## AN ABSTRACT OF THE THESIS OF

Chih-wei V. Tsao for the degree of Master of Science in Horticulture presented on April 27, 1999. Title: *Rubus* Leaf Regeneration and Micropropagation of Virus Infected Raspberry.

Abstract approved: ______________________________
Barbara M. Reed

An *in vitro* regeneration system was developed for leaf explants of *Rubus* cultivars. Adventitious shoots were successfully produced on thirteen economically important cultivars with varying ploidy levels: Cherokee (4X), Chester (4X), Hull Thornless (4X), Kotata (7X), Marion (6X), Navaho (4X), Shawnee (4X), Thornless Evergreen (4X), and Waldo (6X) blackberries and Autumn Bliss (2X), Heritage (2X), Latham (2X), and Watson (Ruby™) (2X) raspberries. *In-vitro* cultured plantlets were pretreated with 1 µM thidiazuron (TDZ) on NCGR-RUB medium for three weeks. The top four to six young leaves with petioles removed were placed on regeneration medium (RM) with indole-3-butyric acid (IBA) and either $N^6$-benzyladenine (BA) or TDZ for one week in the dark followed by 16-hour light. RM without IBA induced a significantly higher percentage of regeneration, more shoots per explant, and less callus compared to 0.5 µM IBA. BA (10 µM) was more effective than TDZ for leaf regeneration *in vitro* from most *Rubus* cultivars.

The addition of the iron chelate, sequestrene, to the second RM increased regeneration of heptaploid 'Kotata' and hexaploid 'Marion' by 60%. Agar, Gelrite, and agar-Gelrite combined had no significant effects on regeneration of 'Kotata', 'Marion', and 'Navaho' blackberries. Silver nitrate ($AgNO_3$) did not influence shoot regeneration for four blackberry cultivars, but when included in the first RM significantly reduced callus

formation. *In vitro* shoot regeneration of *Rubus* is highly genotype dependent, but berry type and ploidy level of 13 cultivars we screened did not influence the process.

Virus infected plants are often symptomless and explant sources used for tissue culture research are rarely tested for viruses. Single and multiple virus infected greenhouse-grown plants were graft-inoculated and maintained as virus stock cultures. Raspberry bushy dwarf virus (RBDV), tomato ringspot virus (TomRSV), and tobacco streak virus (TSV) infected *in vitro* 'Malling Landmark' raspberry cultures were tested for multiplication and growth. Virus status was determined by enzyme-linked immunosorbent assay (ELISA) and bioassay, and reconfirmed before and after multiplication tests. No symptoms were observed on greenhouse stock plants except one with three-virus infection (RBDV + TomRSV + TSV) which was stunted and had yellow leaves. *In vitro* multiplication of plantlets with multi-virus infections (2- and 3-virus combinations) was significantly reduced. No obvious differences were displayed for *in vitro* shoot height and morphology. We recommend using virus-free plantlets for *in vitro* experiments.

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# *RUBUS* LEAF REGENERATION AND MICROPROPAGATION OF VIRUS INFECTED RASPBERRY

by

Chih-wei V. Tsao

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Approved:

Major Professor, representing Horticulture

Chair of Department of Horticulture

Dean of Graduate School

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

Chih-wei V. Tsao, Author

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## CONTRIBUTION OF AUTHORS

Dr. Barbara M. Reed was involved in the experimental design, discussion, support, and critical editing. Plant pathologist Joseph D. Postman contributed his knowledge and assisted in virus indexing ELISA tests and writing of materials and methods for virus cultures and infections.

## TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW 1

INTRODUCTION 1

TAXONOMY 2

*RUBUS* DIVERSITY 3

*RUBUS* PROPAGATION 3
- Asexual propagation 4
- Sexual propagation 4

BREEDING PROBLEMS IN THE GENUS *RUBUS* 5
- Mitotic instability 5
- Incompatibility 5
- Polyploid, pseudogamy, and apomixis 5

MICROPROPAGATION OF *RUBUS* 6

*IN VITRO* PLANT REGENERATION 7
- Definition of regeneration 7
- Factors affecting *in vitro* regeneration – *Rubus* spp. 8
  - Explants 9
    - Sources 9
    - Age 9
    - Tissues 9
    - Excision and orientation 10
  - Medium 10
    - Basal media 10
    - Carbon sources 11
    - Growth regulators 11
    - Pretreatments 12
    - Gelling agents 12
    - Other medium additions 13
  - Environmental conditions 13
    - Temperature, light intensity, and photoperiod 13

*ROSACEOUS* PLANT REGENERATION 14

MISCELLANEOUS PLANT REGENERATION 15

THE APPLICATION OF *IN VITRO* REGENERATION 16
- Biotechnology of *Rubus* spp. 16

Transformation 17

*Rubus* VIRUS DISEASES 18
Field-grown plants 18
Virus incidence in *Rubus* collections 19
*In vitro* plants 19
Virus elimination 20

REFERENCES 21

CHAPTER 2. IRON CHELATE SEQUESTRENE IMPROVES ADVENTITIOUS SHOOT PRODUCTION FROM WHOLE-LEAF EXPLANTS OF *RUBUS* CULTIVARS 34

SUMMARY 35

INTRODUCTION 36

MATERIALS AND METHODS 37
Establishment of *in vitro* cultures 37
Pretreatment 38
Basic regeneration procedure and data analysis 38
Regeneration screening 39
Half leaf 39
Whole leaf 39
Effect of explant types, berry types, and ploidy levels on regeneration 39
Gel firmness test 40
Effect of gelling agents on regeneration 41
Effect of silver nitrate/sequestrene on regeneration 41

RESULTS 41
Pretreatment 41
Regeneration screening 42
Half leaf 42
Whole leaf 42
Effect of explant types, berry types, and ploidy levels on regeneration 45
Gel firmness test 52
Effect of gelling agents on regeneration 52

Effect of sequestrene on regeneration 56
Effect of silver nitrate on regeneration 56

DISCUSSION 59

ACKNOWLEDGEMENTS 63

REFERENCES 64

CHAPTER 3. VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY 68

SUMMARY 69

INTRODUCTION 70

MATERIALS AND METHODS 71
Plant materials and virus cultures 71
Establishment of virus infected 'Malling Landmark' 72
Establishment of *in vitro* cultures 74
Virus indexing of *in vitro* cultures 74
Multiplication test 74
Experimental design and statistical analysis 75

RESULTS AND DISCUSSION 75

ACKNOWLEDGEMENTS 82

REFERENCES 83

CHAPTER 4. CONCLUSIONS 86

## TABLE OF CONTENTS, CONTINUED

BIBLIOGRAPHY 87

APPENDICES 102

APPENDIX A. IRON CHELATE SEQUESTRENE IMPROVES ADVENTITIOUS SHOOT PRODUCTION FROM WHOLE-LEAF EXPLANTS OF *RUBUS* CULTIVARS 103

APPENDIX B. VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY 121

## LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 2-1 | *In vitro* leaf regeneration of blackberry shoots from petiole or whole leaf explants. *A*. 'Marion' blackberry petiole produced adventitious shoots after 42 days on regeneration medium with 10 μM BA and 0.5 μM IBA; magnification, X1.2. *B*. The upper surface of 'Marion' whole-leaf explant produced multiple shoots after 30 days on regeneration medium with 10 μM BA; magnification, X1.2. *C*. 'Kotata' produced multiple adventitious buds from the leaf-petiole junction after 21 days on regeneration medium with 10 μM BA; magnification X2. *D*. Multiple shoots produced from leaf-petiole junction of 'Kotata' after 42 days on regeneration medium with 10 μM BA; magnification, X2. *E*. 'Thornless Evergreen' produced multiple shoots from the lower surface of the leaf-petiole junction after 42 days on regeneration medium with 5 μM BA; magnification, X1.2. *F*. Callus produced on 'Marion' leaf after 63 days on regeneration medium with 10 μM BA and 0.5 μM IBA; magnification, X0.8. | 43 |
| 2-2 | The number of explants regenerating shoots on RM with three IBA concentrations from 30 whole-leaf explants of eight blackberry cultivars: Kotata (KO), Marion (MA), Waldo (WA), Cherokee (CH), Chester Thornless (CT), Navaho (NA), Shawnee (SH), Thornless Evergreen (TE); and four raspberry cultivars, Autumn Bliss (AB), Heritage (HE), Latham (LA), Ruby (RU). | 47 |
| 2-3 | The number of regenerating explants produced at the $84^{th}$ day on regeneration medium with a range of IBA and either BA or TDZ, from 30 whole-leaf explants of eight blackberry and four raspberry cultivars. *A*. Benzyldenine (BA) *B*. Thidiazuron (TDZ). Blackberries: 'Kotata' (KO), 'Marion' (MA), 'Waldo' (WA), 'Cherokee' (CH), 'Chester Thornless' (CT), 'Navaho' (NA), 'Shawnee' (SH), 'Thornless Evergreen' (TE); and raspberries: 'Autumn Bliss' (AB), 'Heritage' (HE), 'Latham' (LA), 'Ruby' (RU). | 49 |
| 2-4 | Linear regressions of gel firmness $[g/(1.1\ cm)^2 \bullet \pi]$ tested using NCGR-RUB medium with plant growth regulators. *A*. Linear regression lines and formulas from eight concentrations of Difco granulated agar and Schweizerhall Gelrite solidified media. *B*. Linear regression lines and formulas from media solidified with gelling agent combinations of 0.3%, 0.35%, and 0.4% agar with 5 concentrations of Gelrite. | 53 |

## LIST OF FIGURES, CONTINUED


| Figure | | Page |
|---|---|---|
| 3-1 | Multiplication of virus-infected ‘Malling Landmark’ raspberry shoot cultures at four culture periods. Explants were cultured for 12 weeks on Anderson’s medium with 8.89 μ M BA in Magenta GA 7 besides with transfer at three weeks intervals. Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom). | 76 |

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| 2-1 | Analysis of variance of number of regenerants from whole-leaf explants of blackberry and raspberry cultivars | 46 |
| 2-2 | Effect of gelling agents and growth regulators on *in vitro* regeneration from whole leaves of three blackberry cultivars | 55 |
| 2-3 | Response of whole-leaf explants of 'Kotata' and 'Marion' blackberries to sequestrene in regeneration medium (RM) | 57 |
| 2-4 | Effect of IBA concentration on whole-leaf regeneration of 'Kotata' and 'Marion' blackberries in regeneration medium with sequestrene | 58 |
| 3-1 | Virus combinations and number of replicates used for 'Malling Landmark' raspberry *in vitro* multiplication tests | 73 |
| 3-2 | Mean multiplication of virus-infected 'Malling Landmark' raspberry cultures at four culture periods | 79 |
| 3-3 | Analysis of variance of mean multiplication of 'Malling Landmark' raspberry cultures for culture periods and virus infection | 80 |
| 3-4 | Analysis of variance of mean height of virus infected 'Malling Landmark' shoot cultures during a 12 week-multiplication cycle | 81 |

# LIST OF APPENDICES

APPENDIX A. IRON CHELATE SEQUESTRENE IMPROVES ADVENTITIOUS SHOOT PRODUCTION FROM WHOLE-LEAF EXPLANTS OF *RUBUS* CULTIVARS

APPENDIX B. VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY

## LIST OF APPENDIX FIGURES


| Figure | | Page |
|---|---|---|
| A-13 | *Rubus* leaf regeneration treatments on agar-Gelrite regeneration medium with combinations of IBA, and either BA or TDZ. | 116 |
| A-14 | The number of half-leaf explants of eight blackberry cultivars that produced shoots on regeneration medium with growth regulators IBA and either *A*. BA. or *B*. TDZ. | 118 |
| B-1 | The mean number of shoots produced on single- or multi-virus infected 'Malling Landmark' raspberry cultures after 17 months in culture (the fourth culture period). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom). | 122 |
| B-2 | The multiplication of virus-infected 'Malling Landmark' raspberry over four data collection times in the fourth culture period (the 17$^{th}$ to 19$^{th}$ month in culture). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom). | 124 |
| B-3 | The mean height of virus infected 'Malling Landmark' raspberry at the third culture period (the 13$^{th}$ to 15$^{th}$ month in culture). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom). | 126 |

## LIST OF APPENDIX TABLES


| Table | | Page |
|---|---|---|
| A-1 | Ploidy level, cane characteristics, and origin of thirteen commercially important blackberry and raspberry cultivars used for leaf regeneration trials | 104 |
| A-2 | Analysis of variance of number of regenerants from half-leaf explants of eight blackberry cultivars | 105 |
| A-3 | Analysis of variance of comparing the number of regenerants from half- and whole-leaf explants of seven blackberry cultivars | 106 |
| A-4 | Analysis of variance of the effect of three gelling agents in the regeneration medium on *Rubus* whole-leaf explants | 107 |
| A-5 | Analysis of variance of the effect of sequestrene in the regeneration medium on *Rubus* whole-leaf explants | 108 |
| A-6 | Contrasts of the effect of sequestrene treatments on the of regeneration response of *Rubus* whole-leaf explants | 109 |
| A-7 | Response of 'Kotata' and 'Marion' blackberries on regeneration medium with IBA and with or without sequestrene | 110 |
| A-8 | A comparison of the percentage of regeneration from three regeneration experiments with 'Kotata' and 'Marion' blackberries | 111 |
| A-9 | Analysis of variance of the effect of silver nitrate in the regeneration medium on *Rubus* whole-leaf explants | 112 |
| A-10 | The effect of IBA concentration on the regeneration of four blackberry cultivars on regeneration medium with silver nitrate | 113 |
| A-11 | Response of four blackberry cultivars to silver nitrate in the regeneration medium | 114 |
| A-12 | Response of whole blackberry-leaf explants to silver nitrate in the regeneration medium | 115 |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| ANOVA | Analysis of Variance |
| BA | $N^6$-benzyladenine |
| BRNV | Black Raspberry Necrosis Virus |
| CIVV | Citrus Infectious Variegation Virus |
| CTV | Citrus Tristeza Virus |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| $GA_3$ | Gibberellic acid $A_3$ |
| GLM | General Linear Model |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| MS | Murashige and Skoog medium |
| MSV | Maize Streak Virus |
| NAA | $\alpha$-naphathaleneacetic acid |
| NCGR | National Clonal Germplasm Repository |
| RAPD | Random amplified polymorphic DNA |
| RBDV | Raspberry Bushy Dwarf Virus |
| RM | Regeneration medium |
| TobRSV | Tobacco Ringspot Virus |
| TomRSV | Tomato Ringspot Virus |
| TSV | Tobacco Streak Virus |
| WPM | Woody Plant Medium |

## DEDICATION

To the love of my Father, Mother, and dog Winnie

# *RUBUS* LEAF REGENERATION AND MICROPROPAGATION OF VIRUS INFECTED RASPBERRY

## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

### INTRODUCTION

Blackberries (*Rubus* spp.), raspberries (*R. idaeus* L.), and blackberry-raspberry hybrids are the brambles or caneberries. The term "bramble" denotes thorniness because of the prickles on main stems and lateral branches, but there are thornless or smooth-stemmed *Rubus* species or cultivars. Wild *Rubus* species are native to six continents and distributed from sea level to 4,500 meters (Hummer, 1996).

Through the centuries, brambles were valued for their flavor as well as highly nutritious content, soluble fiber, vitamins, and minerals. The berries also contain many natural substances and were used for medicinal purposes by the ancient Greeks as early as 370 BC (Pritts and Handley, 1994). The fresh and processed fruits are in high demand for products such as yogurt and juice blends, so supplying the market is difficult. Improvements incorporated by researchers over the past several decades include improved quality, cold hardiness, pathogen, and pest resistance, mechanical harvesting, and thornless plants. Machine harvesting, improved production, and rapid marketing are the goals of modern bramble breeding. Plant genetic engineering could hasten genetic improvement in *Rubus*. A system for regenerating *Rubus* plants from vegetative tissues or cells is a prerequisite for making use of gene transfer technology.

# TAXONOMY

*Rubus* belongs in the family *Rosaceae*, along with apples, pears, quinces, cherries, peaches, and strawberries. Focke (1910) divided the genus *Rubus* into 12 subgenera. Six of these subgenera are not cultivated and have no value as edible fruit (Jennings, 1988). Three subgenera are cultivated: *Rubus* (blackberries), *Idaeobatus* (raspberries), and *Cylactis* (arctic berries). *Malachobatus* subgenus contains no important fruit-bearing species, but includes some ornamentals. Additionally, the subgenera *Chamaemorus*, and *Orobatus* were included in the genus *Rubus*.

The subgenus *Idaeobatus* is characterized by aggregate fruit with many drupelets that adhere to one another, but separate from the receptacle at maturity. Today's cultivated red raspberries (*R. idaeus* L.) were developed from two subspecies. One is the red raspberry of Europe and Asia, *R. idaeus* var. *vulgatus* Arrhen., that has thimble-shaped and conical fruit with few or no glandular hairs. The other is from North America, *R. idaeus* var. *strigosus* Michx, which is more winter hardy, lighter red, rounder, and has more glandular hairs than *R. vulgatus*. *R. occidentalis* L. without genetic variation is a widely cultivated black raspberry (2X) from eastern North America (Jennings, 1988; Ourecky, 1975). Generally, black raspberries are less cold hardy than red raspberries. Yellow (golden or amber) raspberries are the result of recessive mutations in both red and black raspberries. They are quite sweet, but soft, so are suited for local markets and home gardens. Purple raspberries, sometimes given the designation *R. neglecfvs* Peck., are hybrids between black and red raspberries and are tart with strong flavor; they are good for making preserves (Pritts and Handley, 1994).

Blackberries were domesticated from many wild European and American species. The subgenus *Rubus*, formerly known as *Eubatus*, contains all the blackberries. It is highly complicated and includes types with chromosome numbers from 2X to probably 18X (Thompson, 1997). *Rubus* species are often classified according to their growth habit as erect, semi-erect, or trailing, and either with or without thorns. The aggregate fruit has many attached drupelets that do not separate from the receptacle at maturity (Pritts and Handley, 1994).

Blackberry-raspberry hybrids have played an important part in the development of 'Logan', 'Young', and 'Boysen' from the US, and 'Tayberry', 'Sunberry', 'Fertodi Botermo', and 'Tummelberry' from Europe (Pritts and Handley, 1994).

Arctic berries such as *R. stellatus*, *R. occfices* and their hybrids all have a dwarf herbaceous form, are spine-less and mostly dioecious, and occur in north arctic or alpine regions. Several are regularly harvested from the wild in Scandinavia (Jennings, 1988).

## *RUBUS* DIVERSITY

*Rubus* is one of the largest genera in the rose family with more than 740 described species (Gu *et al.*, 1990). Evolving over a wide geographical distribution nearly all over the world, *Rubus* developed many variations in leaf shape, fruit color, seed mass, and growth habit (Hummer, 1996). The basic chromosome number of *Rubus* is seven (X = 7) and their ploidy ranges from diploid (2n = 14) to 2n = 14X = 98 or possibily 2n = 18X = 126) (Jennings, 1988; Thompson, 1997). Most raspberries are diploid. Blackberry ploidy has a wide range and includes many odd ploids (3X, 5X, or 7X, etc.) and aneuploids (6X+2, 8X-3, etc.) (Thompson, 1995a and 1995b).

