, and as good as 20mg/l.

SECTION I

INTRODUCTION

The blueberry, Vaccinium spp., is a variable and wide-ranging genus (95). Many species are indigenous to North America (12) and the genus is especially diverse in the southeastern United States, with Florida alone having as many as 10 species (102). The basic chromosome number of the genus is usually considered to be 12 (23), with the 26 (36) North American species having one of 3 ploidy levels, 2x=24, 4x=48, or 6x=72.

Commercial blueberry production began in Florida with the transplanting of wild plants to cultivated conditions in the late 1800's (15). Controlled hybridizations were started in the early 1900's with the first cultivars from controlled crosses being released in 1920 (69). Breeding efforts have made considerable progress since these initial crosses (21, 39) and several scientists are currently involved with blueberry improvement.

Interspecific hybridization in Vaccinium has been responsible for evolution of the 4x and 6x complexes (39). It has also formed the basis for improvement in most breeding programs (70). The first hybrid cultivars were a result of crosses between V. corymbosum L. and V. australe Small (19). Coville (19) recognized the importance of considering chromosome number when hybridizing. Others have substantiated his observation, and it is generally accepted that only

weak crossing barriers exist between most Vaccinium species of equal ploidy. Crosses between species of unequal polidy are generally more difficult, with the resulting progeny having lowered fertility (19, 22).

All cultivated blueberries are either 4x or 6x. The 4x highbush complex (V. corymbosum and V. australe) is cultivated from North Carolina northward, while the 6x rabbiteye (V. ashei Reade) is limited to North Carolina southward. Traits of horticultural merit exist at all 3 ploidy levels, with each level having beneficial characteristics not found at the others (69). Because of this, interploid crosses have been used in breeding despite the known low success rate.

The interspecific cross of greatest commercial interest would be highbush x rabbiteye. This cross could combine low chilling requirement, vigor, high yield, and tolerance to drought, disease and heat of rabbiteye with early ripening and high fruit quality of highbush (11, 24). These crosses have been made and are one of the easiest interploid crosses. However, the progeny are 5x and have reduced fertility, complicating improvement at this level. Backcrossing to both highbush and rabbiteye has been done, but most of the progeny are noticeably lower in vigor than the parental species and the original F1 population (Lyrene: personal communication). It was therefore recognized that manipulation of the rabbiteye gene pool to create 4x plants, or manipulation of highbush to create 6x plants, needed to be done before the best qualities of each could be combined (24, 26).

Manipulation of the 6x rabbiteye gene pool to create 4x plants has been pursued through 6x x 2x crosses. V. darrowi Camp, V. tenellum Aiton, and V. elliottii Chap. have been the primary diploids used (26, 84). Some success has been attained when using V. darrowi, with

the Florida blueberry breeding program releasing 3 tetraploid cultivars which are complex hybrids of V. darrowi, V. ashei and V. corymbosum (85, 86). For the most part the 6x-2x hybrids have been used only to a limited extent in breeding cultivars.

Manipulation of the highbush to the 6x level is of more recent interest and involves doubling 4x highbush to 8x through the use of chemical agents. The 8x is then crossed to highbush, theoretically creating a 6x plant. Since the genome of the 6x highbush consists exclusively of highbush chromosomes, these may prove more useful in breeding.

Since 8x plants do not naturally occur in Vaccinium, potential plant vigor and fertility cannot be determined conclusively from observations on a few individuals. Several 8x clones should be produced before evaluation of the 8x and the 6x highbush produced from them.

This study describes research aimed at facilitating 4x-6x gene transfers, including 1) evaluation of the fertility of V. darrowi x V. ashei hybrids, with meiotic investigation and crossability studies between the hybrids and 4x highbush; 2) production of 6x highbush-types from 4x x 8x crosses; 3) meiotic investigation of 8x colchiploids; and 4) development of a rapid and efficient method for producing and screening 8x breeding lines from 4x plants.

SECTION II

LITERATURE REVIEW

The blueberry (Vaccinium spp.) probably served as a minor food source for native North American peoples long before its domestication. There is little doubt of its importance as a food source for wildlife (especially birds) who aid in seed dispersion of the various species. Orderly cultivation of blueberries began in Florida (15) with the transplanting of V. ashei Reade plants dug, often indiscriminately from the woods of northwest Florida (65). When these plants came into production they averaged low in yield and fruit quality. During the 1940's improved selections of the northern highbush blueberry (V. corymbosum L. and V. australe Small) were widely planted and have become commercially successful.

Since realization of their commercial potential, blueberries have rapidly become one of the major temperate fruits in North America (39). Research on blueberries has largely been conducted in the United States and Canada, but interest is increasing in Europe (81), Australia (Lyrene: personal communication), and New Zealand (79), as the fruit and its potential become more familiar. Blueberry research in the United States is carried on at both USDA and state experiment stations and much has been determined about its anatomy (36), morphology (36, 88, 97), breeding (21, 39, 69, 70), cultural practices (7, 67, 68, 100),

propagation (67), taxonomy (12, 36, 39, 99, 102), fruit processing (94) and marketing (78).

The person initially responsible for blueberry improvement was the USDA scientist F. V. Coville. He determined many of the cultural requirements and propagation practices necessary for development of the highbush blueberry industry (17, 18) and started the first breeding program. He made the first crosses in 1911 and the first highbush cultivars from controlled hybridization were released in 1920 (39). Breeding of rabbiteye (V. ashei) and lowbush (V. angusifolium Ait.) has received less attention, with the first hybrid cultivars released in 1950 (25) and 1975 (1), respectively.

Blueberries belong to the family Ericaceae, genus Vaccinium, section Cyanococcus and are generally considered to have a base chromosome number of x=12 (73). The diversity of the genus and rate at which gene exchange occurs between species have created much taxonomic controversy. Camp's (12) 1945 taxonomic treatment of the genus is the most extensive. More modern studies have been done (36, 99, 102) but are generally not well accepted, or are based largely on Camp's classifications.

Camp recognized 24 species in Cyanococcus: 9 diploids, 12 tetraploids, and 3 hexaploids. Polyploid species tend to be more widespread, better adapted, and of greater horticultural significance than diploids. Blueberry cultivars are classified commercially into 3 types; lowbush, highbush, and rabbiteye. Traits of horticultural merit exist in several other species (39), and interspecific crossing is important in most breeding programs (70).

Polyploidy and Wide Hybridization

Both wide hybridization and polyploidy play major roles in plant evolution and crop improvement. The 2 processes are often interrelated: in some cases polyploidy makes wide hybridization possible; in others wide hybridization leads to polyploidy (32). Both processes are so important to the development of modern crops that it would be hard to imagine the type of plants that would be cultivated if the 2 processes did not occur.

Stebbins', as cited in Goldblatt (40), estimated that 30-35% of the angiosperms were polyploid. White's (103) estimate was 40% and Grant's (42) 47%. Estimates differed primarily due to different ways of defining polyploidy and different estimation procedures (40). Stebbin's estimate includes as polyploids those species having gametic chromosome numbers that are multiples of the basic diploid number of their genus (intrageneric polyploidy). White's 40% is based on the observation that even haploid numbers exceed odd by 40% and he assumes that this number is largely attributable to polyploidy. Grant postulated that species with haploid numbers over 13 would mainly be polyploid, and those with 13 or less, predominantly diploid. Grant also stated that based on 17,138 species of angiosperms, 43% of the Dicotyledonae and 58% of the Monocotyledonae were polyploid.

A 1980 discussion (40) suggests that almost all angiosperms with haploid numbers above 9 and 10 probably have polyploidy in their evolutionary history and those with 11 or higher almost certainly do. However, plants of lower haploid numbers may also be derived from polyploid ancestors. At least 70% to 80% of the monocots may in some sense be polyploid. Lewis (61), in a similar survey of dicots,

concludes that 70% to 80% of all dicots are polyploid, indicating that incidence of polyploidy in flowering plants has been underestimated, and its significance in evolution is greater than previously assumed.

Polyploids are traditionally classified according to their assumed mode of origin into autoploids or alloploids. These 2 concepts were first proposed by Kihara and Ono (57). They describe autoploidy as a doubling of the diploid genome and alloploidy as hybridization followed by doubling of 2 different haploid genomes. These definitions were sufficient for their time, but as polyploidy has become better understood the definitions proved simplistic and often misleading, since most natural polyploids fall somewhere between autoploidy and alloploidy (32).

Polyploidy has been described as being a process rather than an event (32). The evolutionary and genetic implications of polyploidy are often discussed (31, 47, 55, 92) but modes of origin are not well understood. DeWet (32) discussed 3 ways polyploids might originate: zygotic chromosome doubling, meristematic chromosome doubling, and gametic chromosome nonreduction.

Zygotic chromosome doubling was first proposed by Winge in 1917 (104). It was his belief that when zygotic chromosomes were sufficiently different they failed to pair and the zygote died. However, if the chromosomes of the hybrid zygote split longitudinally, each chromosome had an homologous mate, permitting development of a hybrid individual with double the compliment of parental chromosomes. Supporting evidence for this method is lacking, and evidence exists which contradicts it (32).

Meristematic chromosome doubling as a means of polyploid formation is better supported by evidence (74) but because spontaneous somatic chromosome doubling is rare (31), it is probably not a major contributor to polyploid development.

Gametic chromosome non-reduction is currently thought to be the mode by which most polyploids have arisen (32, 46). Polyploid formation by this process involves a failure of chromosome reduction in Meiosis I, or a failure of cytokinesis in Meiosis II. Occurrence of functional, unreduced gametes has been reported in several plant families (32), with 2x hybridizations frequently resulting in polyploids (30).

Polyploids derived from non-reduced gametes are more commonly 3x than 4x. This is because the probability of fertilization of a rare unreduced female gamete by a rare unreduced male gamete is small. Also 2x pollen does not compete well with haploid pollen (32). The proposed sequence for polyploidization according to this process is for an unreduced gamete to be fertilized by a reduced gamete producing a triploid (2x x x = 3x). The triploid then produces an unreduced gamete which is fertilized by a normal gamete yielding a tetraploid (3x x x = 4x). Another possible route is the natural 4x x 2x interspecific hybridization with a 2n gamete from the 2x parent. This may be more important than the 3x route since sympatric 4x and 2x species are often represented by large populations whereas 3x plants are usually rare. Higher ploidies can then be derived from the 4x plants.

The probability of survival of a newly arisen polyploid is small and has been compared to that of a newly arisen mutant allele in a population of self-fertilizing individuals (105). Few of these polyploids survive beyond one generation and are usually eliminated in

competition with their parents. Success of new polypolids therefore depends on their ability to compete with their parents in similar habitats, in which case they will be sympatric, or to exploit areas not favorable to either parent, exhibiting allopatry. The competition polyploids face is further enhanced by the fact that they are generally less fertile than their parents. DeWet (32) states that competition for habitat has 2 components, ability of newly formed polyploid seedlings to become established, and ability of these seedlings to produce adapted offspring. He further states that such competition favors perennials, since once established they have several years to form desirable gene combinations capable of competing. Among modern genera, the highest frequency of polyploids occurs among herbaceous perennials, lowest among annuals, and woody perennials are intermediate (59).

The reduced fertility of newly produced polyploids compared to their diploid parents is especially evident among autoploids which are often characterized by chromosomal pairing irregularities and cytologically unbalanced gametes (32). Pairing irregularities in autopolyploids result because formation of trivalents and quadrivalents is now possible due to the pairing of homoeologous chromosomes. This is not the case in alloploids since the genomes are dissimilar enough to reduce or eliminate homoeologous chromosome pairing. Fertility of polyploids may be improved by selection. DeWet (32) reports that Gillie and Randolph noted a significant reduction of quadrivalents in tetraploid maize after 10 generations of sexual reproduction.