## *RUBUS* PROPAGATION

Traditional propagation methods for *Rubus* vary with the growth habit and species. Regardless of the methods used, healthy, vigorous plant stocks are essential for successful *Rubus* propagation.

*Asexual propagation*

Vegetative plant tissues like stem tips, root or stem cuttings, dormant suckers, and *in vitro* cultures are the most common ways to propagate caneberries. Pritts and Handley (1994) found that the rooted suckers, which arise from adventitious root buds, are the most useful for red raspberries. Tip layering and root suckers are traditional methods of propagating purple and blackberries. Root cuttings are commonly used to propagate red raspberry. Most commercial *Rubus* nurseries use tissue culture for large-scale production of pathogen-free plants.

*Sexual propagation*

Because of the hard, impermeable endocarp and dormant embryo, seeds of many *Rubus* species are difficult to germinate (Schopmeyer, 1974). Jennings and Tulloch (1965) found that concentrated sulfuric acid and chemical pre-germination treatments are useful to erode or soften the endocarp for seed germination. The time and temperature of acid pre-germination treatment is also important. Blackberry seeds treated longer than one hour with sulphuric acid had lower germination and injured seeds. Acid treatment at 50 $^0$C injured embryos (Heit and Slate, 1950). Soolova and Kichira (1971) found that stratification of raspberry seeds for six months followed by soaking in 1.0% sodium hypochlorite and calcium hydroxide for four days greatly improved germination. A pre-treatment of 0.06% potassium iodide also increased raspberry seed germination (Tomanova, 1971). Moist stratification eliminates growth inhibitors in the endocarp, testa, and embryo (Lasheen and Blackhurst, 1956). The disappearance of inhibitors is correlated with breaking embryo dormancy and allows germination. Jennings and Tulloch (1965) report that concentrated sulfuric acid treatment followed by 1% calcium hypochlorite could eliminate the need for subsequent stratification. Light also plays an important role in *Rubus* seed germination and total darkness almost completely inhibits germination (Scott and Draper, 1967). *In vitro* germination resulted in 57 to 81% germination and bypassed the need for cold stratification and air-dried seed (Ke, *et al.*, 1985).

*Mitotic instability*

*Rubus* plants with chromosome numbers higher than diploid are mitotically unstable. Mitotically unstable cells have more than two metaphase plates, each with its own spindle. Britton and Hull (1956 and 1957) found 28 mitotically unstable plants among 10 progenies, most of them vigorous and indistinguishable phenotypically from stable plants. Crane and Thomas (1949) found that the pentaploid 'Merton' arose from the triploid 'Mahdi', apparently as a somatic mutation. Haskell and Tun (1961) suggest that mitotic instability might have an evolutionary role. Zielinski and Thompson (1966) pointed out the mitotic instability in pollen mother cells of certain high chromosome-number hybrids.

*Incompatibility*

Self-incompatibility is common in many diploid *Rubus* species and occurs in some other rosaceous species. The incompatibility system found in the rose family is homomorphic gametophytic with multi-allelic oppositional type S alleles at one locus (Keep, 1968). Keep (1968) also noticed that self-incompatibility occurs in the wild form of four species from the *Rubus* subgenus *Idaeobatus*, seven species from the subgenus *Eubatus*, and *R. odoratus* from the subgenus *Anoplobatus*. All of these species are diploids except *R. baileyanus* of subgenus *Eubatus*. Incompatibility was caused by the inhibition of pollen tube growth in the styles of seven members from the *Idaeobatus*, one from the *Eubatus*, and four from the *Anoplobatus*. Tammisola and Ryxjnänen (1970) proposed that at least five self-incompatibility genes were in the genus *Rubus*.

*Polyploid, pseudogamy, and apomixis*

All diploid species of *Rubus* are sexual and polyploid species frequently show either facultative (sexual and apomictic reproduction), or obligate (apomictic reproduction only) pseudogamy that indicates pollination without fertilization is required for the formation of apomictic seedlings (Einset, 1951). Facultative pseudogamy is most common in the

blackberries. Jennings *et al.* (1990) found poor seed germination in species with high ploidy levels. High ploidy levels have the advantage of allowing wide crosses (Jennings *et al.*, 1990). Apomictic embryos arise from maternal tissues and resemble the mother plant (Asker and Jerling, 1992). Apomixis can also complicate breeding programs by making recovery of sexual hybrids difficult. Nybom (1980) and Jennings (1988) indicated various forms of apomixis are common in *Rubus*.

## MICROPROPAGATION OF *RUBUS*

Plant micropropagation is useful for rapidly increasing the stock of new cultivars, producing virus-free materials, and maintaining of germplasm. Shchelkunova and Popov (1970) developed a raspberry cutting system using *in vitro* shoot tips for off-season propagation and virus-free plant production. Harper (1978) showed a five to eight-fold increase in plantlet production using micropropagation for a raspberry-blackberry hybrid as compared to leaf-bud cuttings. Kiss and Zatykó (1978) reported that a culture medium with auxins and no cytokinins increased the number of plants growing from etiolated shoot tips of a blackberry-raspberry hybrid. Broome and Zimmerman (1978) applied $N^6$-benzylaminopurine (BA), gibberellic acid ($GA_3$), and indole-3-butyric acid (IBA) to stimulate multiple shoot formation and achieved a 20- to 40-fold increases in shoots. Murashige and Skoog (MS) medium (1962) is widely used for *Rubus in vitro* propagation. Zimmerman *et al.* (1980) successfully propagated thornless blackberry on MS medium containing 0.1 mg per l $GA_3$, 1.0 mg per l BA, and 1.0 mg per l IBA. Rapid proliferation of axillary buds of trailing blackberry, thornless 'Boysen', and thornless 'Young' was achieved after four to six weeks on a modified MS medium, containing 2.0 mg per l BA and 0.1 mg per l β-naphthaleneacetic acid (NAA) (Skirvin *et al.*, 1981). BA in the medium at 1.0, 2.0, and 5.0 mg per l did not significantly affect shoot proliferation, but $GA_3$ at 0.5 mg per l and 1.0 mg per l significantly increased the number of blackberry shoots (Slivinski *et al.*, 1983). Phloroglucinal (PG) also enhanced the number of shoots

(James *et al.*, 1980). Carrillo and Mendoza (1979) indicated that 5% (v/v) pure honey induced 69% well-formed adventitious buds in *Rubus* spp.

James *et al.* (1980) found that Linsmayer and Skoog (LS) medium (Linsmaier and Skoog, 1965) with 0.1 mg per l BA and 0.1 mg per l IBA was optimal for micropropagation of red raspberry. Anderson's (1980) medium produced two fold increases in shoot multiplication of red and black raspberries compared to MS medium with 1.0 mg per l IBA and 600 mg per l of activated charcoal. Other researchers also found Anderson's medium to be superior to MS medium for *in vitro* culture of raspberry (Pyott and Converse, 1981; Borgman and Mudge, 1986; Hoepfner, 1989). Reed (1990) screened 256 accessions of *Rubus* spp. and found that most blackberries grew well on MS medium with 1.0 mg per l BA and 0.1 mg per l IBA, but many raspberries did not grow well on MS medium even with BA, IBA, and $GA_3$. Many raspberries grew better on Anderson's basal medium with 1 or 2 mg per l BA.

Removing cytokinin and increasing IBA to 1.0 mg per l resulted in adventitious root formation from *in vitro*-cultured plantlets (James *et al.*, 1980). Skirvin *et al.* (1981) demonstrated that MS medium consisting of higher mineral salts, vitamins, and myo-inositol at 100 mg per l induced root formation. Proliferated *Rubus* shoots planted directly into Jiffy 7 pellets displayed 100% rooting efficiency (Snir, 1981). Under mist conditions, 75% of the microshoots rooted in sand medium with 0.1% IBA (Pyott and Converse, 1981).

## *IN VITRO* PLANT REGENERATION

### *Definition of regeneration*

Organized growing points on plant primary meristems are at the shoot apex, leaf axils, and root tip. Regeneration denotes the development of growing points or meristems adventitiously derived from somatic cells by organogenesis or embryogenesis.

Therefore, regenerants are plants arising adventitiously via regeneration (Dhir *et al.*, 1992).

Little is known about adventitious shoot development from the initial stimulus to the final regenerants. Broertjes and Keen (1980) suggest that adventitious shoots arise from a single cell or a small group of cells consisting of a single initial cell and a few adjacent cells. Arisumi and Frazier (1968) induced polyploidy in diploid African violet leaves by colchicine and concluded that the epidermis is the only layer involved in adventitious shoot formation. However, Norris *et al.* (1983) concluded that all leaf layers of African violet were involved in adventitious bud formation. Skene and Barlass (1983) showed that injured root apices of grapevine produced some periclinal chimeras, indicating new meristems are organized from several cells not just single cells. The regeneration and adventitious shoots from an *in vitro* culture is greatly influenced by plant genotype, and different response to regeneration within cultivars result from quantitative or qualitative genetic differences (Henry *et al.*, 1994).

*Factors affecting in vitro regeneration – Rubus spp.*

In the last decade, researchers developed several successful methods for *in vitro Rubus* regeneration and genetic transformation (Fiola *et al.*, 1990; Swartz *et al.*, 1990; McNicol and Graham, 1989; Cousineau and Donnelly, 1991; Owens y de Novoa and Connely, 1992; Cantoni *et al.*, 1993; Turk *et al.*, 1994; Gingas and Stokes, 1993; Mathews *et al.*, 1995; Wang and Wang, 1995; Hoepfner *et al.*, 1996; Mezzetti *et al.*, 1997; Graham *et al.*, 1997; Millan-Mendoza, 1998). People found that plants exhibit genotype dependent regeneration from different types of explants, various combinations of plant growth regulators, and different growth environments. Cantoni *et al.* (1993) observed that genotype and culture medium resulted in varied morphogenic processes, and shoots induced even from a single *Rubus* seed showed visible variation. Graham *et al.* (1997) regenerated adventitious shoots from eight genotypes of *Rubus* spp., but the efficiency varied greatly among genotypes. The explant, medium composition, and *in vitro* environment are the three major factors affecting plantlet regeneration.

*Explants*

Sources

*In vitro*-cultured explants from nodal cutting or meristem culture are the most common and successful materials used for *Rubus* regeneration (Fiola *et al.*, 1990; Swartz *et al.*, 1990; McNicol and Graham, 1989; Cousineau and Donnelly, 1991; Owens y de Novoa and Conner, 1992; Cantoni *et al.*, 1993; Graham *et al.*, 1997; Millan-Mendoza, 1998). Cousineau and Donnelly (1991) showed that greenhouse-grown raspberry 'Heritage' had a significantly higher percent regeneration than tissue-cultured plantlets.

Age

Explant age is an important factor affecting regenerability. In general, young leaves of *Rubus* regenerate better than older ones. However, no direct data and reports indicate which factors allow young tissues to regenerate easily. Usually, *in vitro*-cultured plantlets from 10 days (Owens y de Novoa and Conner, 1992) up to eight weeks (Graham *et al.*, 1997) after subculturing are used.

Gingas and Stokes (1993) studied asexual embryogenesis of *Rubus* using cotyledon explants. Three stages of cotyledons of *R. occidentalis* 'Bristol' and 'Jewel', and *R. idaeus* 'Exp-72' were tested on modified MS medium containing 200 mg per l casein hydrolysate, 3% sucrose, and 0, 0.45, and 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Only 11 regenerants were produced. They proposed that *in vitro* regeneration was influenced more by the developmental stage of the explant than by the addition of growth regulators.

Tissues

Tissue type influences regenerability. Ovules, embryos, cotyledons, internodal segments, petioles, and leaves of *Rubus* spp. are used for regeneration. Hall *et al.* (1986) reported that two of three regenerants from meristematic tissue died in culture. Fiola and Swartz (1986) observed somatic embryogenesis in *Rubus* crosses among 'Black Satin', 'C1', 'Cheyenne', 'Thornless Boysenberry', 'Marion', and 'Tayberry'. Adventitious shoots were obtained only from mature embryos, and most somatic embryos that formed

were morphologically abnormal. Fiola *et al.* (1990) found cotyledons represent a segregating generation from heterozygous material in which segregate is unable to form a whole plant. Organogenesis was induced from embryos (0.6 to 1.0 mm) of the *Rubus* cultivar 'Bulgarski Rubin' on MS medium with 0.3 mg per l BA and 0.05 mg per l $GA_3$.

McNicol and Graham (1989) tested six *Rubus* cultivars, including red raspberries 'Autumn Bliss' and 'SCRI selection 8242E6'; red raspberry X blackberry hybrids 'Tayberry', 'Tummelberry' and 'Sunberry'; and the blackberry 'Loch Ness' for regeneration potential. Modified MS medium with 0.1 mg per l IBA and 2.0 mg per l BA produced adventitious shoots from leaf disc explants. Medium with 0.2 mg per l BA and 0.2 mg per l 2,4-D was effective for regeneration of internodal segments, and the internodal segments had significantly higher regeneration if the epidermis was peeled off. Regeneration from internodal segments was less variable than from leaf disc explants. Cousineau and Donnelly (1991) indicated the leaf or petiole separately regenerated no differently than the leaf-petiole explant of raspberry. Hoepfner *et al.* (1996) obtained adventitious shoots from *in vitro*-cultured leaf segments of two red raspberry (*R. idaeus* L.) selections.

Excision and orientation

Wounding leaf explants promoted the percentage of explants regenerating, but the orientation of leaf explants touching the medium did not affect regeneration (Wang and Wang, 1995). Cousineau and Donnelly (1991), however, observed no regeneration difference in raspberries with scoring and with different leaf orientation, either adaxial or abaxial, facing the medium. Graham *et al.* (1990) used epidermal-peeled internodes to obtain higher regeneration. Hassan *et al.* (1993) regenerated plants from leaves with cross cuts on the mid-vein.

*Medium*

Basal media

MS is the most widely used basal medium for *in vitro* culture of many kinds of plants. However, different amounts and sources of ingredients such as nitrogen and iron may

affect plant regeneration *in vitro*. Turk *et al.* (1994) found that blackberry and raspberry genotypes responded differently to MS, Anderson's, N6, and woody plant (WPM) media as measured by the percentage of regeneration and shoots per regenerating explant.

Carbon sources

Carbon source, vitamins, and amino acid contents affect *in vitro* regeneration. Carbon sources like sucrose, glucose, sorbitol, or mannitol are thought related to osmotic potential in the culture medium. Generally, 2 to 3% sucrose is used for *in vitro* regeneration of most *Rubus* cultivars. Mathews *et al.* (1995) replaced 3% sucrose with 3% D-glucose and obtained effective regeneration of 'Meeker' raspberry. Other blackberry and red raspberry cultivars seemed to regenerate well in medium with sucrose.

Growth regulators

The concentration and combination of plant growth regulators required for regeneration greatly depends on the explant and plant genotype. Thidiazuron (TDZ) is more effective than BA for regenerating shoots of many woody plants (Huetteman and Preece, 1993). Mok *et al.*, (1982) reported that TDZ has cytokinin-like activity and is effective for stimulating axillary shoot proliferation or forming adventitious buds in many plant species. Fiola *et al.* (1990) found that TDZ is more effective than BA for inducing shoot organogenesis from excised cotyledons of *Rubus* seedlings. Cousineau and Donnelly (1991) found only eight of 22 red raspberry cultivars regenerated from leaf-petiole explants. TDZ was more effective than BA for promoting shoot regeneration on some cultivars.

Hoepfner *et al.* (1996) used MS medium with the combinations of IBA (4.9 μM) plus low (2.2 μM) and high (4.4 μM) BA concentrations, and 2.5 μM of IBA combined with low (4.5 μM) and high (9.1 μM) concentrations of TDZ to show that BA was more effective than TDZ for shoot regeneration. Shoots regenerated on TDZ medium were brittle with thickened, translucent stems and leaves. Wang and Wang (1995) indicated the optimal combination, 1.0 mg per l BA and 0.1 mg per l IBA, produced 79.17% blackberry leaf regeneration. The addition of 2,4-D in the regeneration medium induced callus formation in blackberry, but it was toxic to 'Autumn Bliss' raspberry (Mezzetti *et*

*al.* 1997). Morphogenesis of 'Autumn Bliss' was improved with 5 µM BA and 2.5 µM IBA in the regeneration medium. Leaf and petiole regeneration of blackberries 'Hull Thornless' and 'Chester Thornless' showed higher regeneration with BA (5 to 20 µM) and no auxin. BA (< 20 µM) combined with 2.5 µM IBA decreased morphogenesis, and MS basal medium with 10 µM IBA induced rooting. Graham *et al.* (1997) tested MS medium with combinations of auxin and cytokinin, and found that leaf disc regeneration required at least 9 µM TDZ, and that NAA (0.067 to 0.27 µM) helped induce adventitious shoots.

Pretreatments

TDZ and colchicine pretreatment of source plants caused an increase in the leaf and petiole size of differentiated shoots (Swartz *et al.*, 1990). Owens y de Denovoa and Conner (1992) examined the regeneration methods of Swartz *et al.* (1990), McNicol and Graham (1990), and Owens y de Denovoa (1992) for shoot regeneration from *Rubus* leaf explants and found that preculturing explants in TDZ was effective for producing more shoots, but the genotype effect was dominant. Mezzetti *et al.* (1997) indicated that pretreatment with 2,4-D promoted shoot differentiation in blackberry 'Hull Thornless'.

Gelling agents

The effects of gelling agents on micropropagation are documented on apples, pears, and 'Autumn Bliss' and 'Canby' red raspberries (Zimmerman *et al.*, 1995; Chevreau *et al.*, 1997). Researchers usually use 0.7 to 0.8% agar or 0.2 to 0.25% Gelrite to solidify tissue culture medium. Those concentrations are most useful for a wide range of species (Anderson, 1980; Reed, 1990). Debergh (1983) found the agar brand will affect the solidity of media, and low concentration or high matrix potential of agar media may interact with cytokinins to induce hyperhydricity. Cousineau and Donnelly (1991) noted that agar concentration ranging from 2.0 to 10.0 g per l did not affect *Rubus* regeneration *in vitro*. Gelrite induced higher regeneration frequency in pear leaves (Chevreau *et al.*, 1997) and more callus from banana leaves (Huang and Chi, 1988). Zimmerman *et al.* (1995) found medium solidified with a mixture of corn starch and Gelrite produced similar or better shoot proliferation than on an agar medium for two pear cultivars.

Starch-Gelrite medium was significantly better than the agar medium for shoot proliferation, for some apple cultivars and 'Canby' red raspberry.

Other medium additions

Adding chemicals such as silver nitrate or sequestrene to regeneration medium is useful for a range of crops (Taylor *et al.*, 1994; Hyde and Phillips, 1996; Castillo *et al.*, 1997). Mathews *et al.* (1995) added 10 mg per l silver nitrate to their culture medium for regeneration and transformation of raspberry 'Meeker' with the S-adenosylmethionine (SAMase) gene. Hyde and Phillips, (1996) examined four developmental stages of chili pepper (*Capsicum annuum* L.) organogenesis: bud induction, bud enlargement, shoot elongation, and root development. They found that silver nitrate at 5 mg per l promotes shoot development and regeneration from cotyledon explants on the second stage, bud enlargement. Silver nitrate is also necessary for multi-shoot production and elongation in the third culture stage. Silver nitrate, an ethylene inhibitor, helped protoplast isolation, ethylene production, and shoot regeneration from sugarcane cell suspension cultures (Taylor *et al.*, 1994).

While sequestrene, an iron chelator, is commonly used in raspberry regeneration medium, there are no published reports that indicate its usefulness. Some researchers report a positive effect from the iron chelate on micropropagation of rose (Castillo *et al.*, 1997; Van de Salm *et al.*, 1994). Because the production of chelator and membrane proteins is controlled by iron availability, reduced iron can inhibit growth.

*Environmental conditions*

Temperature, light intensity, and photoperiod

Cousineau and Donnelly (1991) concluded that the percentage of *Rubus in vitro* regeneration was not affected by temperature (25 $^0$C to 21 $^0$C) or darkness (one to three weeks). Turk *et al.* (1994), however, concluded that a 20 $^0$C incubation temperature with a one-week dark treatment followed by low illumination (40 $\mu mol \bullet m^{-2} \bullet s^{-1}$) increased regeneration in *Rubus* spp. compared to light at 80 $\mu mol \bullet m^{-2} \bullet s^{-1}$. Most studies use a 16-hour photoperiod for incubating explants (Hoepfner *et al.*, 1996; Graham *et al.*, 1997).

Continuous darkness greatly improved regeneration compared to only one, two, or three weeks in darkness following light treatment for *Rubus* spp. (Cousineau and Donnelly, 1991; Wang and Wang, 1995). Fiola *et al.* (1990) found incubating explants in the dark increased regeneration, but light induced more shoots.

## *ROSACEOUS* PLANT REGENERATION

There are many recent reports of *in vitro* regeneration of apple, pear, and strawberry. Almost every tissue of apple can be successfully regenerated. Liu *et al.* (1983) found an initial dark treatment of cotyledons and leaves followed by light produced from five to 20 times more adventitious shoots than culture in light alone. Far-red light promotes regeneration, while red light suppresses regeneration. Kouider *et al.* (1984) found that a dark treatment is essential for apple regeneration, and the age of the explant is important. The regenerability of immature apple embryos peaked at 2.5 months after pollination; if embryos were stored longer than six months, mature cotyledons lost their ability to form adventitious shoots (Kouider *et al.*, 1985). TDZ improved apple adventitious shoot formation from leaf discs as compared to BA (Van Nieuwkerk *et al.*, 1986; Fasolo *et al.*, 1989; Sriskandarajah *et al.*, 1990).

Regeneration in pear was improved by a dark treatment, the addition of TDZ (Chevreau *et al.*, 1989), and a 3:1 ratio or more of $NO_3^-$ to $NH_4^+$ (Abu-Qaoud *et al.*, 1991). BA is the primary cytokinin used for strawberry regeneration (Liu and Sanford, 1988; Nehra *et al.*, 1989). Miller (1990) developed a rapid regeneration method, which included excising and culturing cotyledon explants from mature strawberry achenes (*Fragaria ananassa* Duch.). Cotyledon explants formed callus with multiple buds on MS medium, and whole plantlets were produced by transferring callus to medium without plant hormones. Lis (1993) indicated that the percentage of regenerating strawberry stipule callus was better than from other explants, such as the leaf lamina, petiole, or root. Sorvari *et al.* (1993) showed that pre-culturing strawberry leaf discs on medium with

growth regulators promoted direct shoot regeneration. Infante and Rosati (1993) regenerated strawberry (*F. vesca* L.) plants from protoplasts. Effective shoot regeneration directly from stolon internodes and peduncle of strawberry was cultivar-dependent. Infante (1996) obtained the highest regeneration rate, 10 regenerants per petri dish, from the callus of long-term cell suspension cultures after transferring callus to shoot regeneration medium. Damiano *et al.* (1995) developed a regeneration protocol using cell suspension cultures of strawberry and evaluated the variability of regenerants through enzyme analysis. Liu and Sanford (1988) observed that leaf explants of strawberry from *in vitro*-cultured plants regenerated more efficiently than did leaf explants from greenhouse grown plants. Dark treatment for two or three weeks produced more regenerants in apple (Fasolo *et al.*, 1989). Nehra *et al.* (1989) reported that low light enhanced strawberry regeneration. Finally, fully mature strawberry embryos did not produce embryogenic tissues (Wang *et al.*, 1984) and apple testa and endosperm did not grow *in vitro* (Kouider *et al.*, 1985).

## MISCELLANEOUS PLANT REGENERATION

In general, young tissues of many kinds of plants regenerate better than old tissues (Niederwieser and Van Staden, 1990; Brand and Lineberger, 1991). No direct data or reports indicate which factors determine the cause of regeneration. Possible explanations include high concentrations of endogenous cytokinins in young tissue and DNA methylation related to transcription in cell differentiation, but these have not been proven. Low endogenous cytokinins in the leaves of *Lachenalia* are beneficial for increasing bud numbers (Niederwieser and Van Staden, 1990), and the level of DNA methylation observed was similar in embryogenic and non-embryogenic callus (Morrish and Vasil, 1988). Chaudhury and Signer (1989) compared leaves, cotyledons, stem segments, and epidermal peels of five *Arabidopsis* genotypes and found cotyledons gave consistently better regeneration than other explants.

Kaul *et al.* (1990) studied *Chrysanthemum* and found differences in regenerability among 11 cultivars on regeneration medium with a wide range of BA and NAA combinations. Jourdan and Earle (1989) stated that some cytoplasmic factors might control regenerability; a cytoplasmic male-sterile line of *Brasica* regenerated less frequently than its corresponding male-fertile line. Nehra *et al.* (1989) reported that more shoots regenerated when the adaxial surface of the leaf explant was placed in touch with the medium.

B5 medium has a lower concentration of ammonium than MS medium (Gamborg *et al.*, 1968). A change of basal medium from MS to B5 resulted in sub-epidermal regeneration rather than the usual epidermal regeneration in African violets (Bilkey and Cocking, 1981). Abu-Qaoud *et al.* (1991) pointed out that ammonium is required for regeneration, and the best ratio for ammonium to nitrate was 1:2 or 1:3. Rice callus regenerated shoots when sorbitol or mannitol was applied, and lost regenerability once the sugar-alcohols were taken away (Kavi Kishor and Reddy, 1986).

Rowland and Ogden (1992) indicated that zeatin riboside is more efficient than zeatin in stimulating adventitious shoot formation of blueberry. A possible explanation for the effectiveness of zeatin riboside may be its slow and steady release from the medium. Calvo and Segura (1989) pointed out that light is essential for lavender shoot regeneration and high auxin concentration combined with dark treatment inhibited adventitious bud formation.

## THE APPLICATION OF *IN VITRO* REGENERATION

### *Biotechnology of Rubus spp.*

The introduction of new biotechnologies such as genetic markers and gene transformation has allowed new directions in *Rubus* research (Graham *et al.*, 1994; Graham and McNicol, 1990; Hassan *et al.*, 1993). β-glucuronidase (GUS) and SAMase genes were inserted into some *Rubus* genotypes by transformation of somatic tissues (Mathews *et al.*, 1995). Graham *et al.* (1994) determined the relationships of red

raspberry cultivars using DNA fingerprints. When DNA fingerprinting was used to compare regenerants with control plants, no genetic variations were detected (Hoepfner *et al.*, 1996). Randomly amplified polymorphic DNA (RAPD) marker analysis was carried out and demonstrated the genetic integrity of regenerated *Rubus* cultivars (Graham *et al.*, 1997).

*Transformation*

Due to recent advances, transgenic plants are now part of many important crop lines. Bacterial genes are now being integrated into plants (Barton *et al.,* 1983; Fralay *et al.,* 1983). Fillatti *et al.* (1987) did the first *Agrobacterium tumefaciens*-mediated transformation of a woody plant (poplar). The β-glucuronidase (GUS) reporter gene is a simple, quick, and reliable genetic transformation detection method (Jefferson, 1987). A GUS gene constructed into a plasmid known as pBI121 as part of a binary vector system with the NPTII (neomycin phosphotransferase II) gene conferring kanamycin resistance. A variety of antibiotics like carbenicillin (400 mg/l) are used for controlling *Agrobacterium* contamination in putative transformants. Selection pressure is applied after whole plantlets are regenerated.

Graham *et al.* (1990) developed regeneration systems for *Rubus* and *Ribes* spp. starting from leaf discs and internodal stem segments and using *A. tumefaciens* with the binary vector pBI121 plus the GUS marker gene. Excised leaf discs or internodal segments were held for 20 minutes in a suspension of the disarmed *Agrobacterium* isolate containing the binary vector pBI121. A dot blot assay for the NPTII gene confirmed that a gene was transferred to the plant material. Graham (1990) examined isolates of *A. rhizogenes*: Ar 2628, Ar 2629, Ar 9402, and of *A. tumefaciens*: Ach 5, Ach 516, T 37, and A 6, to determine the efficiency of infection in blackberries, raspberries, and blackberry-raspberry hybrids. The two isolates that consistently produced the best results were Ach 5 (*A. tumefaciens*) and Ar 9402 (*A. rhizogenes*).

Hassan *et al.* (1993) also developed a protocol for *in vitro Rubus* transformation and regeneration. They incubated leaves, internodes, and cotyledons with four *A. tumefaciens* strains. The blackberry ('Cherokee' X 'VSPB-1') was tested using leaf and internode inoculations, and seeds of 'Austin Thornless' X 'Tayberry' were used for cotyledon

analysis and verified the presence of GUS in the plant genome. In addition to *Agrobacterium* transformation, several vectorless gene-transfer systems, i.e. particle bombardment (biolistics), electroporation, membrane permeabilization, and protoplast fusion, are also available for use.

Once *Rubus* transformation and regeneration systems are developed, gene transfer in germplasm will be a reality. Exogenous genes for specific traits isolated from other genera may be inserted into popular cultivars to produce superior genotypes.

## *RUBUS* VIRUS DISEASES

Twenty-six distinct viruses and virus-like diseases are reported for *Rubus* crops throughout the world (Jones, 1986). They are classified into four groups according to their mode of transmission: large aphids (*Amphorophora* sp.), small aphids (*Aphis* sp.), nematodes, and infected pollen. In addition, there are some viruses or virus-like diseases of minor importance whose mode of transmission is unknown. Studies of *Rubus* virus diseases are facilitated by the use of enzyme-linked immunosorbent assay (ELISA). Pollen-borne raspberry bushy dwarf virus (RBDV) and aphid-borne black raspberry necrosis virus (BRNV) are detected by ELISA techniques (Jones, 1986). Kurppa and Martin (1986) introduced a double-stranded RNA technique to detect virus (RLSV), an aphid-borne virus.

### *Field-grown plants*

Virus diseases damage many crops in the field and sensitive cultivars may be killed or severely weakened by virus infection. Latent infections on tolerant cultivars may shorten the planting life and reduce yield and fruit quality (Converse, 1963). Blackberry and raspberry cultivars are susceptible to several virus diseases (Frazier, 1970). Raspberry bush dwarf idaeovirus (RBDV) and tobacco streak ilarvirus (TSV) are distributed worldwide. RBDV is transmitted by pollen; it is usually symptomless in either naturally or experimentally infected red raspberries (Murant, 1987). Under most circumstances,

RBDV caused "Crumbly" fruit of red raspberry and "yellows" symptoms may occur during late spring in sensitive cultivars or doubly infected (Jones *et al.*, 1982). Jones (1979) found that RBDV co-infected with black raspberry necrosis virus (BRNV) caused "bushy dwarf" symptoms in the field. TSV-infected *Rubus* plants do not generally show foliar symptoms; however, TSV may produce a chlorotic leaf pattern, chlorotic ringspot, or line-pattern symptoms when mixed with other viruses (Stace-Smith, 1987).

Tomato ringspot nepovirus (TomRSV) is soil transmitted via nematodes. Symptoms of TomRSV in raspberry may be visible throughout the growing season. New leaves may show yellow rings, line patterns, or a fine yellow vein chlorosis in the spring following the year of infection (Stace-Smith and Converse, 1987); however, symptoms may be unclear or absent in hot weather and following a recent TomRSV infection. Stace-Smith (1987) indicated that the infection of plants with TomRSV and a complex of other viruses induced a fernleaf mosaic symptom not seen in single infections. Chronic infection with TomRSV causes dwarfing and slow growth in the spring. Spring cane death may occur and surviving canes produce small leaves with early fall abscission (Freeman and Stace-Smith, 1968).

*Virus incidence in Rubus collections*

RBDV is an important raspberry virus disease and was found in 25 of 75 cultivars maintained in the British Columbia red raspberry breeding program (Daubeny *et al.*, 1978). Virus incidence in the *Rubus* accessions received at the National Clonal Germplasm Repository (NCGR) in Corvallis, OR included: TomRSV in 0.44% of 455 accessions, TSV in 2.98% of 436 accessions, and RBDV in 5.82% of 447 accessions (Postman, 1989). Presently, virus incidence is: TomRSV in 0.31% of 637 accessions, TSV in 2.76% of 724 accessions, and RBDV in 5.77% of 745 accessions. There is also one combined infection of RBDV and TSV (Postman, unpublished).

*In vitro plants*

The virus status of explants for *in vitro* culture is often unknown, but may play an important role in micropropagation. Inconsistent results and loss of plant material might be due to undetected virus infections.

De Vries-Paterson *et al.* (1992) first reported the effects of single and two-virus infections in reducing asparagus *in vitro* root development, culture survival, and fresh and dry weights in micropropagated plants. Only 8.6% of the asparagus clones were virus-free following *in vitro* shoot-tip culture alone, without other virus-elimination treatments. Establishment of *in vitro* sugarcane cultures was more successful from virus-free material than from material showing symptoms of maize streak virus (MSV) (Peros *et al.*, 1990). Most explants used for *in vitro* culture are not tested for virus infection and are not produced from heat-treated meristems. Combined heat treatment and meristem-culture are successfully used to initiate virus-free raspberry cultures (Pyott and Converse, 1981). There are no reports on the effects of viruses on raspberry shoot cultures.

*Virus elimination*

Viruses can be eliminated from raspberries and blackberries by micropropagation of meristems, either with or without thermotherapy (Shchelkunova and Popov, 1970). In thermotherapy, the heat treatment slows the growth of virus in the shoot tips, while the plant continues to grow. Very small apices (0.5 mm or less) are excised, then transferred to tissue culture, and grown into virus-free plants (Pyott and Converse, 1981). After obtaining virus-free clones, they should be tested and maintained as stock plants.

# References


Abu-Qaoud, H.; Skirvin, R. M.; Below, F. E. Influence of nitrogen from and $NH^{4+}/NO^{3-}$ ratios on adventitious shoot formation from pear (*Pyrus communis*) leaf explants *in vitro*. Plant Cell Tiss. Org. Cult. 27:315-319; 1991.

Anderson, W. C. Tissue culture propagation of red and black raspberry, *R. idaeus* and *R. occidentalis*. Acta Hort. 112:13-20; 1980.

Arisumi, T.; Frazier, C. C. Cytological and morphological evidence for the single-cell origin or vegetively propagated shoots in thirteen species of *Saintpaulia* treated with colchicine. Proc. Amer. Soc. Hort. Sci. 93:679-685; 1968.

Asker, S. E.; Jerling, L. Apomixis in Plants. Boca Raton Fla: CRC Press; 1992.

Barton, K. A.; Binns, A. J.; Matzke, A. J. M.; Chilton, M. D. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033-1043; 1983.

Bilkey, P. C.; Cocking, E. C. Increased plant vigor by *in vitro* propagation of *Saintpaulia ionantha* Wendl. from sub-epidermal tissue. HortScience 16:643-644; 1981.

Borgman, C. A.; Mudge, K. W. Factors affecting the establishment and maintenance of 'Titan' red raspberry root organ cultures. Plant Cell Tiss. Org. Cult. 6:127-137; 1986.

Brand, M. H.; Lineberger, R. D. The effect of leaf source and development stage on shoot organogenic potential of sweet gum (*Liquidambar styraciflua* L.) leaf explants. Plant Cell Tiss. Org. Cult. 24:1-7; 1991.

Britton, D. M.; Hull, J. W. Miotic instability in blackberry seedlings from progenies of Boysen and of Young. J. Hered. 48, 11-20; 1956.

Britton, D. M.; Hull, J. W. Miotic instability in *Rubus*. J. Hered. 48:11-20; 1957.

Broertjes, C. B.; Keen, A. Adventitious shoots: Do they develop from one cell? Euphytica 29:73-87; 1980.

Broome, O. C.; Zimmerman, R. H. *In vitro* propagation of blackberry. HortScience 13:151-153; 1978.

Calvo, M. C.; Segura, J. Plant regeneration from cultured leaves of *Lavandula latifolia* Mediaes: Influence of growth regulators and illumination conditions. Plant Cell Tiss. Org. Cult. 16:75-87; 1989.

Cantoni, G.; Berardi, G.; Rosati, P. Organogenesis and somatic embryogenesis from immature embryos of blackberry. Acta Hort. 352:37-42; 1993.

Carrillo, C. G.; Mendoza, J. L. Effect of natural products on *in vitro* development of *Rubus* spp. buds. Turrialba 29:299-303; 1979.

Castillo, B.; Smith, M. A. L.; Madhavi, D. L. Interactions of irradiance level and iron chelate source during shoot tip culture of *Carica papaya* L. HortScience 32:1120-1123; 1997.

Chaudhury, A. M.; Signer, E. R. Relative regeneration proficiency of *Arabidopsis thaliana* ecotypes. Plant Cell Rep. 8:368-369; 1989.

Chevreau, E.; Skirvin, R. M.; Abu-Qaoud, H. A.; Korban, S. S.; Sullivan, J. G. Adventitious shoot regeneration from leaf tissue of three pear (*Pyrus* sp.) cultivars *in vitro*. Plant Cell Rep. 7:688-691; 1989.

Chevreau, E.; Mourgues, F.; Neveu, M.; Chevalier, M. Effect of gelling agents and antibiotics on adventitious bud regeneration from *in vitro* leaves of pear. *In vitro* Cell. Dev. Biol. – Plant 33:173-179; 1997.