Polyploid plants, if successful, can offer several advantages over their parental diploid's. Polyploidy can lead to a loss or decrease in self-incompatability. Thus, some cross-fertilizing plants can become

self-fertilizing, making it possible for a single plant to reproduce (105). Polyploidy also has the effect of buffering the shock of absorbing foreign genomes as well as masking deleterious alleles. Also, species that are genetically isolated as diploids may cross as auto-tetraploids to produce amphidiploids.

Natural polyploids have been shown in some cases to have a wider range of adaptability than their parents. Hagberg and Akerberg (44) report an allotetraploid Galeopsis species having a distribution range 3 to 4 times greater than its parents. An increase in polyploids is seen with increases in latitude and altitude. This suggests that polyploids may be better adapted to withstanding severe climates. However, it is possible that these environments select for characters associated with polyploidy and not for polyploidy itself (53).

Allopolyploids are unique in that they maintain a state of permanent heterosis (44), even in self-pollinating species. If strict preferential pairing occurs in these polyploids heterozygosity is maintained between the homoeologous chromosomes. Therefore, a plant such as bread wheat (Triticum aestivum L.), even though inbred, maintains a large amount of heterosis between the A, B, and D genomes. Gene exchange through crossing over seldom occurs between homoeologous genomes. Thus, heterosis is not only beneficial for species survival but can also be exploited by the breeder.

Polyploids have several characteristics which make them commercially desirable. The principal characteristics that make them more attractive are that they generally have larger dimensions and greater adaptability (105). Larger dimensions include increased size of flowers, fruits, and seeds as well as roots, stems, and leaves. Larger

plant size may increase food and forage production as well as aesthetic value. Adaptability is important because it allows the same crop to be grown over a wide range of environments. Polyploids originating from domesticated crops may have higher value than domesticated crops that are polyploids since they may combine the advantage of first being a desirable crop as a diploid with the advantages of polyploidy (105).

The success and importance of polyploids are indicated by the large number of cultivated crops which are polyploid. The frequency of polyploidy seems to be higher in cultivated plants than in wild species. Also, in general where the species form a polyploid series, the species with higher chromosome numbers often have the greatest agricultural importance (43). Many agronomic crops such as wheat, tobacco, sugar cane, oats, cotton, and alfalfa are polyploids (105). Horticultural crops such as banana, many Brassicas, blueberries, sweet and Irish potatoes, and many members of the Rosaceae are important polyploids (20, 105). Readers who wish more information on polyploidy may start with the 1980 publication, Polyploidy: Biological Relevance, edited by W.H. Lewis (60).

An evolutionary process that probably equals polyploidy in importance is wide hybridization. Many cultivated crops are a result of past wide hybridizations (4). Extensive review articles (43, 82, 91, 98) concerning the subject have been published. The scope of this review will not be as broad as previous reviews, and readers who have a special interest in this subject should refer to them.

As mentioned earlier, polyploidy and interspecific hybridization are often related processes; one is successful because the other also occurs. Progeny of wide hybridizations are often infertile due to

dissimilar genomes. However, if the genome undergoes a doubling, fertility can be restored (2, 5, 6, 56, 83). In return, wide hybridizations promote formation of cytologically unreduced gametes (the primary cause of polyploid development) because many times the only functional gametes produced from these hybrids are unreduced (32).

The most familiar examples of wide hybridizations are probably those of Triticale ($2n = 56$), an alloploid between bread wheat ($2n = 42$) and rye ($2n = 14$) (72) and Raphinobrassica ($2n = 36$) an alloploid of radish ($2n = 18$) and cabbage ($2n = 18$) (13). Until recently both were considered only curiosities and of no practical importance. Raphinobrassica has been improved to the point of being useful as a forage crop (66) and Raphinus - Brassica crosses have been used by Bannerot et al. (8) to obtain male-sterile cabbage. Within the last 10 years Triticale has been improved to where it is commercially grown in Hungary, Spain, Canada, United States and China (72).

The importance of wide hybridization in plant evolution and speciation may best be demonstrated by the resynthesis of species from their parental species. Tetraploid Galeopsis tetrahit was resynthesized from its two parental species, G. speciosa and G. pubescens. Other plants that have been resynthesized include wheat, rape, tobacco, cotton, rutabaga, plum, and tart cherry (8, 13, 44, 77). Origins of the many polyploid species of Vaccinium have been presumed (12) but actual proof by resynthesis has not yet been done.

The role of wide hybridization in current breeding programs is significant. For breeding purposes wide hybridization may be defined as any hybridization in which the production of viable seed is difficult when only traditional methods of pollination and seed germination are

used (98). Wide crosses include crosses between species within a genus, crosses between cultivars or species within a genus that differ in ploidy, crosses between cultivars or species from different genera within a family, and crosses between cultivars or species from genera in different families (98).

Uhlinger (98), in a recent review, gave 6 reasons for making wide hybridizations: 1) they present a challenge; 2) to incorporate known useful characteristics; 3) to develop new genetic combinations or to permit expression of latent genes; 4) to broaden the genetic base or germplasm pool; 5) to assist in taxonomic or phylogenetic studies; and 6) to provide a bridge between incompatible species. He also discusses, as do others (42, 91), barriers to wide hybridizations and methods to overcome them.

Wide hybridization as a breeding tool has been used most successfully in clonally propagated plants (10). Cultivars of many popular perennial herbs and shrubs (rhododendrons, irises, orchids, cannas, dahlias, gladioli, roses, poppies, and violets) are of wide hybrid origin and it is among ornamentals that wide hybridization is most important (4).

After ornamentals, wide hybridization has been most important among fruit and nut crops. It has been important in the evolutionary history of these crops and is important as a breeding method for introgression of desirable traits from wild species into cultivars (38, 48, 89).

Seed propagated crops, which include most agronomic and vegetable crops, have also benefitted from wide hybridization. As Briggs and Knowles (10, pp. 334-335) point out, "Except for hybrid corn, no plant breeding accomplishment has had more impact on agricultural production

in the United States than the transfer of genes for stem rust resistance to bread wheat from other species." In seed propagated crops the process is essentially one of backcrossing, where genes or small chromosome segments are transferred so that the cultivated parent is left substantially unaltered except for the desired trait (90). In asexually propagated crops, the original hybrid or hybrids from 1 or 2 backcross generations may be economically useful.

Blueberries have greatly benefitted from the processes of polyploidy and wide hybridization, interspecific hybridization in their case. All cultivated blueberries are either auto- or allopolyploids. All 15 polypolids present in Cyanococcus are presumed, but unproven, to have originated from currently existing diploids (39). Wide hybridizations are an important part of current breeding programs (70) since several noncultivated species possess desirable horticultural traits (38). Moore (70) lists several interspecific blueberry crosses which have been made under controlled conditions. Artificial ploidy manipulations to assist in wide hybridization in blueberries are also of interest to present day breeders, as will be discussed in the next section.

Artificial Polyploid Induction

In the early and middle part of this century researchers believed that if it were possible to polyploidize plants at will, it would revolutionize breeding methods (28). Because of this thinking great interest was aroused among plant breeders when Blakeslee announced the discovery of colchicine as a powerful agent in inducing polyploidy (37). Within 20 years after this announcement Allard (4) reported that the

idea of revolutionizing breeding methods through polyploidy has been "thoroughly dispelled." Nevertheless, much literature has been published concerning colchicine and its effects (27, 28, 37). Even though artificial polyploid induction has not revolutionized plant breeding, it remains a useful breeding tool. Other chemical (10) and physical treatments (4) have been used to induce polyploidy, but because of its physical properties and mode of action, colchicine is by far the most effective (4).

Colchicine is an alkaloid derived from the autumn crocus, Colchicum autumnale L. It appears to be effective only in rapidly dividing cells and acts by upsetting normal nuclear division. The main action of colchicine is interruption of spindle fibers so that karyokinesis occurs but a cell plate is not formed. Therefore, cytoplasm does not divide and the cell is left with a doubled chromosome number (37). Several articles have been published concerning techniques of colchicine application as well as enhancement of its effect (2, 9, 28, 76).

The most valuable use of colchicine has been in restoring fertility in wide hybrids (2, 5, 6, 55, 83). Genome doubling often results in homologous pairing and restored fertility. Also, plants that will not cross as diploids may cross as induced tetraploids (32). Induced polyploidy can also be used in studies aimed at determining species ancestory (32).

Levan (58) listed conditions which lead to successful autopolyploidization: 1) number of chromosomes should be suboptimal; 2) chromosomes should not be too large; 3) species should be a cross-fertilizer; and 4) the plant should be grown especially for its

vegetative parts. Zeven (105) listed several successful natural and artificial polyploids some of which do not meet these conditions.

Even though blueberries do not meet all the conditions for polyploid induction, induced polyploids have been used in breeding and meiotic studies. Moore et al. (71) and Jelenkovic and Draper (50) report on development and potential of a derived decaploid. Autotetraploid blueberries have been produced and utilized by Draper et al. (35) and Rousi (81). Autoploids are also being used by Lyrene (personal communication) and his colleagues as a means of gene exchange between diploids and tetraploids, and tetraploids and hexaploids.

Meiotic Pairing in Blueberries

After surveying existing literature on meiosis of blueberries one can conclude that for a largely polyploid genus, meiotic pairing is surprisingly regular. Few pairing abnormalities or fertility problems are found at natural ploidy levels (16, 52, 62, 97) or in induced polyploids (35, 81). Abnormalities that have been observed generally occur in interspecific crosses or polyploids and include univalents (49, 97), laggards (49, 50, 97), unequal chromosome distributions (49), polyspory (62), and multivalent and secondary associations (16, 51, 57, 97).

The reason for regularity in meiotic pairing seems to be the small size of the chromosomes. Fully constricted blueberry chromosomes range from 1.1 to 2.3 microns (45). With frequency of multivalent associations depending on frequency of chiasmata and chromosome size (51), and with chromosome size influencing chiasma frequency (87) the small size of blueberry chromosomes effectively reduces meiotic pairing abnormalities.

SECTION III

ANALYSIS OF THE BREEDING POTENTIAL OF TWO *Vaccinium ashei* (READE) x *V. darrowi* (CAMP) HYBRIDS

Introduction

Interspecific hybridization has played an important role in *Vaccinium* evolution and in most blueberry breeding programs. Several cultivars, especially highbush types, have been developed from 1 or more interspecific crossings. Since there are as many as 19 *Vaccinium* species indigenous to eastern North America (12) great potential exists for interspecific hybridization.

Of the indigenous species described by Camp (12), 8 are diploid ($2n = 24$), 8 tetraploid ($2n = 48$), and 3 hexaploid ($2n = 72$). These ploidy differences present a challenge to blueberry breeders. Several workers (19, 39) have reported that hybrids between species of equal chromosome number are generally easier to obtain than hybrids of species differing in chromosome number.

Cultivated blueberries are primarily tetraploid (highbush complex; *V. corymbosum* L. and *V. australe* Small and lowbush; *V. angustifolium* Ait.) or hexaploid (*V. ashei* Reade). However, desirable horticultural traits exist at all 3 ploidies, with each level possessing traits not found at others (69). For this reason interploid crosses are actively pursued despite low success rates.

Moore (70) lists several successful interspecific crosses made under controlled conditions. Some of these crosses have been repeated and progeny evaluated by several blueberry breeders. Intraploid hybrids led to release of several cultivars (70), but only 3 interploid hybrids have been released (85, 86).

Since commercial cultivation of blueberries is limited primarily to tetraploids and hexaploids, crosses between these groups are of greatest interest, especially between highbush and rabbiteye. Direct hybridization between the 2 levels results in pentaploid ($2n = 60$) plants that have not proven useful in further breeding. Therefore, 6x highbush-types or 4x rabbiteye-types have been proposed to facilitate gene exchange between the 2 groups (24, 26). The procedure that was first pursued was development of 4x rabbiteye-types from 6x-2x crosses (24, 26).