Converse, R. H. Influence of heat-labile components of the raspberry mosaic complex on growth and yield of red raspberries. Phytopathology 53:1251-1254; 1963.

Cousineau, J. C.; Donnely, D. J. Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss. Org. Cult. 27:249-255; 1991.

Crane, M. B.; Thomas, P. T. Reproductive versatility in *Rubus*. III Raspberry-blackberry hybrids. Heridity 3:99-107; 1949.

Daminano, C.; Ascarelli, A.; Frrattarelli, A.; Lauri, P. Adventitious regeneration and genetic variability in strawberry. Acta Hort. 392:107-114; 1995.

Daubeny, H. A.; Stace-Smith, R., Freeman, J. A. The occurrence and some effects of raspberry bushy dwarf virus in red raspberry. J. Amer. Soc. Hort. Sci. 103:519-522; 1978.

De Vries-Paterson, R. M.; Evans, T. A.; Stephens, C. T. The effect of asparagus virus infection on asparagus tissue culture. Plant Cell Tiss. Org. Cult. 31:31-35; 1992.

Debergh, P. C. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant 59:270-276; 1983.

Dhir, S. K.; Dhir, S.; Widholm, J. M. Regeneration of fertile plants from protoplasts of soybean (*Glycine max* L. Merr.): genotypic differences in culture response. Plant Cell Rep. 11:285-289; 1992.

Einset, J. Apomixis in American polyploid blackberries. Am. J. Bot. 38:768-772; 1951.

Fasolo, F.; Zimmerman, R. H.; Fordham, I. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16:75-87; 1989.

Fillatti, J. A. J.; Sellmer, J.; McCown, B., Haissig, B., Comai, L. *Agrobacterium* mediated transformation and regeneration of *Populus*. Molecular General Genetics 206:192-199; 1987.

Fiola, J. A.; Swartz, H. J. Somatic embryogenesis, organogenesis and proliferation *in vitro* from *Rubus* embryos. Acta Hort. 183:91-98; 1986.

Fiola, J. A.; Hassan, M. A.; Swartz, H. J.; Bors, R. H.; McNicol, R. J. Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss. Org. Cult. 20:223-228; 1990.

Focke, W. O. Species Ruborum: Monographiae generis Rubi Podromus, part I. Biblioth. Bot. 17:1-120; 1910.

Fralay, R. T.; Rogers, S. G.; Horsch, R. B.; Sanders, P. R.; Flock, J. S.; Adams, S. P.; Bittner, M. L.; Brand, L. A.; Fink, L. L.; Fry, J. S.; Galluppi, G. R.; Goldberg, S. B.; Hoffmann, N. L.; Woo, S. C. Expression of bacterial genes in plant cells. Proceedings National Academy Science USA 80:4803-4807; 1983.

Frazier, N. W., Ed. Virus Diseases of Small Fruits and Grapevines. Univ. of Calif. Div. of Agric. Sci., Berkeley, Calif. 290 pp; 1970.

Freeman, J. A.; Stace-Smith R. Effect of tomato ringspot virus on plant and fruit development of raspberries. Canadian J. of Plant Science 48:25-29; 1968.

Gamborg, O. C.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50:148-151; 1968.

Gingas, V. M.; Stokes, B. D. *Rubus* plant regeneration via asexual embryogenesis. HortScience 28:58; 1993.

Graham, J. The development and application of methods for using *Agrobacteium* spp. as DNA vectors in soft fruit. Ph.D. Thesis, St. Andrews University; 1990.

Graham, J.; McNicol, R. J. Plant regeneration and genetic transformation in soft fruit species. Acta Hort. 280:517-522; 1990.

Graham, J.; Iasi, L.; Millam, S. Genotype-specific regeneration from a number of *Rubus* cultivars. Plant Cell Tiss. Org. Cult. 48:167-173; 1997.

Graham, J.; McNicol, R. J.; Kumar, A. Use of the GUS gene as a selectable marker for *Agrobacterium*-mediated transformation of *Rubus*. Plant Cell Tiss. Org. Cult. 20:35-39; 1990.

Graham, J.; McNicol, R. J.; Greig, K.; Van de Ven W. T. G. Identification of red raspberry cultivars and an assessment of their relatedness using fingerprints produced by random primers. J. Hort. Sci. 69:123-130; 1994.

Gu, Y.; Zhao, C. M.; Jun, W. O. *Rubus* resources in Hunan and Fujian provinces. XXIII Intl. Hort. Congr. 4014; 1990 (abst.).

Hall, H. K.; Quazi, M. H.; Skirvin, R. M. Isolation of a pure thornless loganberry by meristem tip culture. Euphytica 35:1039-1044; 1986.

Harper, P. C. Tissue culture propagation of blackberry and tayberry. Hort. Res. 18:141-143; 1978.

Haskell, G.; Tun, N. N. Developmental sequence of chromosome number in a cytologically unstable *Rubus* hybrid. Genet. Res. 2:10-24; 1961.

Hassan, M. A.; Swartz H. J.; Inamine G., Mullineaux P. *Agrobacterium tumefaciens*-mediated transformation of several *Rubus* genotypes and recovery of transformed plants. Plant Cell Tiss. Org. Cult. 33:9-17; 1993.

Heit, C. E.; Slate, G. L. Treatment of blackberry seed to secure first year germination. Proc. Amer. Soc. Hort. Sci. 55: 297; 1950.

Henry, Y.; Vain, P.; Buyser, J. D. Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. Euphytica 79:45-58; 1994.

Hoepfner, A. S. *In vitro* propagation of red raspberry. Acta Hort. 262:285-288; 1989.

Hoepfner, A. S.; Nestby, R.; Nybom, H. Genetic deviation initiated by adventitious shoot regeneration from tissue cultured red raspberry. J. of Hort. Sci. 71:71-79; 1996.

Huang, L. C.; Chi, D. L. Pivotal roles of picloram and gelrite in banana callus culture. Environmental and Experimental Botany 28:249-258; 1988.

Huetteman, C. A.; Preece, J. E. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33:105-119; 1993.

Hummer, K. E. *Rubus* diversity. HortScience 31:182-183; 1996.

Hyde, C. L.; Phillips, G. C. Silver nitrate promotes shoot development and plant regeneration of chili pepper (*Capsium annuum* L.) via organogenesis. *In vitro* Cell. Dev. Biol.- Plant 32:72-80; 1996.

Infante, R. Long-term cell suspension culture and regeneration of the single-leafed strawberry *Fragaria vesca* monophylla. J. Sci. Food Agric. 72:196-200; 1996.

Infante, R.; Rosati, P. *Fragaria vesca* L. 'Alpine' protoplast culture and regeneration. Acta Hort. 348:432-434; 1993.

James, D. J.; Knight, V. H.; Thurbon, I. J. Micropropagation of red raspberry and the influence of phloroglucinol. Sci. Hort. 12:313-319; 1980.

Jefferson, R. A. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter 8:387-405; 1987.

Jennings, D. L. Raspberries and blackberries: Their Breeding, Diseases and Growth. Academic Press, London; 1988.

Jennings, D. L.; Tulloch, B. M. M. Studies on factors which promote germination of raspberry seeds. J. Exp. Bot. 16:329-340; 1965.

Jennings, D. L.; Daubeny, H. A.; Moore, J. N. Blackberries and raspberries (*Rubus*). Acta Hort. 290:331-389; 1990.

Jones, A. T. The effects of black raspberry necrosis and raspberry bushy dwarf viruses in Lloyd George raspberry and their involvement in raspberry bushy dwarf disease. J. Hortic. Sci. 54:267-272; 1979.

Jones, A. T.; Murant, A. F.; Jennings, D. L.; Wood, G. A. Association of raspberry bushy dwarf virus with raspberry yellows disease; reaction of *Rubus* species and cultivars, and the inheritance of resistance. Annals of Applied Biology 100:135-147; 1982.

Jones, A. T. Advances in the study, detection and control of viruses and viruse diseases of *Rubus* with a particular reference to the United Kingdom. Crop Res. 26:127-171; 1986.

Jourdan, P. S.; Earle, E. D. Genotypic variability in the frequency of plant regeneration from leaf protoplasts of four *Brassica* spp. and of *Raphanus sativus*. J. Amer. Soc. Hort. Sci. 114:343-349; 1989.

Kaul, V.; Miller, R. M.; Hutchinson, J. F.; Richards, D. Shoot regeneration from stem and leaf explants of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell Tiss. Org. Cult. 21:21-30; 1990.

Kavi Kishor, P. B.; Reddy, G. M. Retention and revival of regenerating ability by osmotic adjustment in long-term culture of four varieties of rice. J. Plant Phys. 126:49-54; 1986.

Ke, S. Q.; Skirvin, R. M.; McPheeters, K. D.; Otterbacher, A. G.; Galletta, G. *In vitro* germination and growth of *Rubus* seeds and embryos. HortScience 20:1047-1049; 1985.

Keep, E. Incompatibility in *Rubus* with special reference to *R. idaeus* L. Can. J. Genet. Crytol. 10:253-262; 1968.

Kiss, F.; Zatykó, J. Vegetative propagation of *Rubus* species *in vitro*. Bot. Koxlem 65:65-68; 1978.

Kouider, M.; Korban, S. S.; Skirvin, R. M.; Chu, M. C. Influence of embryonic dominance and polarity on adventitious shoot formation from apple cotyledons *in vitro*. J. Amer. Soc. Hort. Sci. 109:381-385; 1984.

Kouider, M.; Korban, S. S.; Skirvin, R. M.; Joung, H. The relationship of apple embryos and their cotyledons to maturity, dormancy, and the potential to form shoots *in vitro*. J. Amer. Soc. Hort. Sci. 110:93-96; 1985.

Kurppa, A.; Martin, R. R. Use of double-stranded RNA for detection and identification of virus diseases of *Rubus* species. Acta Hort. 186:51-62; 1986.

Lashecn, A. M.; Blackhurst, H. T. Biochemical changes associated with dormancy and after-ripening of blackberry seeds. Proc. Amer. Soc. Hort. Sci. 67:331-340; 1956.

Linsmaier, E. M.; Skoog, F. Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum 18:100-127; 1965.

Lis, E. K. Strawberry plant regeneration by organogenesis from peduncle and stolon segments. Acta Hort. 348:435-438; 1993.

Liu, J. R.; Sink, K. C.; Dennis, F. G. Plant regeneration from apple seedling explants and callus culture. Plant Cell Tiss. Org. Cult. 2:293-304; 1983.

Liu, Z. R.; Sanford, J. C. Plant regeneration by organogenesis from strawberry leaf and runner tissue. HortScience 23:1057-1059; 1988.

Mathews, H.; Wagoner, W.; Cohen, C.; Kellogg, J., Bestwick, R. Efficient genetic transformation of red raspberry, *Rubus idaeus* L. Plant Cell Rep. 14:471-476; 1995.

McNicol, R. J.; Graham, J. Genetic manipulation in *Rubus* and *Ribes*. Acta Hort. 262:41-46; 1989.

McNicol, R. J.; Graham, J. *In vitro* regeneration of *Rubus* from leaf and stem segments. Plant Cell Tiss. Org. Cult. 21:45-50; 1990.

Mezzetti, B.; Savini, G.; Carnevali, F.; Mott, D. Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biologia-Plantarum 39:139-150; 1997.

Millan-Mendoza, B. Regeneration of *Rubus in vitro* using forchlorfenuron (CPPU). Rev. Fac. Agron. 15:242-248; 1988.

Miller, A. R. Plant regeneration from excised cotyledons of mature strawberry achenes. HortScience 25:569-571; 1990.

Mok, M. C.; Mok, D. W. S.; Dixon, K. W.; Armonstrong, D. J.; Shaw, G. Cytokinin structure-activity relationships and the metabolism of N6-(Delta 2-Isopentenyl) Adensine-8-14C in callus tissues. Plant Physiol. 70:173-178; 1982.

Morrish, F. M.; Vasil, I. K. DNA methylation and embryogenic competence in leaves and callus of napiergrass (*Pennisetum purpureum* Schum.). Plant Physiol. 90:37-40; 1988.

Murant, A. F. Raspberry bushy dwarf. pp. 229-234. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol. Plant 15:473-479; 1962.

Nehra, N. S.; Stushnoff, C.; Kartha, K. K. Direct shoot regeneration from strawberry leaf disks. J. Amer. Soc. Hort. Sci. 114:1014-1018; 1989.

Niederwieser, J. G.; Van Staden, J. The relationship between genotype tissue age and endogenous cytokinin levels on adventitious bud formation on leaves of *Lachenalia*. Plant Cell Tiss. Org. Cult. 22:223-228; 1990.

Norris, R.; Smith, R. H.; Vaughn, K. C. Plant chimeras used to establish *de novo* origin of shoots. Science 220:75-76; 1983.

Nybom, H. Chromosome numbers in *Rubus* species from Sri Lanka. Bot. Notiser 133:47-48; 1980.

Ourecky, D. K. Brambles. In: Advances in Fruit Breeding. eds. Janick J. and Moore N. Purdue University Press, West Lafayette, Indiana, USA; 1975.

Owens y de N, C. Empirical evaluation of *in vitro* media components for cell growth and shoot regeneration from *Rubus* explants. New Zealand Natural Sciences 19:79-86; 1992.

Owens y de N, C.; Conner, A. J. Comparison of *in vitro* shoot regeneration protocols from *Rubus* leaf explants. New Zealand Journal of Crop and Horticultural Science 20:471-473; 1992.

Peros, J. P.; Bonnel, E.; Reynaud, B. *In vitro* culture of sugarcane infected with maize streak virus (MSV). Plant Cell Tiss. Org. Cult. 23:145-149; 1990.

Postman, J. D. Incidence of viruses in the U.S. National Clonal Germplasm Repository *Ribes* and *Rubus* collections. Acta Hort. 262:217-222; 1989.

Pritts, M.; Handley, D. Bramble Production Guide. Northeast Regional Agricultural Engineering Service. New York; 1994.

Pyott, J. L.; Converse, R. H. *In vitro* propagation of heat-treated red raspberry clones. HortScience 16:308-309; 1981.

Reed, B. M. Multiplication of *Rubus* germplasm *in vitro*: a screen of 256 accessions. Fruit Var. J. 44:141-148; 1990.

Rowland, L. J.; Ogden, E. L. Use of a cytokinin conjugate for efficient shoot regeneration from leaf sections of high bush blueberry. HortScience 27:1127-1129; 1992.

Schopmeyer, C. S. Seeds of Woody Plants in the United States. Agric. Handbook No. 450:738-742; 1974.

Scott, D. H.; Draper, A. O. Light in relation to seed germination of blueberry, strawberry, and *Rubus*. HortScience 2:107-108; 1967.

Shchelkunova, S. E.; Popov, Y. G. Effect of growth regulator differentiation of isolated stem apexes of raspberry plants. Fiziol. Rast. 17:618-622; 1970.

Skene, K. G. M.; Barlass, M. Studies on the fragmented shoot apex of grapevine IV. Separation of phenotypes in a periclinal chimera *in vitro*. J. Exp. Bot. 34:1271-1280; 1983.

Skirvin, R. M.; Chu, M. C.; Gomez, E. *In vitro* propagation of thornless trailing blackberries. HortScience 16:310-312; 1981.

Slivinski, J. A.; Preece, J. E.; Myers, O. *In vitro* micropropagation of thornless blackberries utilizing single node explants. Plant Propagator 30:4-5; 1983.

Snir, I. Micropropagation of red raspberry. Sci. Hort. 14:139-143; 1981.

Soolova, V. A.; Kichira, V. V. Increasing the germination rate of raspberry seeds. Vestnik Sel Skok Hozyaistvennol Nanl. 16:87-90; 1971.

Sorvari, S.; Ulvinen, S.; Hietaranta, T.; Hiirsalmi, H. Preculture medium promotes direct shoot regeneration from micropropagated strawberry leaf disks. HortScience 28:55-57; 1993.

Sriskandarajah, S.; Skirvin, R. M.; Abu-Qaoud, H.; Korban, S. S. Factors involved in shoot elongation and growth of adventitious and axillary shoots of three apple scion cultivars *in vitro*. J. Hort. Sci. 65:113-121; 1990.

Stace-Smith, R. Tobacco streak virus in *Rubus*. pp. 235-239. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Stace-Smith, R.; Converse, R. H. Tomato ringspot virus in *Rubus*. pp. 223-227. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Swartz, H. J.; Bors, R.; Mohamed, F.; Naess, S. K. The effect of pretreatment on subsequent shoot organogenesis from *Rubus* and *Malus* leaves. Plant Cell Tiss. Org. Cult. 21:179-184; 1990.

Tammisela, J.; Ryxjnänen, A. Incompatibility in *Rubus arctius* L.. Hereditas 66:269-278; 1970.

Taylor, P. W. J.; Ko, H. L.; Fraser, T. A.; Masel, N.; Adkins, S. W. Effect of silver nitrate on sugarcane cell suspension growth, protoplast isolation, ehylene production and shoot regeneration from cell suspension cultures. J. of Experimental Botany 45:1163-1168; 1994.

Thompson, M. M. Chromosome numbers of *Rubus* species at the National Clonal Germplasm Repository. HortScience 30:1447-1452; 1995a.

Thompson, M. M. Chromosome numbers of *Rubus* cultivars at the National Clonal Germplasm Repository. HortScience 30:1453-1456; 1995b.

Thompson, M. M. Survey of Chromosome numbers in *Rubus*. Annals of the Missouri Botanical Garden 84:128-165; 1997.

Tomanova, R. N. The effect of potassium iodide on raspberry seed germination and on physiological process in the seedlings. ObraZovania 42:169-171; 1971.

Turk, B. A.; Swartz, H. J.; Zimmerman, R. H. Adventitious shoot regeneration from *in vitro*-cultured leaves of *Rubus* genotypes. Plant Cell Tiss. Org. Cult. 38:11-17; 1994 (abst.).

Van der Salm, T. P. M.; Van der Toorn, C. J. G.; Hanisch ten Cate, C. H. Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tiss. Org. Cult. 37:73-77; 1994.

Van Nieuwkerk, J. P.; Zimmerman R. H.; Fordham, I. Thidiazuron stimulation of apple shoot proliferation *in vitro*. HortScience 21:516-518; 1986.

Wang, D.; Wergin, W. P.; Zimmerman, R. H. Somatic embryogenesis and plant regeneration from immature embryos of strawberry. HortScience 19:71-72; 1984.

Wang, G. Y.; Wang, G. H. Regeneration of blackberry plantlets from leaves and stem segments of *in vitro* plants. Acta Agriculturae Universitatis Pekinensis 21:12-16; 1995.

Zielinski, Q. B.; Thompson, M. M. Pollen germination in *Pyrus* species and species hybrids. Euphytica 15:195-198; 1966.

Zimmerman, R. H.; Bhardwaj, S. V.; Fordham, I. M. Use of starched-gelled medium for tissue culture of some fruit crops. Plant Cell Tiss. Org. Cult. 43:207-213; 1995.