Darrow et al. (24, 26) and Sharpe and Sherman (84) have used various 6x-2x crosses in breeding. Darrow et al. crossed V. ashei with a southern lowbush 2x species, V. tenellum Ait., to produce 4x rabbiteye-types which were then crossed to highbush. Tetraploidy of the hybrids was determined by chromosome counts. Draper et al. (34) and Sharpe and Sherman (84) both crossed V. ashei with 2x V. darrowi to develop 4x plants. However, neither group confirmed that their hybrids were 4x, and Draper et al. stated that their hybrids were sterile and possibly 5x. Sharpe and Sherman continued to use their hybrids assuming tetraploidy, eventually releasing 3 cultivars (85, 86). The purpose of this section was to evaluate fertility and breeding potential of 2 V. ashei x V. darrowi clones. Investigations included meiotic observations as well as observations on the crossability between 4x highbush and

V. ashei x *V. darrowi* hybrids. Pollen tube mitosis was also observed to determine ploidy levels of germinating pollen grains.

Materials and Methods

In February 1979 approximately 2,000 pollinations of *V. ashei* were done with a mixture of half *V. darrowi* and half *V. ashei* pollen. From these mentor pollinations approximately 3,000 seedlings were grown. From these seedlings 2 *V. ashei* x *V. darrowi* hybrids were selected, 'Fla. 81-97' ('Powderblue' x *V. darrowi*) and 'Fla. 81-121' ('Climax' x *V. darrowi*). Both had small leaves and were partially evergreen. None of the other seedlings appeared to have any traits from *V. darrowi* and were probably not *V. ashei* x *V. darrowi* hybrids. The 2 hybrids were removed from the field, put in cold (7°C) storage for 1 month and placed in the greenhouse for crossing in February 1982.

To study meiosis of pollen mother cells, developing flower buds were removed and fixed in 3:1 absolute ethanol:glacial acetic acid for 24 hours and then stored in 70% ethanol until studied. Anthers were removed from the flowers, squashed in 1% acetocarmine, destained with 45% acetic acid, and observed using phase contrast at 1000X.

Pollen tube mitosis was studied using techniques outlined by Stushnoff and Feliciano (96) except for an oven temperature of 25°C instead of 20°C. Percent pollen germination was obtained using the methods of Goldy and Lyrene (41).

Indications of fertility and crossability were obtained by comparing percent fruit set, seeds per fruit, and number of seedlings per pollination. Hybrids were used as both females and males. To test

female fertility hybrids were reciprocally crossed and pollinated with pollen from a highbush composite. Male fertility was tested by pollinating 2 highbush clones with each hybrid. Data were analyzed by chi-square (93) for percent fruit set and by t test (93) for seeds per fruit.

'Fla. 78-14', a 13 year old ramet of a V. darrowi x V. ashei (2x x 6x) hybrid, was included in meiotic analysis but not in the fertility study.

Results and Discussions

Meiotic analysis of the 3 V. ashei x V. darrowi hybrids revealed several abnormalities, one of the most interesting of which was their ploidy level. All 3 of the hybrids were 5x (2n = 60), not the expected 4x (2n = 48). They apparently resulted from fusion of an unreduced V. darrowi gamete with a normally reduced V. ashei gamete. V. darrowi has been shown to produce a significant amount of unreduced gametes (16), but why no 4x hybrids were recovered is unclear. It may be that 2 V. darrowi genomes are necessary for intermediate phenotypic expression. One genome may not be enough to make 4x hybrids distinguishable from V. ashei x V. ashei hybrids. However, it is possible that haploid V. darrowi pollen cannot successfully fertilize V. ashei. Wherever V. ashei x V. darrowi hybrids appear in the literature their ploidy level is either not discussed or is uncertain. The only 6x-2x hybridizations that have produced documented tetraploids are V. ashei x V. tenellum (26).

Other abnormalities in meiosis of the 3 hybrids, in their order of occurrence were as follows.

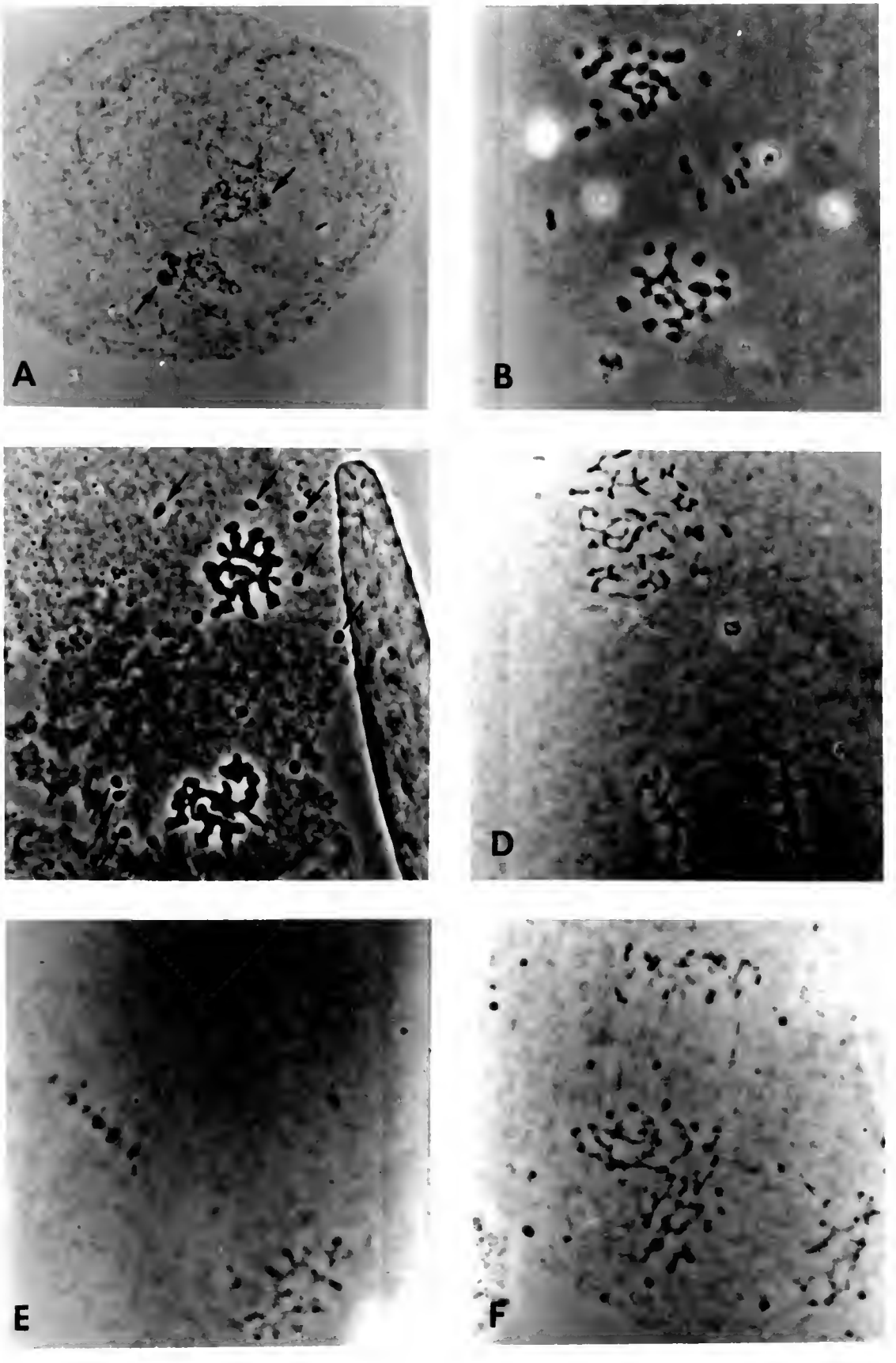
Two Synezetic Knots and Nucleolar Organizing Regions (NOR), Figure 1-a

During meiotic prophase blueberry chromosomes form a structure termed a synezetic knot consisting of condensing chromosomes and a single NOR. Several meiotic figures were observed having 2 synezetic knots and 2 NOR's. It appears that the V. darrowi and V. ashei genomes are in some cases acting independently of each other. Outcome of this type of action could be unequal numbers of chromosomes in the gametes and/or polyspory.

Lagging Chromosomes at Anaphase I, Figure 1-b

Lagging chromosomes were evident in several Anaphase I structures. Previous meiotic investigations of 5x blueberries have also revealed lagging chromosomes during Anaphase I (50). However, previous 5x plants resulted from 6x-4x crosses not 6x-2x. Anaphase I observations of 6x-4x hybrids revealed 24 bivalents and 12 lagging univalents (50). This indicates that either 2 genomes from the 4x parent pair with each other, as do 2 from the 6x parent, leaving one 6x genome unpaired or that 2 genomes from the 4x parent pair with 2 homoeologous 6x parent genomes, again leaving the 6x genome unpaired. In none of the Anaphase I observations from these 3 hybrids were 12 univalents seen and 6 was the highest number of laggards observed, as shown in Figure 1-b. Chromosome pairing during meiosis of these hybrids should theoretically be similar to pairing in 6x-4x pentaploids. V. darrowi genomes should either pair with each other as should V. ashei genomes or 2 V. darrowi genomes pair with 2 V. ashei genomes. Either case leaves 1 odd V. ashei genome. With different species combinations it is difficult to determine how

Figure 1. Meiotic irregularities observed in pollen mother cells of *V. ashei* x *V. darrowi* hybrids. 1-a. Leptotene showing 2 synezetic knots and 2 NOR's (arrows). 1-b. Lagging bivalents during Telophase I. 1-c. Six nonassociating chromosomes (arrows) during Telophase I. 1-d. Nonsynchrony during Meiosis II. 1-e. Three areas of DNA at Telophase I, 2 continuing with Meiosis II, the other apparently through. 1-f. Nonparrallel spindles during Anaphase II.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pairing is actually occurring. Explaining 6 lagging bivalents, however, is more difficult than explaining 12 lagging univalents.

The most interesting explanation is that homology between the 12 extra chromosomes is great enough to allow pairing. This theory would imply that the base chromosome number of the genus is 6, not 12. The theory also raises the question of why most previous researchers have seen 12 univalents rather than 6 bivalents.

Stating that Vaccinium has a base number of 6 is contrary to 60 years of accepted blueberry cytogenetics. However, a search of the literature reveals considerable evidence indicting a base number of 6. Epacridaceae, a family closely related to Ericaceae, or blueberry family, does have members with a base number of 6 and some taxonomists believe that the families separated as a result of an ancient polyploidization giving rise to the Ericaceae (80). Hall and Galetta (45), in studying the blueberry genome, found 2 long, 2 short and 8 intermediate chromosomes. Members of each group were largely indistinguishable from one another and the base genome could possibly be 1 long, 1 short, and 4 intermediate. Newcomer (73) and Ahokas (3) suggested from their studies that blueberries may be secondary polyploids. Lastly students of polyploid evolution have generally believed that species with a base number of 10 or higher probably have a polyploid ancestry (40). The findings of this study help to further substantiate this theory. Definitive proof that $x = 6$ in Vaccinium could be obtained if haploids obtained from a diploid species had bivalent pairing.

The reason that 6 bivalents are observed in these plants rather than 12 univalents may be because of the unique relationship of the

species involved. Previous 5x blueberries were hybrids of V. corymbosum L. x V. ashei and not V. ashei x V. darrowi. If homology exists between V. darrowi and V. ashei genomes, it is possible to get pairing between chromosomes of the respective species, and the 6 lagging bivalents may be 6 V. ashei and 6 V. darrowi chromosomes and not 12 V. ashei.

Nonassociating Chromosomes at Telophase I, Figure 1-c

During Telophase I chromosomes generally form a tight group prior to Metaphase II. Frequently the lagging chromosomes observed in Anaphase I do not associate with the other chromosomes. The 6 non-associating bivalents seen at each pole in Figure 1-c are the most ever seen, but fewer than 6 have been observed. Whether or not these chromosomes are included in the end products of meiosis would depend on whether or not they continue to lag and/or where pollen walls form.

Nonsynchrony of Meiosis II, Figure 1-d

Several meiotic cells were observed where 1 end product of Meiosis I was well into Meiosis II while the other had not begun Meiosis II, or as shown in Figure 1-d, 1 set of chromosomes is uncoiling, apparently indicating a failure of Meiosis II to occur. The latter situation would lead to 2n gamete production which will be discussed later.

More than 2 Nuclei at the End of Meiosis I, Figure 1-e

More than 2 nuclei were observed in some Telophase I products. Figure 1-e shows 1 nuclei with uncoiling chromosomes, indicating that they are through dividing, and 2 areas beginning Anaphase II as

indicated by the presence of the spindle fibers. This situation again could lead to unreduced gametes and/or polyspory.

Nonparallel Spindles at Anaphase II, Figure 1-f

Spindle fibers of Anaphase II normally align parallel to each other and perpendicular to the Anaphase I spindle. This results in the reduced/duplicated chromosomes going to their respective quadrants. Nonparallel Anaphase II spindles are regularly observed as in Figure 1-f. Sometimes they are end to end instead of side by side. When nonparallel spindles occur, even though Meiosis II proceeds normally, pollen wall formation may result in 2 sets of chromosomes being included in the same microspore, again producing unreduced gametes.

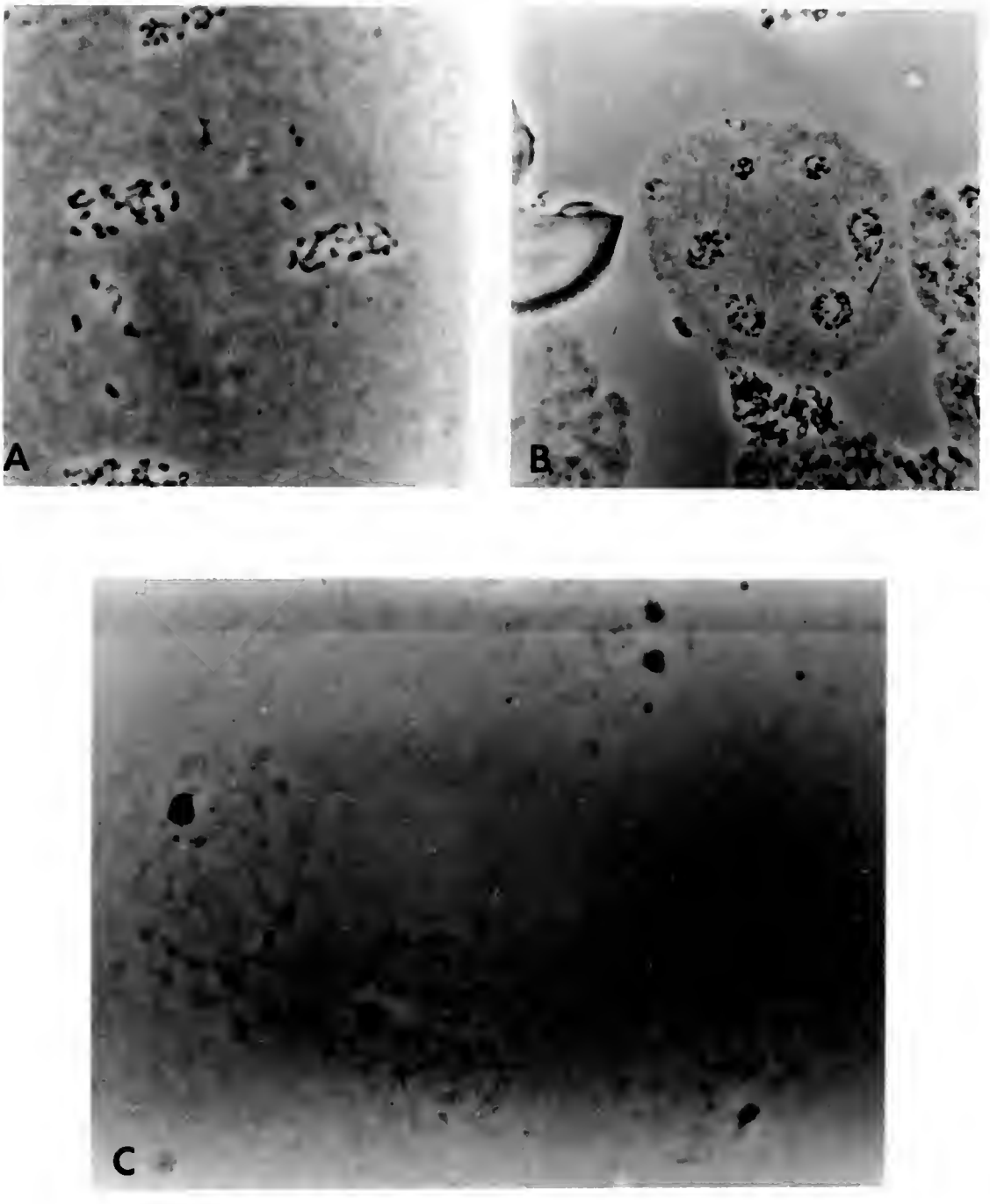
Lagging Chromosomes at Anaphase II, Figure 2-a

The laggards observed at Anaphase and Telophase I continue to lag as shown in Figure 2-a.

More than 4 Nuclei at Telophase II, Figure 2-b

The situation observed in Figure 2-b most likely resulted from that in Figure 1-e. Ploidy level of the resulting pollen grains would vary depending on how many nuclei were included. The pollen could either be unreduced, or more than 4 pollen grains could result. The different sizes of the nuclei in Figure 2-a indicate an unequal distribution of chromosomes, something observed in the hybrids even when the normal 4 nuclei are present.

Figure 2. Meiotic irregularities observed in pollen mother cells of *V. ashei* x *V. darrowi* hybrids. 2-a. Lagging chromosomes at Telophase II. 2-b. Six nuclei at Telophase II. 2-c. Extra nucleoli at Telophase II.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Extra Nucleoli at Telophase II, Figure 2-c

Normal blueberry meiosis results in the formation of 4 nuclei, each containing a single nucleolus. In some cases pollen nuclei from these hybrids are observed having 2 nucleoli as in Figure 2-c. Figure 2-c shows the normal 4 nuclei, 2 having a single nucleolus and 2 having 2 nucleoli each. Size of the nucleoli appears to vary with number; if a single nucleolus is present, it is generally larger than if there are 2.

The origin of these 2 nucleoli may be connected to the condition shown in Figure 1-a. Since nucleoli and nucleolar organizing regions are closely associated, cells having 2 NOR's at Prophase could produce pollen with 2 NOR's.

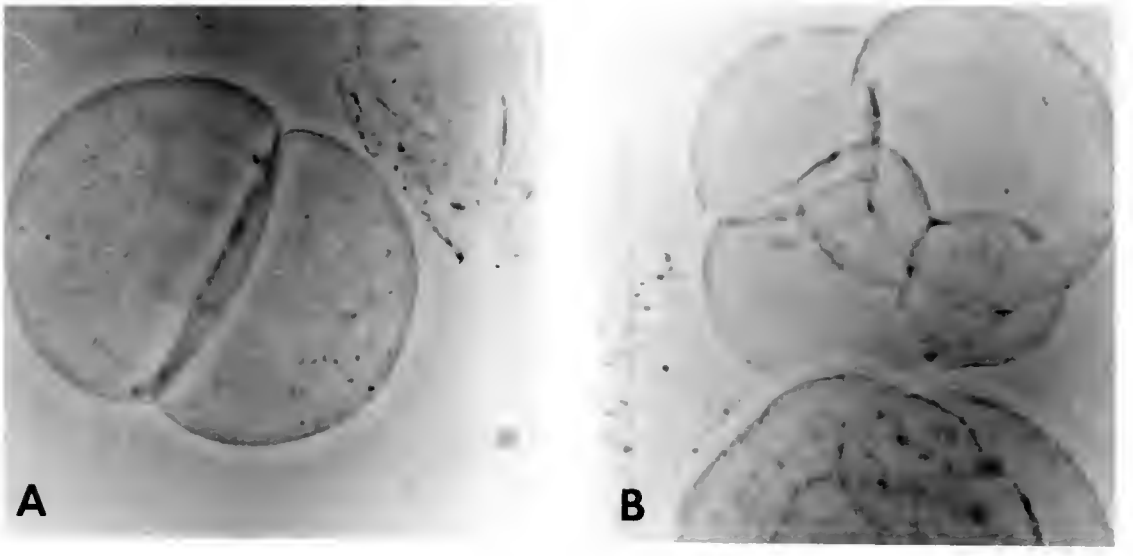
Unreduced Gametes, Figure 3-a

Unreduced gametes have been predicted for these plants as a result of the meiotic figures shown in Figures 1-d, 1-e, 1-f and 2-b and as Figure 3-a shows, they can be found. Frequency of unreduced gametes is very high in these hybrids, with 25% of the sporads from 'Fla. 80-121' containing unreduced gametes compared to 5% for 'Fla. 81-97' and 0 and 2% for V. ashei and V. darrowi, respectively (Table 1). Chromosome number of these "unreduced" gametes could vary depending on location and time of pollen wall formation. If walls formed prior to inclusion of lagging chromosomes they could have 54 chromosomes. If wall formation occurred after inclusion, they could have up to 60 chromosomes.

Mature Pollen Abnormalities; Polyspory, Incomplete Tetrads, Figures 3-b and 3-c

From Figures 1-e and 2-b pentad and hexad sporads were predicted. Pentad sporads were observed in 'Fla. 81-97' but not at the frequency in

Figure 3. Irregularities observed in pollen ov *V. ashei* x *V. darrowi* hybrids. 3-a. A dyad containing 2 unreduced gametes. 3-b. Pentad sporad, difference in size indicates a difference in chromosome number. 3-c. SEM of pollen showing shrunken, poorly filled tetrads.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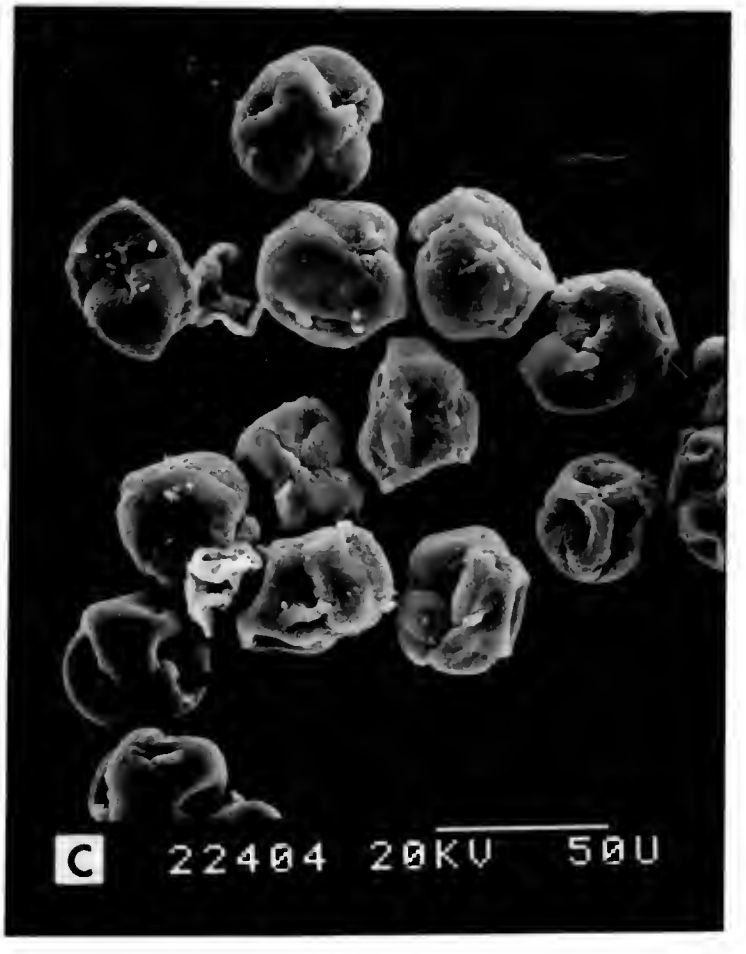
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Table 1

Number of Pollen Grains Per Sporad and their Apparent Chromosome Number[z]

Clones	Pollen Grains per Sporad					Total Sporads	% Normal	Sporads Having 2n Gametes	% Sporads Having 2n Gametes
	1	2	3	4	5				
'Fla. 81-97'	47	172	29	521	1	770	68	42	5
'Fla. 81-121'	179	134	23	155	0	491	32	124	25
V. ashei[y]	15	68	101	457	0	641	71	0	0
V. darrowi[x]	59	208	39	385	0	691	56	16	2

[z]Determined visually according to size.