Zimmerman, R. H.; Galletta, G. J.; Broome, O. C. Propagation of thornless blackberries by one-node cuttings. J. Amer. Soc. Hort. Sci. 105:405-407; 1980.

# CHAPTER 2. IRON CHELATE SEQUESTRENE IMPROVES ADVENTITIOUS SHOOT PRODUCTION FROM WHOLE-LEAF EXPLANTS OF *RUBUS* CULTIVARS

**C. W. V. Tsao[1] and B. M. Reed[2]**

*Department of Horticulture, Oregon State University, Corvallis, Oregon 97331 (C.W.V.T.); USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd. Corvallis, Oregon 97333-2521 (B.M.R.)*

RUNNING HEAD: *RUBUS* REGENERATION FROM WHOLE LEAF EXPLANTS

[1] Part of a thesis submitted by C.W.V.T. in partial fulfillment of the requirements for the MS degree.

[2] To whom correspondence should be addressed. E-mail: reedbm@bcc.orst.edu

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# SUMMARY

Nine commercially important blackberry cultivars: Cherokee (4X), Chester Thornless (4X), Hull Thornless (4X), Kotata (7X), Marion (6X), Navaho (4X), Shawnee (4X), Thornless Evergreen (4X), and Waldo (6X); and four raspberry cultivars: Autumn Bliss (2X), Heritage (2X), Latham (2X), and Watson (Ruby™) (2X), were tested for regeneration using the top 1 to 6 leaves from *in vitro* plantlets. Adventitious shoots were successfully produced from all thirteen cultivars with a range of regeneration treatments at 20 $^{0}$C with a 16-hour photoperiod. Regeneration depended on genotype and plant growth-regulator combinations. The addition of the iron chelate, sequestrene at 200 mg per l, significantly increased regeneration from 30 to 40% for 'Marion' and from 23 to 43% for 'Kotata' when included in the second regeneration medium (RM). Three gelling agents (agar, Gelrite, and a combination) and silver nitrate ($AgNO_3$) did not affect blackberry regeneration. The ploidy level (2X, 4X, 6X, or 7X) and berry type (blackberry or raspberry) of 13 *Rubus* cultivars screened also did not influence regeneration. RM with $N^6$-benzyladenine (BA) induced shoots on more *Rubus* cultivars than did RM with thidiazuron (TDZ), particularly for most blackberries. Explants on RM with 5 µM IBA produced significantly more callus, but fewer shoots, compared to zero or 0.5 µM IBA treatments. 'Heritage' and 'Latham' raspberry produced shoots only on RM with 5 or 10 µM BA. RM with 0.5 to 10 µM TDZ induced shoots on 'Autumn Bliss' raspberry, while 'Ruby' responded to BA and TDZ at 0.5 to 10 µM. We recommend using a regeneration medium with 200 mg per l sequestrene and either 10 µM BA alone or with 0.5 µM IBA for *in vitro* leaf regeneration from most *Rubus* genotypes.


*Key words*: blackberry; raspberry; adventitious shoot; regeneration; cytokinin; silver nitrate; sequestrene; gelling agent.

# INTRODUCTION

*Rubus*, one of the most diverse genera in the plant kingdom, includes two economically important crops, raspberry (*Rubus idaeus* L.) and blackberry (*Rubus* sp.) (Jennings, 1988). The *Idaeobatus* subgenus contains raspberries that are mostly diploids. The subgenus *Rubus* (former *Eubatus*) includes the blackberries that range from diploid (2n = 14) to 2n = 14 X = 98 or possibly 2n = 18X = 126, including odd-ploids and aneuploids (Thompson, 1995a, 1995b, and 1997). Genotypic variability in response to tissue culture is well documented (Walden and Wingender, 1995). It is not known if ploidy level influences regenerability *in vitro*.

More than a dozen studies on *Rubus in vitro* regeneration are reported, but only a small proportion (< 2 %) of *Rubus* cultivars were successfully regenerated and only a few transformed with foreign genes grew into whole plants (Mathews *et al.*, 1995). Adventitious shoots are regenerated from leaves, petioles, internodes, cotyledons, and immature and mature embryos of *in vitro* cultured *Rubus* plantlets. (Fiola and Swartz, 1986; Graham and McNicol, 1990; Cousineau and Donnelly, 1991; Cantoni *et al.*, 1993; Gingas and Stokes, 1993; Hassan *et al.*, 1993; Hoepfner *et al.*, 1996). Murashige and Skoog (MS) medium (1962) has been widely used for regeneration of all *Rubus* genotypes (Turk *et al.*, 1994; Graham *et al.*, 1997). Using $N^6$-benzyladenine (BA) (5 to 10 μM) or thidiazuron (TDZ) (≤ 1 μM) alone or in combination with less than 1 μM of either indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) work well on many *Rubus* genotypes (Norton, 1994; Wang and Wang, 1995; Graham *et al.*, 1997). N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), a synthetic cytokinin-like compound, was first reported useful for *Rubus* regeneration using internodal segments (Millan-Mendoza, 1998). Swartz *et al.* (1990) enhanced organogenesis of *in vitro* leaf explants from two *Rubus* hybrids using a TDZ pretreatment. Additions of chemicals such as silver nitrate or sequestrene to regeneration medium may be useful for a range of crops (Taylor *et al.*, 1994; Hyde and Phillips, 1996; Castillo *et al.*, 1997).

The effects of gelling agents on micropropagation were tested on apples, pears, and 'Autumn Bliss' and 'Canby' red raspberries (Zimmerman *et al.*, 1995; Chevreau *et al.*,

1997). Regeneration medium gelled with agar (0.2 to 1.0%) or gellan gum (Phytagel™) (0.2 to 0.25%) and growth room temperatures ranging from 20 to 25 $^{0}$C had no effect on raspberry regeneration (Cousineau and Donnelly, 1991). Although some protocols produce 50% or greater regeneration frequencies from specific cultivars, no protocol is available for use with multiple *Rubus* genotypes (Owens y de Novoa and Conner, 1992; Gingas and Stokes, 1993; Mezzetti *et al.*, 1997).

Our objective was to develop a leaf regeneration system applicable to many *Rubus* genotypes. Combinations of plant growth regulators, gelling agents, additives, explant types, and incubation conditions were tested on thirteen *Rubus* cultivars, including commercially important blackberries and raspberries.

## MATERIALS AND METHODS

*Establishment of in vitro cultures*

Nine blackberry cultivars: Cherokee (4X), Chester Thornless (4X), Hull Thornless (4X), Kotata (7X), Marion (6X), Navaho (4X), Shawnee (4X), Thornless Evergreen (4X), and Waldo (6X); and four raspberry cultivars: Autumn Bliss (2X), Heritage (2X), Latham (2X), and Watson (Ruby™) (2X), were collected in the summer of 1997 from pot-grown, virus-tested plants in the screenhouse of the National Clonal Germplasm Repository (NCGR) in Corvallis, OR. Cuttings (3 cm) with all leaves removed were washed under running water for 5 minutes, surface-disinfected in 10% bleach solution (0.525% sodium hypochlorite) and 0.1 ml per l of Tween 20 (Sigma Chemical Co., St. Louis, MO), shaken 10 minutes on a rotary shaker, and rinsed twice in sterile water for 5 minutes each. All nodal cuttings were initiated in 100 X 16-mm tubes with 5 ml of ½X MS liquid medium (pH 6.7) without growth regulators for seven days (Reed *et al.*, 1995). Initiated cuttings were transferred into NCGR-RUB medium for blackberries (Reed, 1990) or Anderson's medium (Anderson, 1980) for raspberries. Both media included double MS iron, 0.29 μM gibberellic acid $A_3$ ($GA_3$), 0.49 μM indole-3-butyric acid (IBA), 4.45 μM BA, 0.35%

agar (Difco granulated agar, Chicago, IL) and 0.145% Gelrite (Schweizerhall, Piscataway, NJ). The pH was adjusted to 5.7 before autoclaving. Growth-room conditions were 25 $^{0}$C with a 16-hour photoperiod (cool white fluorescent illumination, 25 $\mu$mol•m$^{-2}$•s$^{-1}$). Plantlets were proliferated and maintained in GA7 Magenta boxes (Magenta Corp., Chicago, IL) with a three- to four-week transfer cycle.

*Pretreatment*

Microshoots (≈ 10 mm long) with the upper four to six leaflets, were subcultured for 21 days on pretreatment medium (NCGR-RUB or Anderson) with 1 $\mu$M TDZ as the only growth regulator and grown at 20 $^{0}$C with a 16-hour photoperiod (cool white fluorescent illumination) in a growth chamber (T173, Hoffman, Albany, OR). Light intensity varied from 15 to 40 $\mu$mol•m$^{-2}$•s$^{-1}$ from the front to the back of the chamber.

*Basic regeneration procedure and data analysis*

Microshoots from proliferated cultures were pretreated for 21 days (three weeks), then leaf explants were placed on regeneration medium (RM) (NCGR-RUB or Anderson's basal nutrients and 2.9 $\mu$M $GA_3$) for one week of darkness followed by three weeks in the pretreatment conditions (RM 1). Explants were then subcultured on fresh RM (RM 2) for three additional weeks. Plant growth regulators and other additives were added to the basic RM formulation. Half- or whole-leaf explants from *Rubus* cultivars were screened for leaf regenerability under a range of growth regulator combinations. Whole-leaf explants of certain blackberry cultivars were then tested on RM with gelling agents, silver nitrate, or sequestrene.

Three replicates (three petri dishes with 10 explants each) were included in a completely randomized block design. Data from each individual experiment were taken at 84 days and analyzed separately. The screening trial for half- and whole-leaf regeneration was not repeated. The number of explants producing shoots, shoots produced per explant, and callus index (0 to 5) were recorded at the 84$^{th}$ day of culture following the one week in darkness. Callus index was reported as no callus (0), 1 to 20% callus on the leaf surface (1), 21 to 40% (2), 41 to 60% (3), 61 to 80% (4), and 81 to 100% (5). The percentage of regeneration was the mean number of explants producing

shoots divided by the number of explants tested (30 or 36 pieces) X 100. All data analysis, including analysis of variance (ANOVA), were made by the general linear model (GLM) procedure using SAS programming. The results of statistical analysis were considered significant at $P \leq 0.05$.

*Regeneration screening*

Half leaf

After shoot pretreatment, leaf explants (≤ 10 mm diameter, without petioles) of eight blackberry cultivars were detached on wet paper towels and cross cut evenly through the midvein. Both sections of the half-leaf explants were randomly placed on RM with 0, 0.5, or 5 μM IBA and either 0.5, 1, 5, or 10 μM BA or TDZ in plastic petri dishes (100 X 15 mm) with abaxial surfaces touching the medium. Explants received a one-week dark treatment, then were incubated for three weeks under pretreatment conditions. Each regeneration treatment included 36 half-leaf explants (three petri dishes with 12 explants each). The design was multifactorial with three IBA concentrations, two cytokinins, and four cytokinin concentrations.

Whole leaf

This experiment followed the same procedure and used similar treatments as the half-leaf screening. However, 30 whole–leaf explants were taken from each of nine blackberry and four raspberry cultivars. Leaf explants (≤ 10 mm diameter, without petioles) were detached without wounding the leaf surface. Five growth regulator combinations (5 μM IBA combined with 0.5 μM BA and 0.5, 1, 5, and 10 μM TDZ) were eliminated from this screening. Therefore, the degree of freedom for explant was nine instead of 14 on the two-factor interaction term (IBA X cytokinin).

*Effect of explant types, berry types, and ploidy levels on regeneration*

The effect of explant type (half vs. whole) on regeneration was analyzed from half- and whole-leaf screening of the seven blackberry cultivars (without Hull Thornless). The effect of berry type, blackberry vs. raspberry, of 12 cultivars (without Hull Thornless)

was analyzed for the whole-leaf data only. The effect of ploidy level, 2X, 4X, 6X, and 7X, was analyzed separately using eight cultivars of half-leaf explants and 12 cultivars of whole-leaf explants.

*Gel firmness test*

NCGR-RUB medium with 0.29 μM $GA_3$, 0.49 μM IBA, and 4.45 μM BA was used for the firmness test. Test plates were prepared with 0.4 to 1.0 % (w/v) agar, 0.1 to 0.5% Gelrite, and all combinations of agar (0.3, 0.35, and 0.4%) and Gelrite (0.045 to 0.235%). Gelling agents were added to the medium after adjusting the pH to 5.7, but before autoclaving. Medium with each concentration of gelling agent was poured into plastic petri dishes (100 X 25 mm) to a depth of 6 mm (approximately 100 ml), air dried uncovered in the laminar flow hood for one hour to evaporate excess water, and held at room temperature overnight before testing.

The firmness tester (Ametek LKg-1, Hatifield, PA) was designed for testing the firmness of fruit. Five positions were tested in each plate (punching order was 12, 6, 9, and 3 o'clock, and the center of plate of the plate). The tester was calibrated to zero and an 11-mm flat disk tip was inserted 5 mm into the medium. Each point was punched at least 20 mm from the next position and 10 mm from the plate edge. The penetrating force in grams was recorded.

The linear regression formulas of gel firmness of the average of five-point firmness data from each concentration of gelling agent were ploted using the SAS procedure gplot (SAS, 1993): Difco granulated agar [Fa (firmness) = -217.09 + 460.49 X agar concentration (%)], Schweizerhall Gelrite [Fg = -222.25 + 1792.88 X Gelrite concentration (%)], and the combination of agar and Gelrite [$F_{(0.35\%a)+g}$ = -88.69 + 1792.13 X Gelrite concentration (%)]. A standard firmness for each medium was set as 110 $g/(1.1\ cm)^2 \bullet \pi$ to calculate the standard concentration (%) by a linear regression formula to provide equal firmness for testing the effect of gelling agents and the additives silver nitrate or sequestrene on *in vitro Rubus* regeneration.

*Effect of gelling agents on regeneration*

Three blackberries; 'Kotata', 'Marion', and 'Navaho', were tested for the percentage of regeneration, shoots per regenerating explant, and callus formation on RM with zero or 0.5 μM IBA, either 5 or 10 μM BA, and gelled with 0.71% agar, 0.19% Gelrite, or 0.35% agar and 0.11% Gelrite combined. Explant preparation was modified by detaching each leaf in liquid MS medium without growth regulators to decrease drying during excision. BA at 5 and 10 μM were chosen because whole-leaf explant regeneration was the best at these concentrations for most cultivars.

*Effect of silver nitrate/sequestrene on regeneration*

Silver nitrate (10 mg per l) or sequestrene (200 mg per l) were each tested separately in the first two transfers on RM containing 10 μM BA and either zero or 0.5 μM IBA. The compounds were added in both RM transfers (+, +), neither (-, -), the first only (+, -), or the second only (-, +). The two compounds were not tested together, and neither was included in the pretreatment medium. Explant preparation was the same as in the gelling agent test. 'Chester Thornless', 'Kotata', 'Marion', and 'Navaho' blackberries were tested for the effects of silver nitrate on organogenesis, and 'Marion' and 'Kotata' were tested with sequestrene. Contrast analysis was conducted after 49 days on regeneration media on six combinations of silver nitrate or sequestrene treatments alone and on their interaction with IBA. Additional statistical analyses were the same as for regeneration screening.

## RESULTS

*Pretreatment*

Most blackberry cultivars multiplied normally during the three-week pretreatment (data not shown). Microshoots of 'Marion', 'Navaho', and 'Thornless Evergreen'

blackberries and the four raspberry cultivars sometimes grew abnormally with long, vitrified shoots and small, yellowing leaves on the pretreatment medium.

*Regeneration screening*

<u>Half leaf</u>

Adventitious shoots were successfully produced on the basal sections of half-leaf explants of all eight blackberry cultivars. Nearly all shoots were observed on the leaf base across the midvein at the leaf-petiole junction. On some growth-regulator combinations and genotypes, a few shoots, along with some callus, arose at the leaf edge. No shoots regenerated on the cut edges of explants. All cultivars except 'Shawnee' produced at least one shoot by the third week on regeneration medium (data not shown). Adventitious buds started regenerating on 'Shawnee' in the fourth week and additional shoots emerged from the other blackberry cultivars. The control and $GA_3$-only treatments had no shoots, but occasionally had callus. Several morphogenic forms of regenerants occurred with the various regeneration treatments and genotypes. BA induced normal shoots, but TDZ sometimes stimulated a few abnormal shoots on some of the cultivars screened. Generally, adventitious shoots induced from BA treatments elongated well, but shoots from some TDZ treatments did not elongate. TDZ with 5 μM IBA induced shoots only on 'Hull Thornless', with one shoot on 5 μM TDZ (data not shown). Tetraploid 'Thornless Evergreen' had the highest percentage of explant regeneration (25%), while heptaploid 'Kotata' (16.67%) was the second. 'Thornless Evergreen' produced the most regenerants per explant (6.71), and tetraploid 'Chester Thornless' had the least (1.78).

<u>Whole leaf</u>

Thirteen cultivars regenerated shoots from whole-leaf explants. Adventitious shoots of the four raspberry cultivars appeared later than most blackberry cultivars but all shoots grew normally. Petiole explants (5 mm long) of 'Marion' blackberry produced multiple shoots on 0.5 μM IBA and 10 μM BA RM (Fig. 2-1A). However, only one small bud was observed on 5 μM BA, and none was produced on TDZ. Many shoots arose from

Fig. 2-1. *In vitro* leaf regeneration of blackberry shoots from petiole or whole leaf explants. *A.* 'Marion' blackberry petiole produced adventitious shoots after 42 days on regeneration medium with 10 μM BA and 0.5 μM IBA; magnification, X1.2. *B.* The upper surface of 'Marion' whole leaf explant produced multiple shoots after 30 days on regeneration medium with 10 μM BA; magnification, X1.2. *C.* 'Kotata' produced multiple adventitious buds from the leaf-petiole junction after 21 days on regeneration medium with 10 μM BA; magnification X2. *D.* Multiple shoots produced from leaf-petiole junction of 'Kotata' after 42 days on regeneration medium with 10 μM BA; magnification, X2. *E.* 'Evergreen Thornless' produced multiple shoots from the lower surface of the leaf-petiole junction after 42 days on regeneration medium with 5 μM BA; magnification, X1.2. *F.* Callus produced on 'Marion' leaf after 63 days on regeneration medium with 10 μM BA and 0.5 μM IBA; magnification, X0.8.