[y]'Climax'.

[x]4-B.

which structures as in Figure 8 were observed. Instead of the expected increase in polyspory, 'Fla. 81-121' had an increase in the number of monads, dyads and triads, with many containing unreduced gametes. Incomplete sporads probably result from a failure of pollen walls to form around a critical amount of DNA, producing pollen that will abort or be nonviable.

Much of the pollen formed by the hybrids was shrunken (Figure 3-b) and not well filled. When pollen was shed it was not fine and granular, but was released in groups that stuck together. Spherical bodies of unknown origin were observed on the surface of the pollen and may be responsible for the stickiness. They have been observed on pollen from other interspecific blueberry hybrids but not pollen from normal plants. Pollen germination was 31% and 8% for 'Fla. 81-97' and 'Fla. 81-121', respectively.

Crossing analysis found 'Fla. 81-97' to be more fertile than 'Fla. 81-121' both as a female and a male (Table 2). This is not surprising considering the larger number of meiotic problems that occur in 'Fla. 81-121' as is shown by lower pollen germination and number of complete tetrads. Meiotic problems observed in PMC's could also be occurring in megaspore mother cells leading to reduced egg fertility.

Chi square analysis revealed that the 2 hybrids differed significantly in both male and female fertility. When pollinated with a highbush composite, 'Fla. 81-97' had 63% fruit set compared to 38% for 'Fla. 81-121'. When used as the pollen parent, 'Fla. 81-97' set 83% on 'Sharpblue', 'Fla. 81-121' set only 14%.

Table 2

Crossability and Fertility Data of 2 *V. ashei* x *V. darrowi* Hybrids

Parents		Flowers Pollinated	% Fruit Set	$\bar{X}$ No. of Seeds/ Fruit	Seedlings	Seedlings/ Pollination
Female	Male					
'Fla. 81-97'[z]	Hb comp.[x]	102	63	13.00	82	0.80
	'Fla. 81-121'	75	1	14.00	7	0.09
'Fla. 81-121'[z]	Hb comp.	145	38	2.50	21	0.14
	'Fla. 81-97'	706	7	1.45	20	0.03
'Fla. 81-194'[y]	'Fla. 81-97'	172	6	14.00	--	--
	'Fla. 81-121'	148	0	--	--	--
'Sharpblue'	'Fla. 81-97'	93	83	15.00	43	0.46
	'Fla. 81-121'	43	14	5.00	5	0.12

[z]Pentaploid *V. ashei* x *V. darrowi* hybrid.

[y]Tetraploid highbush clone.

[x]Composite of 4 tetraploid highbush selections.

A t test was performed on number of plump seeds per fruit data and revealed a significant female difference but not a significant male difference. When pollinated with the hybrids, 'Sharpblue' had fruit containing an average of 15 and 5 seeds per fruit for 'Fla. 81-97' and 'Fla. 81-121', respectively. These values were not significantly different at the 5% level. This lack of difference may be due to the small sample sizes (6 for 'Fla. 81-121' and 23 for 'Fla. 81-97') and the large variance values. Seed number per berry on 'Sharpblue' ranged from 1 to 36 seeds when 'Fla. 81-97' was the pollen parent and from 1 to 11 when 'Fla. 81-121' was the pollen parent.

'Fla. 81-97' gave more seedlings per pollination, both as a male and as a female, than did 'Fla. 81-121'. As a female parent 'Fla. 81-97' produced 0.8 seedlings per pollination compared to 0.14 for 'Fla. 81-121'. As a pollen parent 'Fla. 81-97' produced 0.46 seedings per pollination and 'Fla. 81-121' produced 0.12.

Seedlings from the crosses in Table 2 could have a range in chromosome numbers. In hybrid x hybrid crosses it is possible that 2 unreduced gametes could combine giving 10$\underline{x}$ (2$\underline{n}$ = 120) plants. However, if the 12 extra chromosomes are eliminated and 24 chromosome gametes are produced, seedlings could have 48 (24 + 24) or 84 (24 + 60) chromosomes depending on whether they combine with a reduced or unreduced gamete. Also if the 6 laggards that are observed are lost, "unreduced" gametes may only have 54 chromosomes, in which case a 78 (24 + 54) chromosome plant could be produced. Mitotic chromosome analysis of these populations should be done to determine if they do range in ploidy.

Analysis of pollen tube mitosis was unsuccessful in determining ploidy of germinating pollen. The pollen of the hybrids germinated over a long period of time, beginning after 2 hours and continuing to 20 hours after placement on the media. For doing mitotic studies it is necessary to have a large number of cells undergoing division, something not occurring in these hybrids since germination is not synchronized.

Usefulness of 6x-2x hybrids in transferring genes into cultivars is something that has already been proven, since 3 cultivars exist that have such an ancestry. Since these 3 cultivars are all 4x, the pentaploidy of the initial hybrids must have been reduced with further breeding. The 3 cultivars have not been well accepted by commercial blueberry growers, mostly because of their susceptability to Phytophthora cinnamomi (Rands), a root disease to which V. ashei is tolerant. Since most V. ashei clones are highly tolerant to P. cinnamomi, this approach to combining rabbiteye and highbush gene pools should not be abandoned. However, for it to be successful, breeders should concentrate on selecting hybrids possessing the good traits of the species involved. This may not be easy due to the difficulty in obtaining large numbers of 6x-2x hybrids.

Since the 2 hybrids in this study exhibited a difference in fertility, to insure continued success in breeding, only those plants such as 'Fla. 81-97' should be selected. 'Fla. 81-97' is not only more fertile than 'Fla. 81-121', but also has larger, more attractive fruit, 'Fla. 81-97' also sets a large number of fruit for an interspecific, 5x hybrid. Both plants, however, are interesting from a cytogenetic standpoint because of their large number of meiotic problems.

SECTION IV

USE OF *Vaccinium* OCTOPLOIDS TO FACILITATE 4x-6x GENE TRANSFERS

Introduction

Since 4x-6x *Vaccinium* crosses yield pentaploids of little breeding value, previous efforts to combine 4x highbush (*V. corymbosum* L. and *V. australe* Small) and 6x rabbiteye (*V. ashei* Reade) have been designed to reduce the rabbiteye genome to 4x through 6x-2x crosses (24, 26, 84). This technique incorporates genes of an unimproved diploid into the breeding lines and a pure highbush-rabbiteye hybrid is not obtained.

Another approach to highbush-rabbiteye hybridization would be to raise highbush to the 6x level. This could be done by doubling the 4x genome to 8x and then backcrossing to 4x, theoretically producing 6x plants of entirely highbush genes, which could then be crossed to the 6x rabbiteye. This technique has the advantage that the resulting highbush-rabbiteye hybrids would only contain the genetic information from the 2 gene pools. Also, 6 homoeologous genomes offer more potential for heterosis than 4. One problem that may arise using this method is the existence in blueberries of a 3x block, which keeps 3x plants from being obtained from 2x-4x crosses. Some researchers theorize that 3x plants fail to form because of unfavorable genomic ratios present in 2x-4x hybridizations (54). The genomic ratios of 4x-8x are the same as those of 2x-4x crosses and a 6x block analagous to the 3x block could exist.

This study was conducted to determine the feasibility of using 8x colchiploids to obtain hybrids between highbush and rabbiteye. Investigations included 1) meiotic analysis of the 8x colchiploid; 2) observation of pollen tube mitosis to determine ploidy of germinating pollen; and 3) generation of synthetic 6x highbush plants through 4x-8x crosses.

Materials and Methods

Plant material consisted of a single 8x plant ('Fla 80-46') produced by Chandler (14) from 120 colchicine treated seedlings of a 4x, 'Fla 3-8' x V. fuscatum Ait. cross. 'Fla 80-46' was selected as having larger than normal guard cells and pollen tetrads, indicating that at least the epidermal and gametic tissues were doubled. The plant is also characterized by slow growth and small, misshapen leaves that bear trichromes and often form rosettes.

For study 'Fla. 80-46' was removed from the field in December 1980, put in cold (7°C) storage for 1 month, and then placed in the greenhouse. It produced 50 flower buds in 1981, all of which were used for crossing. In December 1981 it was again placed in cold storage for 1 month, and it was moved to the greenhouse in January 1982. The plant produced 11 flower buds in 1982 which were used for meiotic analysis. Further meiotic, pollen mitotic, and crossing studies were done in 1983. By this time 8 ramets taken in 1980 came into flowering, and limited flower supply was no longer a problem.

For meiotic analysis, flower buds at the appropriate state were removed and fixed in 3:1 absolute ethanol:glacial acetic acid for 24 hours and stored until needed in 70% ethanol. Anthers were removed

and squashed in 1% acetocarmine, destained with 45% acetic acid and observed using phase contrast at 1000X.

Pollen tube mitosis was studied using techniques outlined by Stushnoff and Feliciano (96), except for using a 25°C oven instead of 20°C. Percent pollen germination was obtained using the method of Goldy and Lyrene (41).

For fertility and crossability analysis five 4x plants were pollinated with pollen from 'Fla. 80-46' and 'Sharpblue'. 'Sharpblue' was pollinated with pollen from 'Fla. 80-46' and 'Avonblue'. Parameters analyzed were 1) fruit set; 2) fruit weight; 3) number of well developed seeds/fruit; and 4) number of seedlings/pollination. A 2-way analysis of variance (93) was performed on each parameter and an F value was used to test differences for significance.

Seeds from the crosses were removed from the fruit, disinfested with 30% Clorox (1.6% sodium hypochlorite) for 15 minutes, rinsed twice in sterile distilled water and placed in 50ml screw top vials containing 5ml of 0.45% agar. The agar had been autoclaved at 1.05 kg/cm^2 for 15 minutes. Vials were placed on a window sill where they received several hours of full sunlight each day. After germination, seedlings were removed and rooted under mist in 100% peat. Root or shoot tips from the seedlings were fixed and stored as described for flower buds. In preparation for making chromosome counts, the tips were placed for 15 minutes in a solution of 0.03g pectinase and 0.03g cellulysin dissolved in 2ml water. After softening they were rinsed with water and squashed in 1% acetocarmine. Slides were destained with 45% acetic acid and observed using phase contrast at 1000X.

To test female fertility 10 flowers of 'Fla. 80-46' were selfed in 1981 and 10 were pollinated with pollen from 4x plants in 1981 and 1982. Female fertility was again tested in 1983 using a composite of V. elliottii Chap., V. corymbosum, V. ashei and 'Fla. 80-46' pollen.