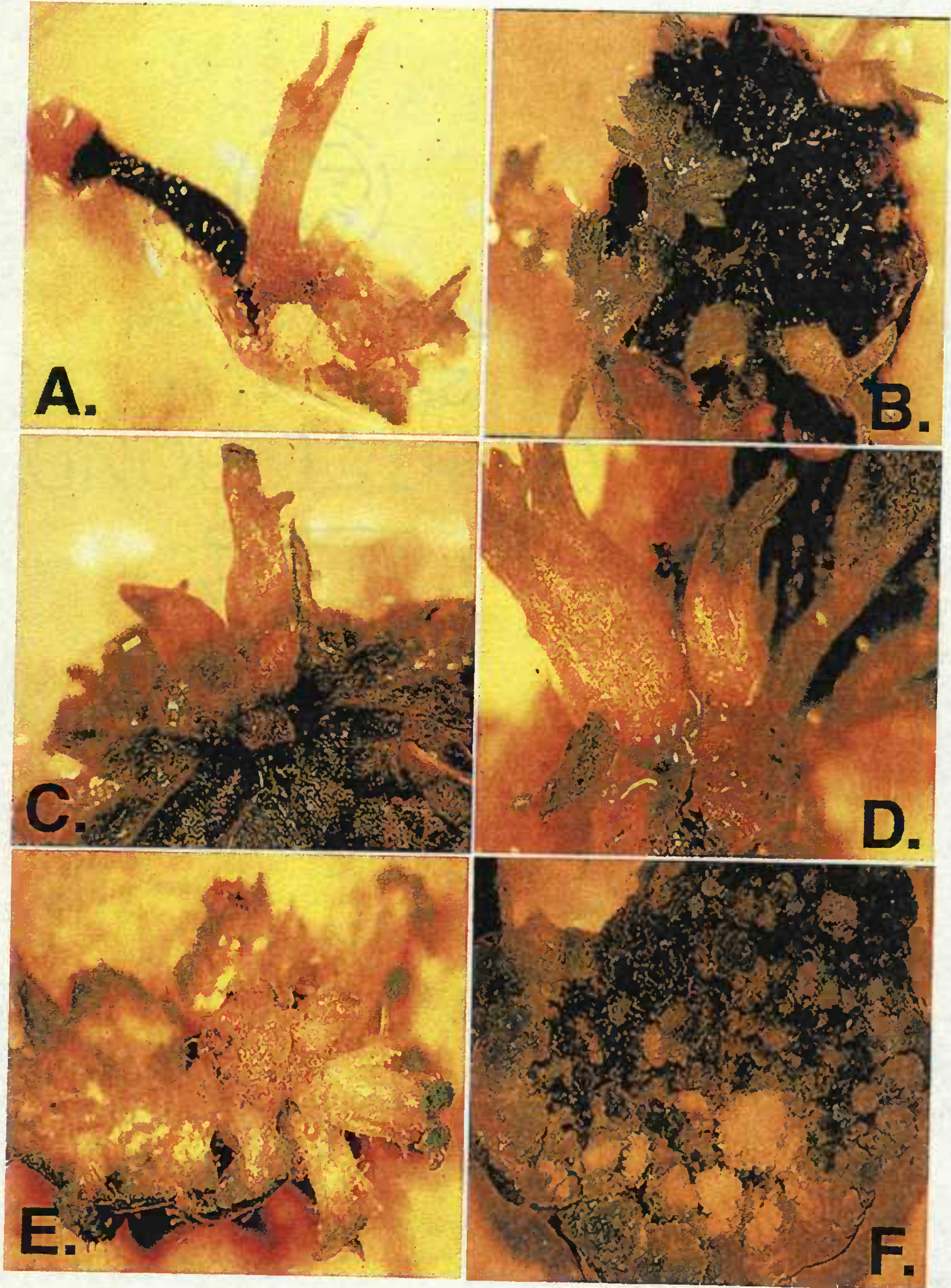


Fig. 2-1

the leaf surface of 'Marion' (Fig. 2-1B) and the leaf-petiole junction of 'Kotata' (Fig. 2-1C), with little or no associated callus. Adventitious shoots of 'Kotata' were not generated from callus (Fig. 2-1D); 'Thornless Evergreen' produced shoots from the lower side of the leaf (Fig. 2-1E). Induced callus varied in color and morphology with growth regulator treatment (Fig. 2-1F). Elongated shoots from 'Marion' blackberry rooted after 80 days on RM with 0.5 μM IBA and 5 μM BA. Most cultivars, except 'Thornless Evergreen', had nearly 100% callus formation on RM with 5 μM IBA. Callus formation was significantly lower on medium without IBA than with either 0.5 or 5 μM IBA (data not shown). Callus died or stopped growing after 3 months on many TDZ treatments.

Cultivar, IBA concentration, and cytokinin concentration were the dominant factors that affected *Rubus* leaf regeneration. The interactions of cultivar X IBA and cultivar X cytokinin concentration influenced *Rubus* regeneration, but the IBA X cytokinin concentration interaction was not significant for the 19 growth-regulator combinations (Table 2-1). Genotype X IBA differences were apparent, as 'Kotata' and 'Ruby' produced many shoots on a broad range of treatments but 'Heritage', 'Latham', and 'Waldo' regenerated only a few shoots on specific treatments (Fig. 2-2). All cultivars produced shoots on RM with 0.5 μM IBA. 'Kotata' blackberry and 'Ruby' raspberry produced the most shoots on RM with 5 μM IBA, but most cultivars had more shoots on RM without IBA (Fig. 2-2). All blackberries and most raspberries produced shoots on RM with 5 and 10 μM BA. Low BA concentrations (0.5 and 1 μM) were not effective for regeneration of most *Rubus* cultivars (Fig. 2-3A). Low TDZ (0.5 and 1 μM) concentrations in RM induced shoots on more cultivars than did high concentrations (5 and 10 μM). Raspberries 'Heritage' and 'Latham' did not produce any shoots on TDZ treatments (Fig. 2-3B). 'Autumn Bliss' responded better to TDZ than BA.

*Effect of explant types, berry types, and ploidy levels on regeneration*

Whole-leaf explants of seven blackberries tested produced significantly more shoots than did half-leaf explants as tested (data not shown), so we used whole-leaf explants for the other regeneration experiments. 'Kotata' was the only heptaploid screened and it had a significantly higher percentage of regeneration than any other cultivars (Fig. 2-2). No ploidy level differences for regeneration were displayed, when 'Kotata' was eliminated

TABLE 2-1

ANALYSIS OF VARIANCE OF PERCENT OF REGENERATION FROM WHOLE-LEAF EXPLANTS OF BLACKBERRY AND RASPBERRY CULTIVARS

| Source of variation | Whole leaf explant | |
|---|---|---|
| | D.F. | Mean square |
| Berry type | 1 | 1.69 ns |
| Ploidy level (Berry) | 2 | 10.53 ** |
| Cultivar[a] (Berry Ploidy) | 8 | 14.67 *** |
| IBA concentration | 2 | 12.67 *** |
| Cytokinin concentration | 7 | 15.18 *** |
| Berry X IBA | 2 | 6.25 * |
| Berry X cytokinin | 7 | 3.86 * |
| Ploidy (Berry) X IBA | 4 | 0.96 ns |
| Ploidy (Berry) X cytokinin | 14 | 4.57 ** |
| Cultivar (Berry Ploidy) X IBA | 16 | 3.31 * |
| Cultivar (Berry Ploidy) X cytokinin | 56 | 2.90 ** |
| IBA X cytokinin | 9[b] | 1.23 ns |
| Error[c] | 99 | 1.62 |

ns, *, **, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001.

[a] 'Hull Thornless' plates were contaminated and, therefore, were not included in this analysis.

[b] Five treatments used for half-leaf explants were not tested on whole-leaf explants (D.F. = 9 instead of 14).

[c] The three- and higher order-interaction terms were used as the error term for the analysis.

Fig. 2-2. The number of explants regenerating shoots on RM with three IBA concentrations from 30 whole-leaf explants of eight blackberry cultivars, Kotata (KO), Marion (MA), Waldo (WA), Cherokee (CH), Chester Thornless (CT), Evergreen Thornless (ET), Navaho (NA), Shawnee (SH), and four raspberry cultivars, Autumn Bliss (AB), Heritage (HE), Latham (LA), Ruby (RU).

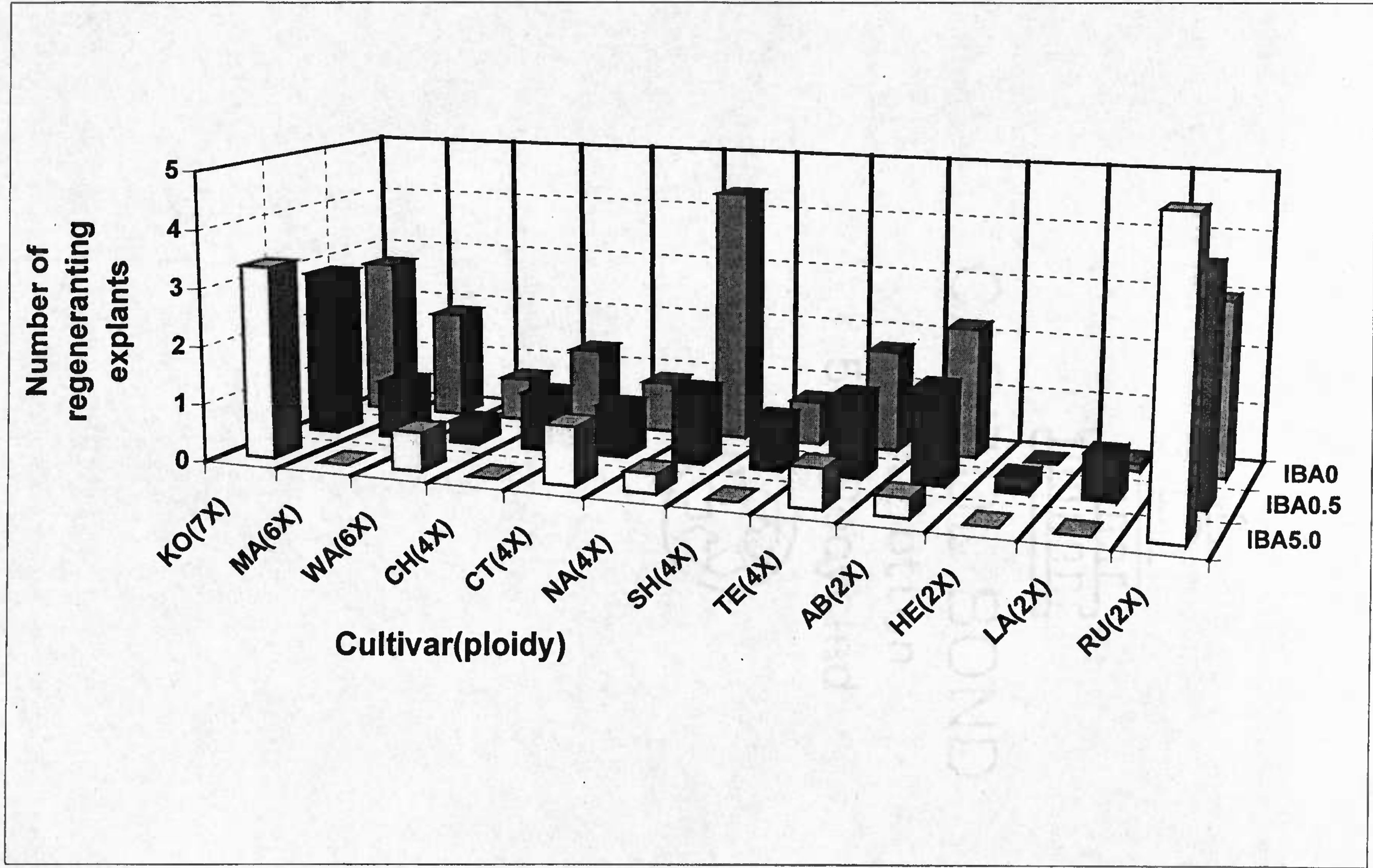


Fig. 2-2

Fig. 2-3. The number of regenerating explants produced at the 84$^{th}$ day on regeneration medium with a range of IBA and either BA or TDZ, from 30 whole-leaf explants of eight blackberry and four raspberry cultivars. *A.* Benzyldenine (BA) *B.* Thidiazuron (TDZ). Blackberries: 'Kotata' (KO), 'Marion' (MA), 'Waldo' (WA), 'Cherokee' (CH), 'Chester Thornless' (CT), 'Evergreen Thornless' (ET), 'Navaho' (NA), 'Shawnee' (SH), and raspberries: 'Autumn Bliss' (AB), 'Heritage' (HE), 'Latham' (LA), 'Ruby' (RU).

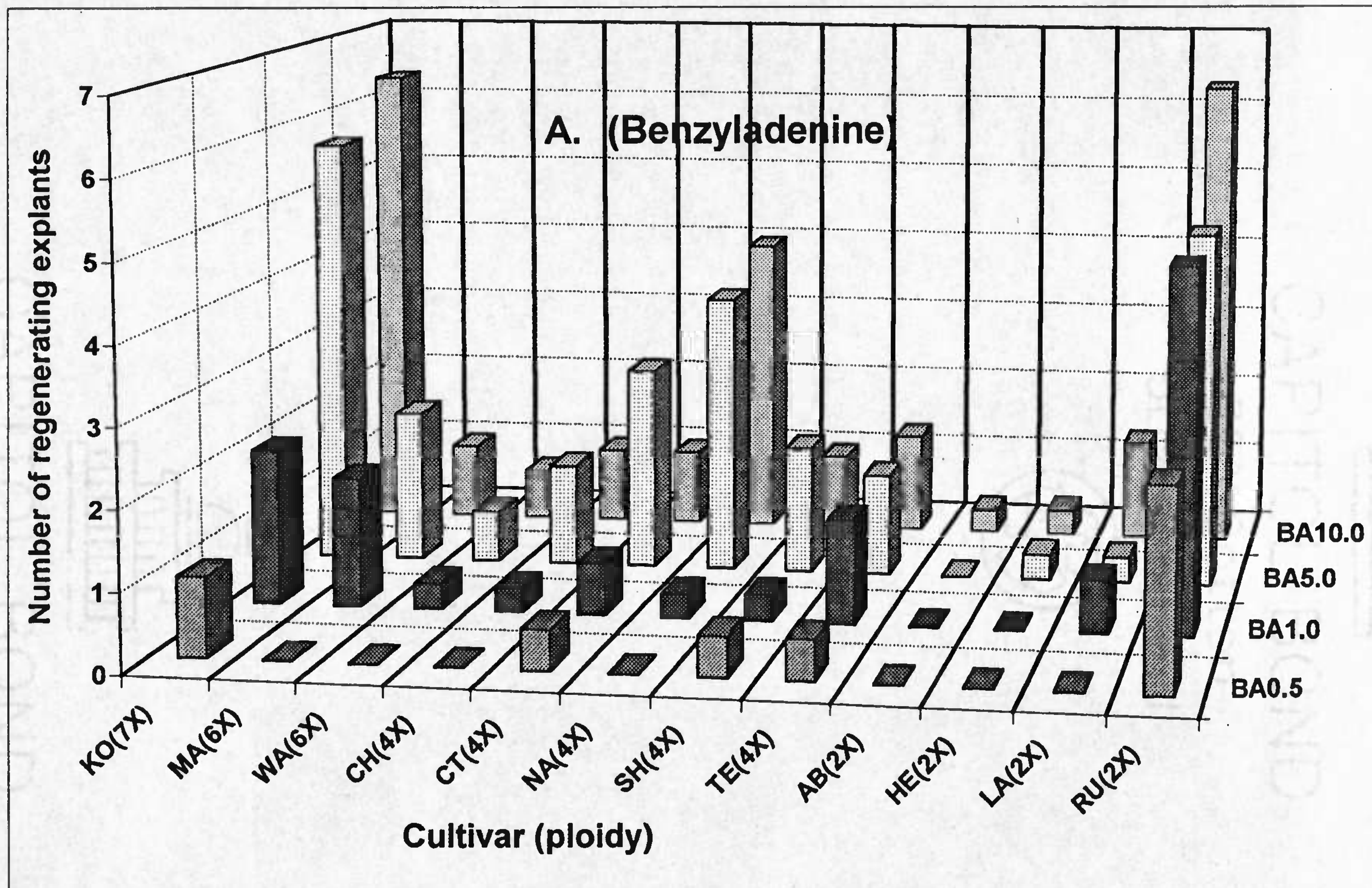


Fig. 2-3 A.

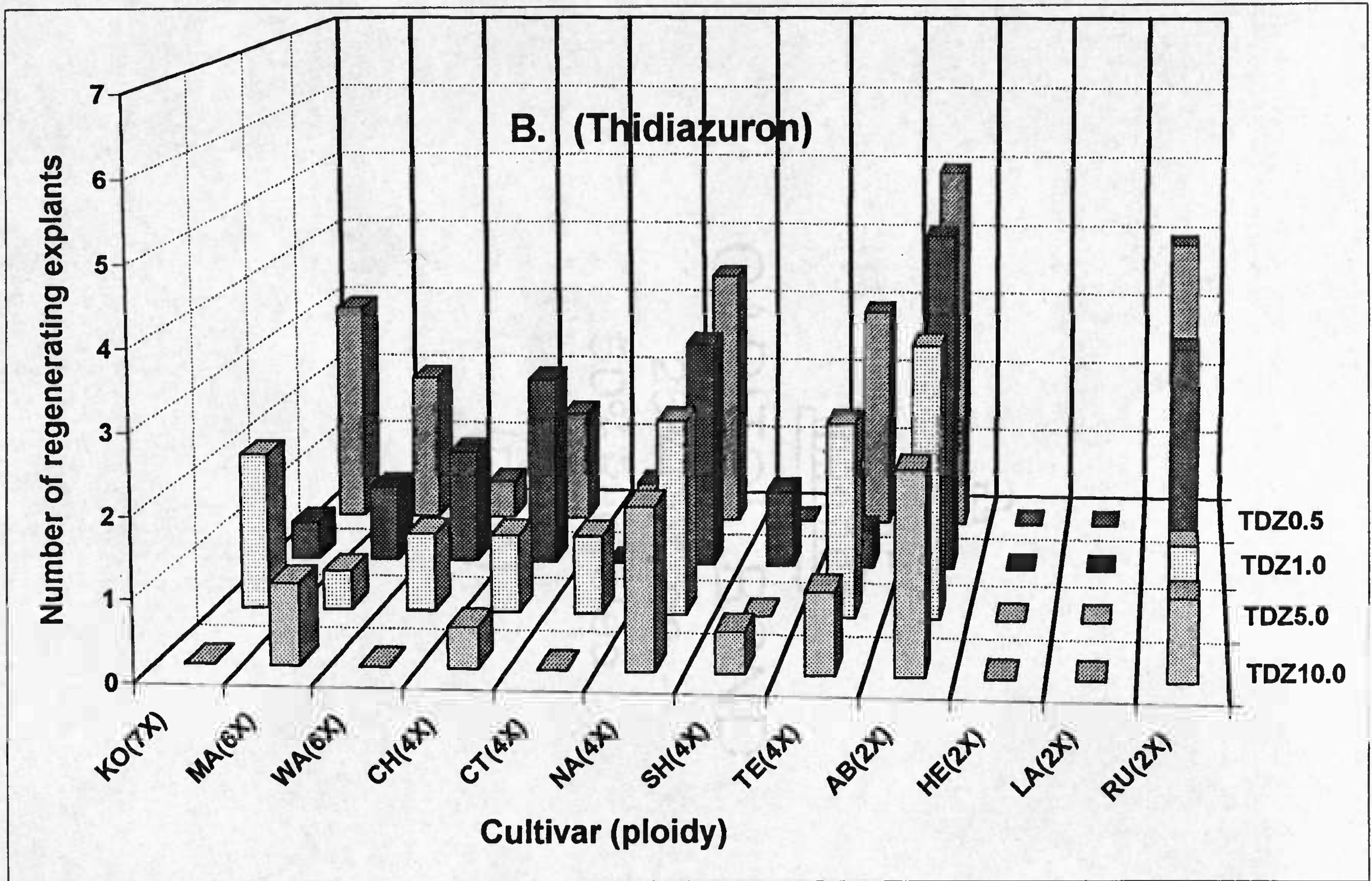


Fig. 2-3 B.

from the analysis. There were only minor differences when the two-factor-interaction terms were eliminated. Significance was similar to results in Table 2-1, with the analysis for regeneration screening data using three variables, cultivar, IBA concentration, and cytokinin concentration (three-way-ANOVA) only. Regenerability of blackberries was never significantly different from raspberries (Table 2-1).

*Gel firmness test*

The linear regression formula for the gel firmness tests showed that medium with 0.71% agar was the same firmness as that with 0.19% Gelrite, and of the agar (0.35%) and Gelrite (0.11%) combination (Fig. 2-4). The standard firmness of 0.71% agar medium was 110 $g/(1.1\ cm)^2 \bullet \pi$, and was used for regeneration tests with three gelling agents and silver nitrate or sequestrene. The gel firmness of medium used for the initial regeneration screening tests (0.35% agar and 0.145% Gelrite) was about 171 $g/(1.1\ cm)^2 \bullet \pi$.

*Effect of gelling agents on regeneration*

Cultivar significantly affected percent regeneration on the three-gelling-agents tested ($P \leq 0.001$). There were no significance differences in the percent of regeneration, shoots per explant, or callus index for the three gelling agents (Table 2-2). The two- and three-factor-interaction terms of cultivar, agent, and treatment were all significant for callus formation ($P \leq 0.001$). The agent X treatment interaction was significant for shoot regeneration ($P \leq 0.01$) and callus formation ($P \leq 0.001$). Callus induction was enhanced by 0.5 μM IBA on all three gelling-agent treatments (Table 2-2). More shoots per explant and higher percent regeneration occurred in the same growth regulator treatments except 5 μM BA on Gelrite RM. RM with 10 μM BA and no IBA had the highest percentage of regeneration and more shoots per explant, but there was no statistical difference to 0.5 μM IBA treatments (Table 2-2). Multiple shoots without callus were produced from the leaf edge and surface of 'Marion' blackberry on Gelrite RM with 10 μM BA (Fig. 2-1B). There were no shoots produced on Gelrite RM with 5 μM BA and combined agar and Gelrite RM with 0.5 μM IBA plus 10 μM BA.

Fig. 2-4. Linear regressions of gel firmness [g/(1.1 cm)$^2$•π] tested using NCGR-RUB medium with plant growth regulators. *A*. Linear regression lines and formulas from eight concentrations of Difco granulated agar and Schweizerhall Gelrite solidified media. *B*. Linear regression lines and formulas from media solidified with gelling agent combinations of 0.3%, 0.35%, and 0.4% agar with 5 concentrations of Gelrite.

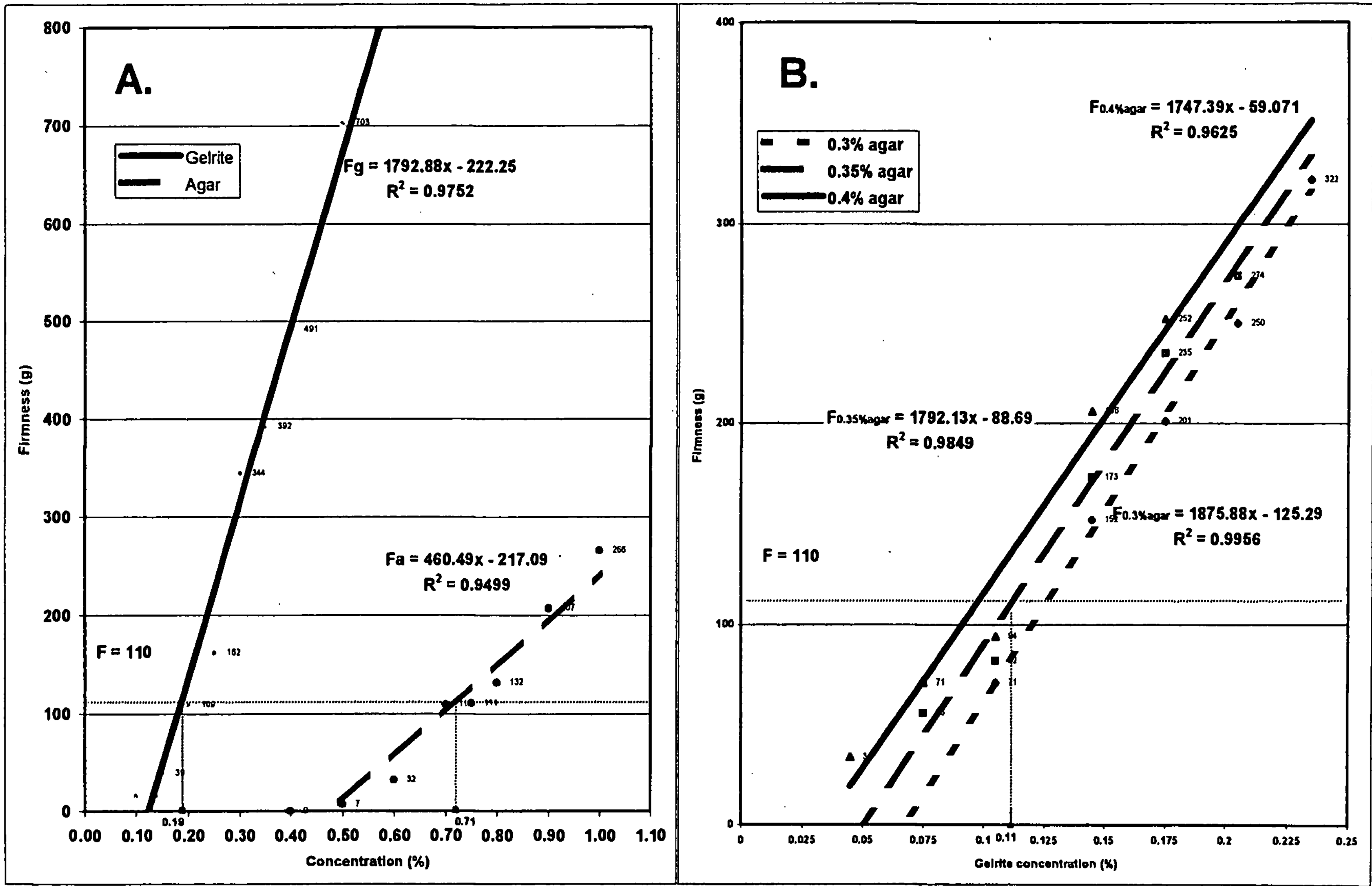


Fig. 2-4

TABLE 2-2

EFFECT OF GELLING AGENTS AND GROWTH REGULATORS ON *IN VITRO* REGENERATION FROM WHOLE LEAVES OF THREE BLACKBERRY CULTIVARS

| Treatment[d] | Least square means | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regeneration (%)[a] | | | Shoots per explant[b] | | | Callus index[c] | | |
| | Agar | Gelrite | Combination | Agar | Gelrite | Combination | Agar | Gelrite | Combination |
| IBA0+BA5 | 10.00 z | 0 y | 13.33 z | 4.39 z | 0 w | 3.42 xy | 2.29 vw | 1.56 u | 2.56 w |
| IBA0+BA10 | 12.22 z | 14.44 z | 14.44 z | 2.95 wxyz | 4.19 yz | 3.68 xy | 2.54 w | 1.93 v | 1.77 uv |
| IBA0.5+BA5 | 8.89 yz | 11.11 z | 6.67 yz | 2.13 wxy | 2.44 wxy | 1.33 wxy | 3.63 y | 4.01 yz | 3.80 xyz |
| IBA0.5+BA10 | 5.56 yz | 10.00 z | 0 y | 0.74 wx | 3.48 xyz | 0 w | 3.59 y | 4.11 z | 3.54 x |

[a] n = 30 whole-leaf explants. % regeneration means with the same letter are not significantly different at $P \leq 0.05$.

[b] Shoots per explant means with different letters are significantly different at $P \leq 0.05$.

[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface). Callus index means with the same letter are not significantly different at $P \leq 0.05$.

[d] The NCGR-RUB medium with 2.9 µM $GA_3$ BA, and IBA as listed.

*Effect of sequestrene on regeneration*

'Kotata' and 'Marion' blackberries had similar responses to RM with IBA and sequestrene (data not shown). In absence of IBA, significantly more explants were regenerated when sequestrene was in only the second RM (RM 2) (Table 2-3). Contrast analysis confirmed that result ($P \leq 0.05$). RM with sequestrene did not induce more shoots per explant or more callus (Table 2-3). The percent regeneration of 'Kotata' increased from 23 to 43% and 'Marion' from 30 to 40% in the sequestrene-free control compared to sequestrene in the RM 2 only (-, +). Whole-leaf explants of 'Kotata' and 'Marion' blackberries produced more shoots, less callus, and had a high percent regeneration with sequestrene in either or both RM, but no IBA (Table 2-4).

*Effect of silver nitrate on regeneration*

Silver nitrate did not improve regeneration for 'Chester Thornless', 'Kotata', 'Marion', and 'Navaho' blackberries (data not shown). Cultivar was the only significant factor for percent regeneration, shoots per explant, and callus index ($P \leq 0.001$). Regardless of IBA content, 'Kotata' produced significantly more shoots than the other three cultivars, and more callus than 'Chester Thornless' and 'Navaho' (callus index 4.4 vs. 3.35 and 3.36) on the 0.5 μM IBA treatments. IBA did not influence the percent regeneration and shoots per explant in any of the four blackberry cultivars, but did induce more callus. Contrast analysis confirmed that result ($P \leq 0.05$). Callus formation was significantly affected by cultivar X IBA concentration X silver nitrate interaction ($P \leq 0.001$). Significantly less callus was produced in the RM 1 with silver nitrate (+); callus index without IBA: 1.89 (+, +) and 1.98 (+, -) vs. 2.47 (-, -); or, with IBA: 3.32 (+, +) and 3.75 (+, -) vs. 4.19 (-, -)]. Silver nitrate in RM 1 reduced callus formation but had no influence on regeneration of blackberries.

TABLE 2-3

RESPONSE OF WHOLE-LEAF EXPLANTS OF 'KOTATA' AND 'MARION' BLACKBERRIES TO SEQUESTRENE IN REGENERATION MEDIUM (RM)

| | RM[a] | | Means[b] | | |
|---|---|---|---|---|---|
| IBA conc. (μM) | RM 1 | RM 2 | Regeneration (%) | Shoots per explant | Callus index[c] |
| 0 | + | + | 28.33 yz | 3.36 yz | 2.77 y |
| | + | - | 25.00 y | 1.96 y | 2.70 y |
| | - | + | 41.67 z | 3.21 yz | 2.73 y |
| | - | - | 26.67 y | 3.95 z | 2.83 y |
| 0.5 | + | + | 15.00 xy | 1.70 y | 3.98 z |
| | + | - | 21.67 xy | 1.72 y | 4.75 z |
| | - | + | 16.67 xy | 1.50 y | 4.72 z |
| | - | - | 10.00 x | 1.47 y | 4.73 z |

[a] The NCGR-RUB medium with 2.9 μM $GA_3$, 10 μM BA, and solidified by 0.19% Gelrite.

[b] Means with different letter in a column are significantly different at $P \leq 0.05$.

[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface). + or – indicates with or without Sequestrene (200 mg per l).

TABLE 2-4

EFFECT OF IBA CONCENTRATION ON WHOLE-LEAF REGENERATION OF 'KOTATA' AND 'MARION' BLACKBERRIES IN REGENERATION MEDIUM[a] WITH SEQUESTRENE

| IBA conc. (μM) | Means[b] | | |
|---|---|---|---|
| | Regeneration (%) | Shoots per explant | Callus index[c] |
| 0 | 30.42 z | 3.12 z | 2.76 y |
| 0.5 | 15.83 y | 1.60 y | 4.55 z |

[a] The NCGR-RUB medium with 2.9 μM GA3, 10 μM BA, and solidified by 0.19% Gelrite.
[b] n = 30 whole-leaf explants and means with different letters in a column are significantly different at $P \leq 0.05$.
[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

# DISCUSSION

Genetic transformation is used to introduce genes into a range of crops (Walden and Wingender, 1995) and gene transfer through *Agrobacterium* is the most commonly used method to genetically engineer dicot plants. An efficient shoot regeneration system is required for successful *Agrobacterium* gene transformation. There are many reports on optimization of culture media and conditions for regenerating new plantlets from single cells or small groups of cells of individual species and cultivars (Huy *et al.*, 1997).

We used diploid raspberries, and blackberries ranging from tetraploid to heptaploid to regenerate new shoots from leaf explants. Although, tetraploid 'Thornless Evergreen' had the best regeneration from half-leaf explants (25%), the average regeneration percentage was near 10% for tetraploid and 6% for hexaploid blackberries. Heptaploid 'Kotata' had about 17% regeneration. Compton and Gray (1993) found that shoot regeneration of diploid watermelon cotyledons was best (57%) and regeneration decreased to 20% when ploidy level increased to tetraploid. Our whole-leaf screening produced about 30% regeneration from the diploid raspberry 'Ruby', tetraploid blackberry 'Navaho' and heptaploid 'Kotata'. Although diploid 'Ruby' and heptaploid 'Kotata' had significantly higher regeneration than other cultivars, there was no significant ploidy effect on regeneration from either half or whole *Rubus* leaves. No other reports indicate a ploidy effect for *Rubus*. Graham *et al.* (1997) produced a similarity matrix and dendrogram from randomly amplified polymorphic DNA (RAPD) markers to discriminate the regenerability of eight *Rubus* genotypes. They found the tetraploid blackberries 'Chester Thornless' and 'Hull Thornless' were closely related and regenerated shoots on similar regeneration media and growth-regulator combinations (0.49 μM IBA with 8.8 μM BA or 9.0 μM TDZ; or, 0.005 μM IBA with 4.5 μM TDZ). Our half-leaf screening found that 'Chester Thornless' and 'Hull Thornless' both produced shoots on RM with 1 μM TDZ. 'Chester Thornless' had shoots on 5 μM IBA with 5 or 10 μM BA, and 'Hull Thornless' had the most shoots on 0.5 or 5 μM IBA with 5 μM TDZ.

Regeneration from leaf explants is becoming popular, because leaf tissue is easy to handle, and produces reasonable regenerability for many crops; also, *Agrobacterium* produces fast and efficient infection (Boxtel and Berthouly, 1996; Gelvin, 1990). Adventitious shoots are successfully produced from almost every plant part: leaves, petioles, internodes, cotyledons, immature, and mature embryos. Tissues may vary in their ability to regenerate, and regeneration from a particular tissue may be genotype dependent (Cousineau and Donnely, 1991; Turk *et al.*, 1994). For *Rubus* regeneration, the most adventitious shoots are produced from the leaf-petiole junction. The regeneration efficiency of this tissue may be too low for good transformation frequency (Graham *et al.*, 1995; Hassan *et al.*, 1993). We observed that green meristematic protrusions along with callus usually originated from the leaf-petiole junction of the base of half-leaf explants, but not from the cut edges or the apical explants. Compton and Gray (1993) induced shoots from watermelon and indicated that adventitious shoots originated solely from the base of the cotyledons. Longitudinal halves or quarters of watermelon cotyledons produced almost twice as many shoots if the basal half was included. Similar observations are reported in tomato for shoot formation at the base of cotyledon explants (Monacelli *et al.*, 1988). Turk *et al.* (1994) found that the uppermost two young expanding leaves produced a higher regeneration frequency than the fourth leaf.

Reports of *Rubus* micropropagation note that $GA_3$ in the medium can cause leaf enlargement and shoot elongation (Zimmerman *et al.*, 1995). We confirmed earlier reports that 5 to 10 μM BA combined with 0.5 μM IBA induced shoots from some *Rubus* cultivars (Graham *et al.*, 1997; Mezzetti *et al.*, 1997; Wang and Wang, 1995). We also found that low TDZ (0.5 or 1 μM) induced many shoots on certain cultivars like 'Thornless Evergreen' as has been reported by Norton (1994). Higher concentrations of TDZ induced more shoots on other cultivars and sometimes stimulated abnormal shoots or hyperhydricity, as reported by Hassan *et al.* (1993). Swartz *et al.* (1990) pretreated source shoots *in vitro* with either colchicine (75 to 250 μM) or TDZ ($\geq$ 5 μM); this had the potential to enhance the organogenesis from detached leaves in two *Rubus* hybrids. We found that a 1 μM TDZ pretreatment produced leaf hyperhydricity, extended stems, and small yellowing leaves on raspberry and some blackberry cultivars. A few TDZ

treatments regenerated long microshoots with needle-like new leaflets or slow-growing adventitious buds. Abnormal microshoots grew normally after transfer back onto RM. Abnormal adventitious shoots are usually attributed to the stronger cytokinin effect of TDZ (Swartz *et al.*, 1990). Hoepfner *et al.* (1996) indicated that after growth on RM with 4.4 μM BA and 4.5 μM TDZ regenerants had divergent phenotypes compared to control plants, but DNA fingerprinting detected no genetic variation.

In our screening tests, adventitious shoots were regenerated via organogenesis. Adventitious shoots were produced directly from leaf explants, but callus was also present sometimes. Shoots of 'Marion' regenerated on 5 or 10 μM BA with only occasional callus, and multiple shoots were directly produced from the whole-leaf surface with no callus (Fig. 2-1B). Some pale, fragile callus induced adventitious shoots in a study by Graham *et al.* (1997); however, all RM we examined induced callus, but few shoots were associated with callus. Callus sometimes was observed after the second week of culture on RM before the formation of microshoots. The growth regulator combinations we applied did not induce adventitious shoots through callus; and, shoots were rarely produced on RM with 5 μM IBA, which produced a large amount of callus (data not shown). Callus produced on RM with 5 μM IBA and 5 or 10 μM TDZ was firm and hard and did not regenerate. Mezzetti *et al.* (1997) found that explants with the most callus formation had very poor shoot regeneration. TDZ induced more and harder callus than BA, because TDZ also has auxin activity (Millan-Mendoza, 1998). Soft, proembryo-like white or light-colored callus regenerated under some plant growth-regulator combinations (Compton and Gray, 1993). Hall *et al.* (1986) used higher $GA_3$ (5 mg per l) and lower 6-benzylaminopurine (0.05 mg per l) in Anderson's (1980) medium to induce rooted microshoots from meristem-derived callus. Cell suspension culture is another way to induce embryogenic callus (Boxtel and Berthouly, 1996). Huy *et al.* (1997) isolated protoplasts from leaf tissue of 'Autumn Bliss' raspberry and 'Hull Thornless' and 'Chester Thornless' blackberries, but only root formation resulted from the protoplast-induced callus.