Results and Discussion

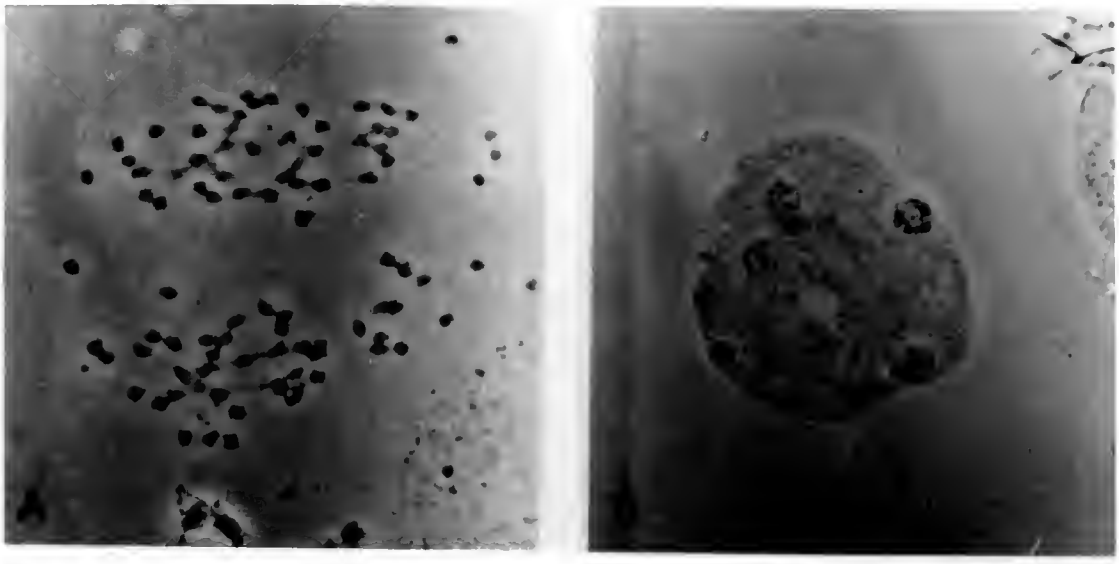
Meiosis appeared relatively normal in 'Fla. 80-46' despite the increased chromosome number. Anaphase I figures were observed with the expected 48 chromosomes at each pole (Figure 4-a). Small amounts of lagging DNA were occasionally observed at Anaphase II, but meiosis for the most part appeared to proceed normally. Chromosomal connections evident in Figure 4-a were often seen in meiosis of 'Fla. 80-46' and have been reported in other plants (101). Whether or not they affect fertility is unknown.

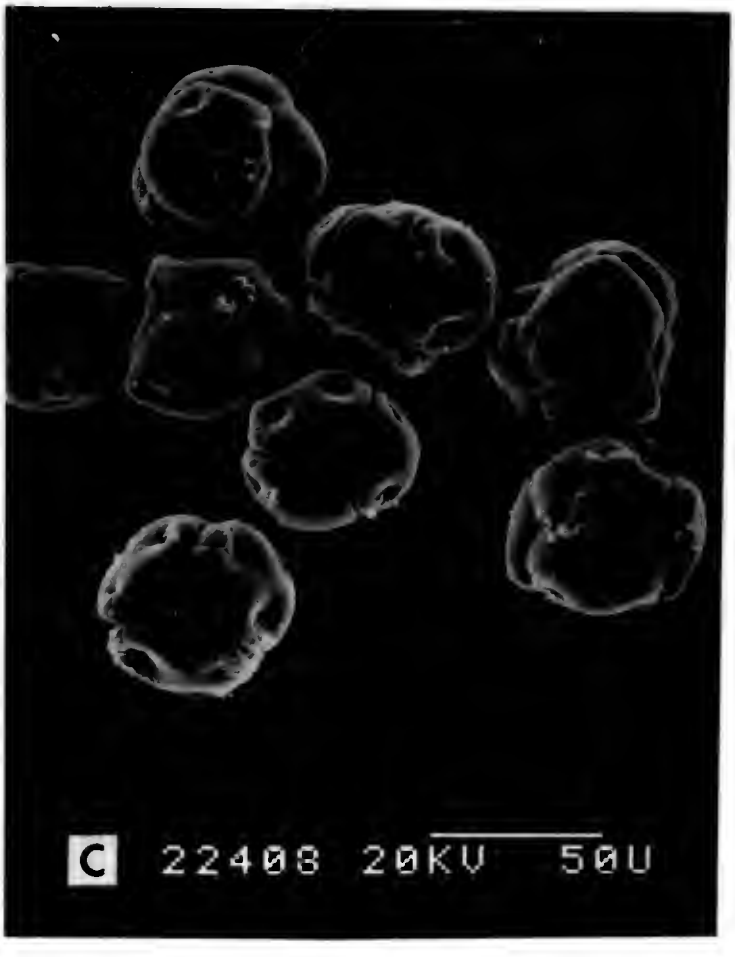
One abnormality evident in some Telophase II structures was 6 nuclei instead of the normal 4 (Figure 4-b). The 2 extra nuclei always appeared smaller and may lead to the formation of pentads and hexads instead of normal tetrads. The fact that they were smaller than normal indicates that an extra reduction of some sort may have occurred.

Scanning electron micrographs (Figure 4-c) of pollen tetrads revealed pollen walls that were somewhat concave, had a large plug in each pore, and showed an increase in diameter of approximately 24% over tetrads from tetraploids. Pollen germination was found to be 26%.

Pollen of 'Fla. 80-46' is sticky and released in groups. The spherical bodies apparent on the pollen in Figure 4-b may be the reason for the stickiness. These bodies are also observed in interspecific crosses whose pollen is also sticky.

Figure 4. Meiosis and meiotic products of an 8x colchiploid highbush blueberry. 4-a. Anaphase I showing 48 chromosomes at each pole and interchromosomal connections. 4-b. Telophase II showing 6 nuclei. 4-c. SEM of tetrads showing pollen that is slightly concave in the pore area and with each pore having a large plug.

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Analysis of crossing data showed that 4x highbush plants were significantly more fertile when pollinated with pollen from other 4x highbush than they were with pollen from 'Fla. 80-46', no matter what fertility parameter was used (Table 3). Average fruit sets on 4x plants pollinated with 8x and 4x pollen were, respectively, 12.5 vs. 72.4%, average fruit weight was 1.12g vs. 1.68g, mean number of seeds per fruit was 2.2 vs. 19.8 and the best indicator of fertility, seedlings per pollination, was 0.15 vs. 2.07.

Although 125 seedlings were obtained from 735 highbush flowers pollinated by 'Fla. 80-46', only 1 seedling had the expected chromosome number of 72, and it was mitotically unstable. Mitotic chromosome counts of shoot tips ranged from 48 to 168. The majority of the cells however contained 72 chromosomes. The other 124 seedlings had 48 somatic chromosomes.

The simplest explanation for the origin of the 4x seedlings is that they resulted from self pollinations. This may or may not have been the case. Several factors from the 4x-8x pollinations indicate that they may be hybrids rather than selfs. Average fruit weight was lower and ripening time peaked 2 weeks later for 'Fla. 80-46' than for control pollination. Smaller fruit and later ripening are characteristic of both interploid crosses and selfs because of their lower seed set (18). The strongest indication that the seedlings were hybrids was the fact that seedling vigor appeared too great for selfs and that when planted in the field, they showed traits characteristic of the pollen parent.

The question then arises as to how these "hybrids" may have formed. As stated earlier some meiotic figures were observed with 6 instead of 4 nuclei in Telophase II. If the theory of a further reduction is

Table 3

Comparative Fertility of 4x x 4x and 4x x 8x Crosses

Parents		No. of Flowers Pollinated	% Fruit Set	Avg. Wt/ Fruit (gm)	$\bar{X}$ No. of Seeds/ Fruit	Seedlings	Seedlings/ Pollination	6x Seedlings
Female	Male							
'Avonblue'	'Fla 80-46'	174	5.2	1.19	2.0	13	0.07	0
	'Sharpblue'	36	88.9	2.09	12.0	67	1.86	
'Fla 80-40'	'Fla 80-46'	170	32.9	1.08	3.0	82	0.48	0
	'Sharpblue'	45	93.3	1.58	16.0	89	1.98	
'Sharpblue'	'Fla 80-46'	92	1.1	0.69	4.0	4	0.04	0
	'Avonblue'	26	34.6	1.30	34.0	54	2.08	
'Fla 4-76'	'Fla 80-46'	167	10.8	1.38	1.7	24	0.14	0
	'Sharpblue'	79	86.1	1.94	19.0	141	1.78	
'Flordablue'	'Fla. 80-46'	136	12.5	1.25	0.4	2	0.01	1
	'Sharpblue'	54	59.3	1.51	18.0	197	3.65	

correct, these smaller nuclei may contain the previously normal number of 24 chromosomes instead of the desired 48. Also, if a 6x block does exist in these crosses, the screen for 24 chromosome gametes in 4x-8x crosses might be as powerful as the screen for unreduced pollen in 2x-4x crosses. Two tests were performed to determine the ploidy of germinating pollen: analysis of pollen tube mitosis and pollinating V. myrsinites Lam. with 'Fla. 80-46' pollen.

Mitotic analysis of pollen tubes was difficult using the techniques of Stushnoff and Feliciano (96) and proved unsuccessful after repeated attempts. Handling and observation of pollen tubes were difficult, and when mitotic figures were observed, it was impossible to get accurate counts because space limitations imposed by the pollen tubes resulted in crowded, poorly spread chromosomes.

V. myrsinites was pollinated so that hybrid seedlings could be phenotypically identified. V. myrsinites is a 4x lowbush blueberry with small evergreen leaves. Tetraploid hybrids between 4x highbush and 'Fla. 80-46' would be difficult to determine phenotypically, but hybrids between V. myrsinites and 'Fla. 80-46' should be readily distinguishable from V. myrsinites selfs. Chromosome counts could then be performed on the hybrids to determine if they are 4x or 6x. The crosses onto V. myrsinites were done in February 1983 and results are not yet available.

The 6x hybrid obtained by pollinating 'Flordablue' with 'Fla. 80-46' resembled the pollen parent in several characteristics and was definitely a true hybrid. As in the pollen parent the leaves are small, misshapen and trichomatous. It was also slow growing and not nearly as vigorous as other seedlings from 'Fla. 80-46' pollinations. As stated earlier the plant is mitotically unstable with mitotic cells containing

48 to 168 chromosomes. This is a phenomenon that has been observed by Lyrene (personal communication) in previously studied interploid hybrids. However, since most cells contained 72 chromosomes the plant may breed as a 6x. The mitotic instability that is observed in the plant may be useful for developing breeding lines at other ploidy levels. By putting the plant in tissue culture, it might be possible to get a series of adventitious shoots with stable chromosome numbers ranging from 4x to 14x. This opens the possibility of exploiting previously nonexisting ploidy levels and making new interploid combinations.

Female fertility was not tested as extensively as male fertility due to the small number of flowers. Because of the large number of pollen grains compared to eggs in each flower, the most efficient use of gametes is as males. Number of flowers available for crossing in 1982 and 1983 was further reduced by meiotic analysis.

Female fertility of 'Fla. 80-46' was not high. No fruit was set from the self and 4x pollinations in 1981 and 1982. The composite cross in 1983 set 2 berries from 15 pollinated flowers. Female fertility of other colchiploids has been reported as questionable (81). The flowers of 'Fla. 80-46' appear to produce the normal number of eggs and the reason for low fruit set is unknown.

Usefulness of 8x plants and hybrids of 4x-8x crosses is difficult to determine from a single plant. Although performance of 'Fla 80-46', phenotypically and in production of 6x hybrids, was not as good as desired, other 8x plants could perform better. Since 8x and 6x plants are to be used only as bridges between highbush and rabbiteye, they need

not have the qualities of cultivars. Development of more 8x colchiploids (as will be discussed in Section V) can play an important role in improving this crossing technique. When more 8x plants become available they can be intercrossed and selected for increased vigor and fertility. This is also true for any subsequent 6x hybrids from 4x-8x crosses. Usefulness of the 6x hybrid has yet to be determined since it has yet to flower. It is hoped that it will produce at least some 3x gametes, but with the mitotic instability it exhibits, this cannot be safely assumed.

With potential to do breeding at these 2 new ploidy levels it is possible that highbush itself could be improved, given enough time and concentrated effort. Development of more 8x and 6x lines containing only highbush genes may pave the way for practical gene transfer from 4x highbush and 6x rabbiteye.

SECTION V

DEVELOPMENT OF 8x HIGHBUSH BLUEBERRY BREEDING LINES USING COLCHICINE AND TISSUE CULTURE

Introduction

The ability to artificially induce polyploidy in plants has long been a desire of plant breeders who believed that if plants could be polyploidized at will, breeding methods would be revolutionized (28). Great interest was therefore aroused when colchicine, an alkaloid from autumn crocus (Colchicum autumnale L.), was discovered to be a powerful agent in inducing polyploidy. Although plant breeding was not revolutionized, the ability to induce polyploidy remains an important tool of plant breeders.

Woody species are more recalcitrant to the effects of colchicine than are herbaceous species (29). One problem arising from treatment of woody plants is the large number of cytochimeras which develop (29). Induced polyploidy is often difficult to detect in early stages of post-treatment growth. Also polyploid tissue often competes poorly with normal tissue and becomes overgrown by normal tissue.

Due to poor competing ability of polyploid tissue it is necessary to provide the most favorable environment possible for treatment, growth,and selection. In vitro tissue culture has therefore been proposed as a means of reducing stress imposed through colchicine treatment (65). The purpose of this section is to report on preliminary

experiments leading to development of a rapid, efficient method for producing and screening 8x blueberry breeding lines from 4x plants.

Many highbush blueberry clones have proven difficult to maintain in tissue culture. The plants grow poorly, producing slow growing shoots with minimal proliferation, many tending to be monopodial. Therefore, before colchicine treatments could be performed it was necessary to 1) determine basic media for best performance; 2) discover media alterations improving performance; and 3) select highbush clones with rapid shoot growth and proliferation. Experiments 1 through 4 deal with establishment of tissue cultures, while 5 through 14 deal with polyploid induction. Unless otherwise indicated all experiments were conducted in 50ml screw top vials containing 10ml of media.

Materials and Methods of Experiments 1-4

Experiment 1

The objective of this experiment was to select highbush clones which would grow well on modified Knop's medium (KM). In November 1980 approximately 400 seeds, from a 4x, 'Fla. 79-12' x ' Sharpblue', cross were surface disinfested with 30% Clorox (1.6% sodium hypochlorite) for 15 minutes, rinsed twice in sterile, distilled water and placed in 40 vials containing a 0.45% agar solution which had been autoclaved at 1.05 Kg/cm^2 for 15 minutes. The vials were then placed on a window sill to receive several hours of full sunlight. After seedlings had germinated and produced several leaves they were transferred to KM (Table 4) containing 5mg/l 2iP (6-gamma-gamma-dimethyl-allyl amino purine). The cultures were incubated between 22 and 32°C with

Table 4

Composition of Modified McCown and Lloyd's Woody Plant Medium (WPM), Modified Anderson's Rhododendron Medium (ARM), and Modified Knop's Medium (KM)

Compound	Modified WPM	Modified ARM	Modified KM
	mg/liter		
NH_4NO_3	400	400	--
KNO_3	--	480	190
K_2SO_4	990	--	--
KH_2PO_4	170	--	170
$Ca(NO_3) \cdot 4H_2O$	556	--	1,140
$CaCl_2 \cdot 2H_2O$	96	440	--
$MgSO_4 \cdot 7H_2O$	370	370	370
$NaH_2PO_4 \cdot H_2O$	--	380	--
Na_2EDTA	74.5	74.5	74.5
$FeSO_4 \cdot 7H_2O$	55.6	55.6	55.6
$MnSO_4 \cdot H_2O$	22.3	16.9	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.025	0.025
KI	--	0.83	0.83
$CoCl_2 \cdot 6H_2O$	--	0.025	0.025
Pyridoxine . HCl	0.5	--	0.5
Thiamine . HCl	1.0	0.4	0.1
Nicotinic Acid	0.5	--	0.5
Myo-inositol	100	100	100
Adenine Sulfate	--	80	--
Glycine	2.0	--	2.0
Casein Hydrolysate	1,000	1,000	1,000
Sucrose	30,000	30,000	30,000
Agar	4,000	4,000	4,000
2ip	10	10	10

pH adjusted to 5.7 with 1N NaOH. Autoclaved at 1.05 Kg/cm^2 for 15 minutes.

illumination of about 1Klx for 16hr/day with Cool-White fluorescent lamps. Genotypes that grew best were selected based on regeneration time and vigor.

Experiment 2

This experiment was performed to determine the effect of 3 levels of 2iP on *in vitro* shoot proliferation. One liter of KM was divided into 3 portions. To each portion 2iP was added at the rate of 5, 10, or 20 mg/l. Each treatment was placed in 30 vials, 90 vials total. Three 2-node cuttings from 5 clones selected from Exp. 1 were placed in each vial. Plants were placed under incubation conditions indicated in Exp. 1. After 60 days plants were visually rated for shoot proliferation.

Experiment 3

This experiment was done to determine shoot production potential from 2 explant sources: hypocotyls and $1cm^2$ leaf sections placed on KM. Leaf sections were obtained from 3 greenhouse-grown highbush sources. Rapidly growing leaves were removed, disinfested in 10% Clorox (0.525% sodium hypochlorite) for 20 minutes and rinsed in sterile, distilled water. Sections were cut on all sides. Midrib tissue was excluded. Hypocotyl sections were taken from a seedling composite obtained by open pollination of several highbush plants. Disinfestation and germination were the same as Exp. 1. Fifty vials for each of 2 explant types were prepared: 1 leaf section per vial or 3 hypocotyls per vial. Explants were incubated as for Exp. 1.

Experiment 4

This experiment was done to compare the effects of *in vitro* multiplication of 3 growth media: Modified Knop's Medium (KM), modified Anderson's Rhododendron Medium (ARM), and modified McCown and Lloyd's Woody Plant Medium (WPM). Composition and preparation of media are the same as that listed in Table 4. Each treatment was placed in 30 vials, 90 vials total. Three 2-node cuttings of 5 clones from Exp. 1 were placed on the media. Plants were placed under the same incubation conditions as Exp. 1. After 60 days plants were visually evaluated for vigor and regeneration.

Results and Discussion of Experiments 1-4

From Exp. 1, 66 seedlings were selected and transferred to KM. After incubation, 10 were selected for further evaluation and transferred to fresh media, with a minimum of 10 replications. Evaluation was repeated after a second 60-day incubation and 6 clones were selected for superior growth in the shortest amount of time.

The experiment indicated that ability to habituate to *in vitro* conditions depended on genotype. However, growth of the selections on KM was still not as good as desired. Growth was more rapid but shoot proliferation was minimal and monopodial cultures quickly outgrew the vials. Since shoot proliferation is dependent on cytokinin activity (33) and 2iP has proven effective on blueberries (63), Exp. 2 was conducted to determine if levels other than 5 mg/l could induce more shoots. A 0mg/l treatment was not included since it was known *a priori* that 2iP was needed for shoot proliferation. Also since shoot

proliferation was not good at 5mg/l only higher levels were tested, with 5mg/l acting as the control.

Visual evaluation after 60 days revealed an apparent increase in shoot proliferation on the 10 and 20mg/l treatments over the 5mg/l. However, there was no apparent difference between 10 and 20mg/l treatments. Therefore, 10mg/l 2iP was determined as the best level for satisfactory shoot proliferation.

Experiment 3 was done to determine if chimerism after colchicine treatment could be avoided through induction of adventitious buds, but first it was necessary to determine if adventitious buds could be obtained in vitro. Since leaf sections have been successful in other plants and blueberries have the ability to produce adventitious buds from hypocotyl tissue (75), both tissues were tried.

The 10% Clorox for 20 minutes gave adequate disinfestation of leaf tissue with minimal damage, but shoot regeneration was never obtained despite repeated experiments and various 2iP levels. Callus formation did occur but cultures died after limited growth. Adventitious shoots do form from juvenile leaf and stem tissue, but this is not helpful when doubling of cultivars is desired.

Hypocotyl tissue also proved unsuccessful in forming in vitro adventitious shoots. The tissue quickly entered a callus phase from which shoots were never obtained. Further experiments with various hormone additions and levels may be useful in obtaining shoots from such callus.

Experiment 4 revealed that both ARM and WPM were superior to KM after 60 days incubation. Out of 30 vials in ARM and WPM treatments,

73% and 60%, respectively, were rated as having medium to good growth, while only 20% of those grown in KM received a similar rating.

As a result of these 4 experiments, 6 superior genotypes were selected (213, 218, 221, 229, 236, and 238); 10mg/l 2iP proved better than 5mg/l and as good as 20mg/l; ARM is better than WPM or KM; and 2-node stem cuttings of *in vitro* grown plant material were found to be the best explant source. Once these factors were determined it was possible to begin polyploid induction through colchicine treatment; and unless otherwise noted the above conditions apply to the following experiments.

Materials and Methods of Experiments 5-14

Experiment 5

This experiment was done to determine the effect of colchicine concentration on highbush blueberry. Colchicine at concentrations of 0.001, 0.010, 0.050, 0.100 and 0.200% was incorporated into solid KM. Three cuttings of clone MHB (a clone that grows well on KM) were placed in vials containing 5ml of media, 20 vials per treatment, 100 vials total. Explants were left on the media for 14 days and then transferred to fresh KM containing no colchicine. Plants were incubated as in Exp. 1. Cultures were visually evaluated for polyploidy every 30 days for 6 months.

Experiment 6

This experiment was performed to determine optimum exposure times to 0.010% colchicine in solid media. Three cuttings per vial of clone

221 were placed in vials containing 0.010% colchicine. Explants were left on the media for 24, 48, 96, 192, or 384 hours. They were then transferred to fresh colchicine-free media. There were 20 vials per treatment, 100 vials total. Evaluation was the same as for Exp. 5.

Experiment 7

This experiment was done to observe the effect of 3 colchicine concentrations on highbush treated in liquid KM. Cuttings of clone MHB were placed in vials containing 5ml liquid KM into which 0.0, 0.05, 0.10, or 0.20% colchicine had been added. The vials were then placed in a rotating wheel at 3 rpm for 48 hours. The explants were triple rinsed in autoclaved, distilled water and planted on fresh media, approximately 10 explants per vial. Evaluation was the same as for Exp. 5.

Experiment 8

The purpose of this experiment was to observe the effect of 0.1% colchicine on 6 clones of highbush. Several cuttings of clones 213, 218, 221, 236, 238, and MHB were placed in 5ml of liquid ARM containing 0.1% colchicine and placed on a rotating wheel for 24 or 48 hours. Further handling and evaluation was similar to Exp. 7.

Experiment 9

This experiment was similar to Exp. 8, except for the addition of 0.02g/l ascorbic acid to determine if tissue browning due to colchicine treatment could be reduced. Plant materials were clones 218, 236, 238, and MHB.

Experiment 10

This experiment was an attempt to induce polyploidy using 0.05% colchicine at exposure times of 72 and 96 hours. Several cuttings of clones 218, 221, 236, and 238 were placed in 5ml liquid ARM containing 0.05% colchicine. Plants were then placed on the rotating wheel for 72 or 96 hours. Further handling and evaluation was the same as for Exp. 7.

Experiment 11

The purpose of this experiment was to induce polyploidy using 0.050 and 0.025% colchicine in liquid ARM at exposure times of 24 and 48 hours. Cuttings of clones 218, 221, 236, and 238 were placed in liquid media containing 0.050 or 0.025% colchicine. Further handling and evaluation was the same as for Exp. 7.

Experiment 12

This experiment was done to determine if providing axillary buds with a growth phase prior to colchicine treatment would facilitate obtaining polyploids. Cuttings of clones 218, 221, 236, and 238 were placed in vials containing 5ml of media. Vials were then placed on a rotating wheel for 5 days, removed, and placed in a 0.1% colchicine solution for 24 or 48 hours. Further handling and evaluation were similar to Exp. 7.

Experiment 13

This experiment was performed to determine if cold and/or darkness prior to colchicine treatment would aid in obtaining polyploids. Two

rapidly growing cultures each of clones 218, 221, 229, and 236 were placed in darkness at 25°C or at 4°C in a refrigerator for 96 hours. After pretreatment the plants were placed under normal incubation for 7 hours, cut into 2-node cuttings and treated 24 or 48 hours in 0.025% colchicine. Handling and evaluation was the same as Exp. 7.

Experiment 14

This experiment combined cold treatment with a pretreatment growth phase. Cuttings of clones 218, 221, 229, and 238 were placed in 100 x 15mm Petri dishes containing solid ARM.

The dishes were taped shut and placed in a 4°C refrigerator for 7 days. The plants were then placed under normal incubation for 24, 48, 96, or 192 hours. After incubation, cuttings were removed and treated for 24 hours in 5ml liquid ARM containing 0.025% colchicine. Further handling and evaluation was the same as Exp. 7.

Detection of Polyploids and Ploidy Determinations

Initial detection of polyploidy was done visually according to the methods of Lyrene and Perry (64). Those shoots which appeared to have unusually large diameter were selected as "visible polyploids", removed from culture and recultured on fresh media. After sufficient regrowth, ploidy level of the plants was determined by removing actively growing shoot tips, fixing them in 3:1 absolute ethanol:glacial acetic acid for 24 hours and storing until needed in 70% ethanol. For slide preparation, shoot tips were placed for 20 minutes in a solution of 0.03g pectinase and 0.03g cellulysin dissolved in 2ml water, rinsed with water, and squashed and stained in 1% acetocarmine. Slides were

destained with 45% acetic acid and observed using phase contrast at 1000X.

Results and Discussion of Experiments 5-14

Experiment 5 indicated that colchicine levels between 0.01 and 0.05% were best for treatment. Higher levels usually resulted in greater shock and death of the explants while lower concentrations had essentially no effect (Table 5). Ideal concentration would be that level giving minimum death with maximum polyploid production; therefore 0.01% was chosen for the next experiment.

Since the action of colchicine depends on cell division, maximum effect occurs at times of rapid mitosis. Exp. 6 was designed to determine a duration of exposure that was best to insure colchicine incorporation during cell division. None of the durations were effective and the explants remained in a nongrowing state when replanted on colchicine-free medium. Few of them were killed by treatment, but few of them recovered enough to grow within 6 months after treatment.

No visible polyploids were detected from either Exp. 5 or Exp. 6. The explants appeared to be severely shocked by the colchicine treatment; they neither died nor grew well after treatment. Treatment on solid media, where the plants were not rinsed of residual colchicine, gave poor results. Treatment in liquid media was therefore suggested.

Experiment 7 tested several procedures for liquid treatment. From this experiment it was determined that colchicine levels between 0.05 and 0.01% gave the best results (Table 6). Shoot regeneration was better and treatment shock was reduced, presumably due to rinsing off of

Table 5

Growth of Highbush Blueberry Clone MHB on Colchicine-Free Medium Following Treatment of Explants with Various Concentrations of Colchicine for 14 Days on Solid Medium

	Number of Vials		
% Colchicine	Dead	Green[z]	Growing
0.200	5	15	0
0.100	1	19	0
0.050	0	18	2
0.010	3	13	4
0.001	0	3	17

[z]Green but lacking signs of growth.

Table 6

Growth of Highbush Blueberry Clone MHB on Colchicine-Free Medium Following Treatment of Explants with Various Concentrations of Colchicine for 48 Hours in Liquid Medium

	Number of Vials		
% Colchicine	Dead	Green[z]	Growing
0.20	12	17	6
0.10	2	11	12
0.05	4	16	10
0.00	1	0	20

[z]Green but lacking signs of growth.

residual colchicine. However, this method requires considerably more handling of the explants introducing a greater possibility of contamination.

Experiments 8-11 attempted to further define the liquid treatment method. Experiment 8 revealed variation among genotypes with respect to sensitivity to treatment (Table 7) and that generally more shoots were regenerated from an exposure time of 24 hours than from 48. Addition of ascorbic acid to the media in Exp. 9 did not appear to reduce tissue browning. Due to the large amount of death in Exps. 8 and 9 the 0.10% colchicine treatment was concluded to be too strong. Treatment was repeated in Exp. 10 using 0.05% colchicine for 72 and 96 hours. This treatment again proved too harsh and many of the regenerated shoots were adventitious. Treatment apparently killed the axillary buds in which colchicine was hoped to have its greatest effect. Shoots arising from adventitious buds were probably not affected by colchicine, since the explants were rinsed to remove residual colchicine before adventitious buds arose. Adventitious buds gave rise to many shoots, none exhibiting signs of induced polyploidy. Because of these findings lower concentrations and/or exposure times were investigated.

Experiment 11 revealed that a 0.050% treatment for both 24 and 48 hours gave good regeneration (up to 60%) but that most of the shoots were again adventitious. The 0.025% treatment gave lower shoot regeneration; however, many of the shoots were from axillary buds. The 0.025% treatment gave lower regeneration than 0.050%, probably because axillary buds grew at this level, suppressing adventitious growth. Neither the 0.025% nor the 0.050% treatment produced visible polyploids.

Table 7

Performance of 6 Highbush Clones on Colchicine-Free Medium After 24 and 48 Hour *in Vitro* 0.1% Colchicine Treatment in Liquid Arm

Clone	Time(hrs)	Number of Vials		
		Dead	Growing	%Growing
213	24	11	2	15
	48	11	1	8
218	24	10	3	23
	48	11	2	15
221	24	5	7	58
	48	13	4	24
236	24	14	2	12
	48	13	6	32
238	24	7	11	61
	48	10	4	29
MHB	24	11	0	0
	48	11	0	0

Findings of the previous induction experiments led to a dilemma: when shoots were treated with low concentrations, no change occurred; if treated with higher concentrations, the tissue was damaged beyond regeneration. Highbush appears to be more sensitive to colchicine than other blueberries similarly treated, and at the same time appears to be more resistant to its chromosome doubling effects. These conclusions led to Exps. 12-14. Since varying colchicine concentrations and exposure times had little effect, it was decided that treatments predisposing the plant material might be beneficial when treating at lower levels. Because colchicine is effective only in dividing cells, several treatments to enhance cell division prior to colchicine treatment were investigated.

In Exp. 12 plants were cut into the usual 2-node cuttings but were allowed to grow for 5 days on colchicine-free medium. The goal was to give axillary buds a chance to begin growth, thereby increasing the mitotic index. A colchicine concentration of 0.10% was used, but again axillary buds were killed and only adventitious growth occurred.

Pretreatments for Exp. 13 were cold and/or darkness. The idea behind these treatments was to stop growth for an extended period of time and then provide optimum growing conditions prior to colchicine treatment. With the extra handling necessary for pretreatment, contamination became a serious problem. However the treatments were successful in inducing polyploid shoots.

From Exp. 13 a total of 14 vials containing visible polyploids were selected, 7 from the cold/dark treatment and 7 from the darkness (Table 8). The 2 pretreatments appeared equally effective, with 15% of the cold/dark treated vials containing visible polyploids and 13% of the

Table 8

Vials Containing Visible Polyploids Following a 96 Hour Darkness or Cold Darkness Pretreatment, 7 Hours at Normal Incubation Conditions and a 0.025% Colchicine Treatment for 24 or 48 Hours

Pretreatment	Clone	Time(hrs)	Total # of Vials	Vials with Fat Shoots
darkness	218	24	0	0
		48	7	5
	221	24	12	0
		48	10	0
	229	24	7	1
		48	6	0
	238	24	6	0
		48	5	1
	total		53	7
cold darkness (7°C)	218	24	8	2
		48	4	2
	221	24	8	0
		48	7	1
	229	24	8	1
		48	0	0
	238	24	7	1
		48	6	0
	total		48	7

dark treated vials with polyploids. The 48 hour colchicine treatment appeared best for polyploid induction following darkness, while 24 hour exposure was best for induction following cold/darkness.

Experiment 14 again revealed that lower concentrations (0.025%) appeared more effective than higher (0.050%) (Table 9). There was also a slight increase in visible polyploids in Exp. 14 over the refrigerator treatment in Exp. 13, 17.5% and 15%, respectively. Experiment 14 also indicated that growth periods between 24 and 48 hours after pretreatment may enhance the effect of colchicine. Further refinement of the technique could increase polyploid induction.

Not all of the shoots that were selected as visible polyploids proved to have doubled chromosome numbers after microscopic observation. From the 24 shoots visually selected, half had the desired 96 chromosomes and the other half had 48.

Experiments of the sort described in this section are difficult to analyze statistically. Strict control of total numbers and replications is difficult due to the type of plant material, difficulty of defining the experimental unit, and losses due to contamination. However, these experiments are necessary and important for directing further research even though the assessment of experimental results is necessarily somewhat subjective. Conclusions from these experiments are: 1) highbush is difficult, but not impossible to propagate in vitro; 2) highbush is more sensitive to the toxic effects of colchicine, and more resistant to its chromosome doubling effects than other blueberries; 3) problems associated with propagation and polyploid induction can be overcome with proper treatment.

Table 9

Vials Containing Visible Polyploids Following 7 Days of Darkness or Cold Darkness, 24-192 Hours at Normal Incubation Conditions and a 0.025% or 0.050% Colchicine Treatment for 24 Hours

% Colchicine	Clone	Time(hrs)[zy]	Total # of Vials	Vials with Fat Shoots
0.025	218	24	6	3
		48	2	1
		96	3	1
		192	4	1
	221	24	6	0
		192	6	0
	238	24	5	1
		48	4	0
		192	4	0
	total		40	7
0.050	218	24	7	0
		48	6	1
		96	5	1
		192	10	1
	221	24	9	0
		48	9	0
		96	8	0
		192	10	0
	229	24	9	0
		96	7	0
	238	192	9	0
	total		40	3

[z]Duration of growing phase prior to colchicine treatment.

[y]Missing treatments were lost due to contamination.

SECTION VI

SUMMARY AND CONCLUSIONS

Interspecific hybridization will no doubt remain an important aspect of blueberry breeding. This method holds much promise for improvement of bush performance and/or fruit quality. Visualizing the types of plants desired from these crosses is easy, but obtaining them is often difficult due to the crossing barriers that exist between species with different ploidy levels.

Currently the most promising way of combining the rabbiteye and highbush gene pools is still through the proven way of developing 4x rabbiteye-types from 6x-2x crosses and crossing these to 4x highbush. This research has shown that the initial hybrids are not 4x but 5x. These plants have numerous meiotic problems which result in lower fertility and an increased percentage of 2n gametes. However, the 5x plants, through further breeding, eventually eliminate the extra genome to become 4x.

Because of the difficulty in obtaining hybrids from 6x-2x crosses, extra care must be taken in using them for further breeding. Approximately 1 hybrid seedling results from 1,000, 6x-2x pollinations, and as a result breeders are reluctant to discard them even though they may have serious flaws. This can lead to development of plants lacking the desirable traits of the parents. Therefore, selection should be not

only for hybrids but those hybrids possessing good fertility and the desired parental traits.

Evaluation of combining rabbiteye and highbush gene pools through developing 6x highbush-types should not be based on the performance of 1 plant. More 8x plants need to be developed, and possibly their fertility increased through intercrossing prior to backcrossing to the 4x level. The production of 8x plants has proven difficult but not impossible. With further refinement of the techniques used in this research, large numbers of 8x highbush plants should be obtainable. The fact that one 6x plant was obtained from a poorly growing, semifertile 8x and from the limited efforts of 1 person should inspire the efforts of more people as soon as the 8x germplasm has been developed and improved.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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