Researchers usually use 0.7 to 0.8% agar or 0.2 to 0.25% Gelrite to solidify tissue culture medium (Anderson, 1980; Reed, 1990); these concentrations are most useful for a wide range of species, and they provide an appropriate firmness. Our firmness data

confirmed that 0.7% agar and 0.2% Gelrite have a similar firmness in MS medium, and 0.8% agar and 0.25% Gelrite are also nearly equivalent (Fig. 2-4). Sigma phytagel™ (0.25%) induced higher regeneration frequency in pear (Chevreau *et al.*, 1997) and 0.2% Gelrite induced more callus in banana (Huang and Chi, 1988) than did the agar medium. Cousineau and Donnelly (1991) showed that 0.2 to 1.0% agar gelled media had no significant effect on raspberry regeneration. In general, we found that the gel type did not significantly influence the percent regeneration, shoots per explant, and callus formation, but significantly more callus was induced with IBA on RM with 10 μM BA and 0.19% Gelrite, than with 0.71% agar (Table 2-2). More shoots per explant and the highest regeneration occurred in the Gelrite RM with 10 μM BA only, but there was no statistical difference from the addition of 0.5 μM IBA. No shoots were produced on Gelrite RM with 5 μM BA and on agar-Gelrite combined RM with 0.5 μM IBA and 10 μM BA.

Mathews *et al.* (1995) added 10 mg per l silver nitrate to shoot regeneration medium for producing S-adenosylmethionine hydrolase (SAMase) transgenic raspberries. We did not find significant improvement in regeneration by adding 10 mg per l silver nitrate. Other additives such as ascorbate (0.1 mM), myo-inositol (550 μM), casein hydrolysate (100 mg per l), extra thiamine or Staba vitamins did not significantly promote *Rubus* regeneration (Fiola *et al.*, 1990; Turk *et al.*, 1994). Hyde and Phillips, (1996) recognized four developmental stages of organogenesis in chili pepper (*Capsicum annuum* L): bud induction, bud enlargement, shoot elongation, and root development; and, they found silver nitrate improved regeneration in the shoot elongation stage. In our experiments, sequestrene significantly increased the percent regeneration only when included in the RM 2 (bud enlargement/shoot elongation stage). Sequestrene may allow previously induced buds to develop further. 'Kotata' and 'Marion' blackberries responded quite differently in the initial screens (26.67% and 6.67%), but their regeneration responses on RM 2 with sequestrene were very similar (43% and 40%). This leads us to think that sequestrene may has the potential to promote leaf regeneration in other *Rubus* cultivars as well.

We screened 13 *Rubus* cultivars for regeneration from leaf explants using our general regeneration procedure. Whole-leaf explants produced more adventitious shoots at the

leaf-petiole junction and on the intact leaf surface than half leaves. The three gelling agents tested did not show significant differences in the percent of regeneration, shoots per explant, or callus formation for 'Chester Thornless', 'Kotata', 'Marion', and 'Navaho' blackberries. Although there were no significant differences among gelling agents on the four blackberries tested, the highest percentage of regeneration and the most shoots per explant occurred on Gelrite RM with 10 μM BA. RM 2 with 200 mg per l sequestrene and 10 μM BA significantly improved the regeneration percentage of 'Kotata' and 'Marion' blackberries. IBA stimulated callus, but did not increase shoot production per explant or regeneration percentage. We suggest testing whole-leaf explants of additional cultivars on sequestrene (200 mg per l) and either 10 μM BA or 1 μM TDZ. This leaf regeneration screening provides an easy and fast procedure from which to begin *Rubus* regeneration with the commercially important cultivars. Applying this regeneration method to transgenic *Rubus* could enrich conventional breeding program and improve the quality of modern *Rubus*.

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# REFERENCES

Anderson, W. C. Tissue culture propagation of red and black raspberry, *R. idaeus* and *R. occidentalis*. Acta Hort. 112:13-20; 1980.

Boxtel, J. V.; Berthouly, M. High frequency somatic embryogenesis from coffee leaves. Plant Cell Tiss. Org. Cult. 44:7-17; 1996.

Cantoni, G.; Berardi, G.; Rosati, P. Organogenesis and somatic embryogenesis from immature embryos of blackberry. Acta Hort. 352:37-42; 1993.

Castillo, B.; Smith, M. A. L.; Madhavi, D. L. Interactions of irradiance level and iron chelate source during shoot tip culture of *Carica papaya* L. HortScience 32:1120-1123; 1997.

Chevreau, E.; Mourgues, F.; Neveu, M.; Chevalier, M. Effect of gelling agents and antibiotics on adventitious bud regeneration from *in vitro* leaves of pear. *In vitro* Cell. Dev. Biol. – Plant 33:173-179; 1997.

Compton, M. E.; Gray, D. J. Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid, and tetraploid watermelon. J. Amer. Soc. Hort. Sci. 118:151-157; 1993.

Cousineau, J. C.; Donnely, D. J. Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss. Org. Cult. 27:249-255; 1991.

Fiola, J. A.; Hassan, M. A.; Swartz, H. J.; Swartz, H. J.; Bors, R. H.; McNicol, R. Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss. Org. Cult. 20: 223-228; 1990.

Fiola, J. A.; Swartz, H. J. Somatic embryogenesis, organogenesis and proliferation *in vitro* from *Rubus* embryos. Acta Hort. 183:91-98; 1986.

Gelvin, S. B. Crown gall disease and hairy root disease. Plant Physiol. 92:281-285; 1990.

Gingas, V. M.; Stokes, B. D. *Rubus* plant regeneration via asexual embryogenesis. HortScience 28:58; 1993.

Graham, J.; Iasi, L.; Millam, S. Genotype-specific regeneration from a number of *Rubus* cultivars. Plant Cell Tiss. Org. Cult. 48:167-173; 1997.

Graham, J.; McNicol, R. J. Plant regeneration and genetic transformation in soft fruit species. Acta Hort. 280:517-522; 1990.

Graham, J.; McNicol, R. J.; Kumar, A. *Agrobacterium*-mediated transformation of soft fruit *Rubus*, *Ribes*, and *Fragaria*. Methods in Molecular Biology. v. 44, pp.129-133; 1995.

Hall, H. K.; Quazi, M. H.; Skirvin, R. M. Isolation of a pure thornless loganberry by meristem tip culture. Euphytica 35:1039-1044; 1986.

Hassan, M. A.; Swartz H. J.; Inamine G.; Mullineaux, P. *Agrobacterium tumefaciens*-mediated transformation of several *Rubus* genotypes and recovery of transformed plants. Plant Cell Tiss. Org. Cult. 33:9-17; 1993.

Hoepfner, A. S.; Nestby, R.; Nybom, H. Genetic deviation initiated by adventitious shoot regeneration from tissue cultures red raspberry. J. of Hort. Sci. 71:71-79; 1996.

Huang, L. C.; Chi, D. L. Pivotal roles of picloram and gelrite in banana callus culture. Environmental and Experimental Botany 28:249-258; 1988.

Huy, B. P.; Landi, L.; Taruschio, L.; Mott, D.; Mezzetti, B.; Rosati, P. Protoplast isolation, culture, and cell differentiation in raspberry and blackberry cultivars (*Rubus* spp.). J. Applied Botany 71:131-137; 1997.

Hyde, C. L.; Phillips, G. C. Silver nitrate promotes shoot development and plant regeneration of chile pepper (*Capsicum annuum* L.) via organogenesis. *In vitro* Cell. Dev. Biol. –Plant 32:72-80; 1996.

Jennings, D. L. Raspberries and blackberries: Their Breeding, Diseases and Growth. Academic Press, London; 1988.

Mathews, H.; Wagoner, W.; Cohen, C.; Kellogg, J., Bestwick, R. Efficient genetic transformation of red raspberry, *Rubus idaeus* L. Plant Cell Rep. 14:471-476; 1995.

Mezzetti, B.; Savini, G.; Carnevali, F.; Mott, D. Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biologia-Plantarum 39:139-150; 1997.

Millan-Mendoza, B. Regeneration of *Rubus in vitro* using forchlorfenuron (CPPU). Rev. Fac. Agron. 15:242-248; 1998.

Monacelli, B.; Altamura, M. M.; Pasqua, G. The histogenesis of somaclones from tomato (*Lycopersicon esculentum* Mill.) cotyledons. Protoplasma 142:156-163; 1988.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol. Plant 15:473-479; 1962.

Norton, M. A. Regeneration and evaluation of first and second cycle somaclones of 'Thornless Evergreen' blackberry (*Rubus laciniatus* Willd.). Ph. D. Thesis, University of Illinois at Urbana-Champaign; 1994.

Owens y de N, C.; Conner, A. J. Comparison of *in vitro* shoot regeneration protocols from *Rubus* leaf explants. New Zealand Journal of Crop and Horticultural Science 20:471-473; 1992.

Reed, B. M. Multiplication of *Rubus* germplasm *in vitro*: A screen of 256 accessions. Fruit Varieties J. 44:141-148; 1990.

Reed, B. M.; Buckley, P. M.; DeWilde, T. N. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In vitro* Cell. Dev. Biol. –Plant 31:53-57; 1995.

SAS Institute, Inc. SAS User's Guide. version 6, 2$^{nd}$ ed. SAS Institute, Inc., Cary, N.C.; 1993.

Swartz, H. J.; Bors, R.; Mohamed, F.; Naess, S. K. The effect of pretreatment on subsequent shoot organogenesis from *Rubus* and *Malus* leaves. Plant Cell Tiss. Org. Cult. 21:179-184; 1990.

Taylor, P. W. J.; Ko, H. L.; Fraser, T. A.; Masel, N.; Adkins, W. Effect of silver nitrate on sugarcane cell suspension growth, protoplast isolation, ethylene production and shoots regeneration from cell suspension cultures. J. of Experimental Botany 45:1163-1168; 1994.

Thompson, M. M. Chromosome numbers of *Rubus* species at the National Clonal Germplasm Repository. HortScience 30:1447-1452; 1995a.

Thompson, M. M. Chromosome numbers of *Rubus* cultivars at the National Clonal Germplasm Repository. HortScience 30:1453-1456; 1995b.

Thompson, M. M. Survey of Chromosome numbers in *Rubus*. Annals of the Missouri Botanical Garden 84:128-165; 1997.

Turk, B. A.; Swartz, H. J.; Zimmerman, R. H. Adventitious shoot regeneration from *in vitro*-cultured leaves of *Rubus* genotypes. Plant Cell Tiss. Org. Cult. 38:11-17; 1994.

Walden, R.; Wingender, R. Gene transfer and plant regeneration techniques. Trends in biotechnology 13:324-331; 1995.

Wang, G. Y.; Wang, J. H. Regeneration of blackberry plantlets from leaves and stem segments of *in vitro* plants. Acta-Agriculturae-Universitatis-Pekinensis 21:12-16; 1995.

Zimmerman, R. H.; Bhardwaj, S. V.; Fordham, I. M. Use of starch-gelled medium for tissue culture of some fruit crops. Plant Cell Tiss. Org. Cult. 43:207-213; 1995.

# CHAPTER 3. VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY

**Chih-wei V. Tsao[1], Joseph D. Postman, and Barbara M. Reed[2]**

*Department of Horticulture, Oregon State University, Corvallis, Oregon 97331-7304 (C.W.V.T.); USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd., Corvallis, Oregon 97333-2521 (J.D.P. and B.M.R.)*

RUNNING HEAD: VIRUS INFECTION IN RASPBERRY

[1] Part of a thesis submitted by C.W.V.T. in partial fulfillment of the requirements for the MS degree.

[2] To whom correspondence should be addressed. E-mail: reedbm@bcc.orst.edu

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## SUMMARY

Virus infected plants are often symptomless and explant sources used for tissue culture research are rarely tested for viruses. Our objective was to determine if virus infection affects micropropagation. We studied the effects of single and multiple infections of three common raspberry viruses on the *in vitro* culture of 'Malling Landmark' red raspberry (*Rubus idaeus* L.). Virus infected plants were produced by leaf-graft inoculation from known infected raspberry plants onto virus-free 'Malling Landmark'. Single-virus source plants were infected with either tobacco streak ilarvirus (TSV), tomato ringspot nepovirus (TomRSV), or raspberry bushy dwarf idaeovirus (RBDV) and were free of other viruses as determined by enzyme-linked immunosorbent assay (ELISA) and bioassay. Virus-free, single, and multiple virus-infected 'Malling Landmark' explants were initiated into culture and multiplied on Anderson's medium with 8.89 μM N6-benzyladenine (BA). At the end of the multiplication tests, virus infections were reconfirmed with ELISA. *In vitro* multiplication of 'Malling Landmark' raspberry was significantly reduced by multiple infections, and multiplication of plants infected with all three viruses (RBDV + TomRSV + TSV) was about 1/3 that of virus-free cultures. Shoot height and morphology of *in vitro* cultures were not influenced by virus infection. The greenhouse stock plant with the three-virus infection was stunted and yellow compared to the control and the other infected plants. We recommend using virus-free plants as explant sources for *in vitro* experiments.


*Key words:* raspberry; *in vitro* culture; multiplication; raspberry bushy dwarf virus; tomato ringspot virus; tobacco streak virus.

## INTRODUCTION

Virus diseases damage many raspberry cultivars, and sensitive cultivars may be killed or severely weakened by virus infection. Latent infections on tolerant cultivars may shorten the planting life of a field through reduced yield and fruit quality (Converse, 1963). Blackberry and raspberry cultivars are susceptible to several virus diseases (Frazier, 1970). Raspberry bushy dwarf idaeovirus (RBDV) and tobacco streak ilarvirus (TSV) are distributed worldwide and RBDV is transmitted by pollen. RBDV is usually symptomless in either naturally or experimentally infected red raspberries (Murant, 1987). "Crumbly fruit" of red raspberry is a symptom of RBDV under some circumstances and a "yellows" symptom may occur in late spring in sensitive cultivars (Jones *et al.*, 1982). Jones (1979) found that RBDV co-infected with black raspberry necrosis virus (BRNV) caused "bushy dwarf" symptoms in the field. TSV infected *Rubus* plants do not generally show foliar symptoms, however, TSV may produce a chlorotic leaf pattern, chlorotic ringspot, or line-pattern symptoms when infection occurs with other viruses (Stace-Smith, 1987). Symptoms of tomato ringspot nepovirus (TomRSV), which is soil transmitted via nematodes, in raspberry may be visible throughout the growing season. New leaves may show yellow rings, line patterns, or a fine yellow vein chlorosis in the spring following the year of infection (Stace-Smith and Converse, 1987). However, hot weather and recent TomRSV infection may result in symptomless or unclear symptoms in the plant. Stace-Smith (1987) indicated that infection with TomRSV and a complex of other viruses induced a fernleaf mosaic symptom not seen in single infections. Chronic TomRSV infection causes dwarfing and slow growth in the spring. Spring cane death may occur and surviving canes produce small leaves with early fall abscission (Freeman and Stace-Smith, 1968). Virus incidence in the *Rubus* accessions received at the National Clonal Germplasm Repository (NCGR) in Corvallis, OR includes TomRSV (0.31% of 637 accessions tested), TSV (2.76% of 724 accessions), and RBDV (5.77% of 745 accessions), with only one combined infection of RBDV and TSV (Postman, unpublished).

De Vries-Paterson *et al.* (1992) first reported the effects of single and two-virus infections in reducing asparagus *in vitro* root development, survival in culture, and the fresh and dry weights of micropropagated plants. *In vitro* shoot-tip culture alone, without other virus-elimination treatments, resulted in only 8.6% virus-free asparagus clones. Establishment of *in vitro* sugarcane cultures was more successful from virus-free material than from material showing symptoms of maize streak virus (MSV) (Peros *et al.*, 1990). Most explants used for tissue culture research are not tested for virus infection and are not produced from heat-treated meristems. Combined heat treatment and meristem-culture are successfully used to initiate virus-free raspberry cultures (Pyott and Converse, 1981). There are no reports on the effects of viruses on raspberry shoot cultures, although it may be known that virus infections decrease micropropagation. 'Malling Landmark' was selected for this study, because it shows mosaic symptoms and is used as a virus indicator plant. Additionally, we were able to multiply it *in vitro*. Virus infected greenhouse-grown stocks, as well as well established virus indexed 'Malling Landmark' raspberry plants were available at NCGR.

The virus status of explants for *in vitro* culture is often unknown, but may play an important role in micropropagation. Inconsistent results and loss of plant material might be due to undetected virus infection. The purpose of this study was to determine the effects of single and multiple virus infections on the *in vitro* propagation of 'Malling Landmark' raspberry.

## MATERIALS AND METHODS

### *Plant materials and virus cultures*

Plants used in this study were grown from root cuttings made from a virus-free clone of raspberry, *Rubus idaeus* L. cv. Malling Landmark (NCGR accession CRUB-96.001). This plant tested negative for tobacco ringspot virus (TobRSV), tomato ringspot virus (TomRSV), and tobacco streak virus (TSV) by standard double-antibody-sandwich

ELISA (Clark and Adams, 1977) and for raspberry bushy dwarf virus (RBDV) by triple-antibody ELISA using a monoclonal antibody to decorate trapped virus followed by alkaline-phosphatase conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). This plant also tested negative for mechanically transmitted viruses using *Chenopodium quinoa* Willd. as the assay host, and for graft-transmitted viruses using *Rubus occidentalis* L. as the assay host with standard bioassays for *Rubus* viruses (Converse 1987).

A collection of *Rubus* plants infected with different viruses is maintained as virus stock cultures at NCGR-Corvallis (Postman, 1998). *Rubus* plants infected with raspberry bushy dwarf (CRUB-9008), tomato ringspot (CRUB-9011), and tobacco streak viruses (CRUB-9003) were used as inoculum sources. These single-virus source plants were free of other viruses as determined by ELISA and by bioassay (unpublished NCGR virus indexing records). We chose three common raspberry viruses (TomRSV, RBDV, and TSV) for this study because antisera for these viruses were available for subsequent analysis. Antiserum PVAS-239 against TomRSV was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Polyclonal and monoclonal antisera against RBDV, and polyclonal antiserum against TSV were provided by Dr. R. Martin (USDA-ARS Corvallis, OR).

*Establishment of virus infected 'Malling Landmark'*

Daughter plants of virus-free 'Malling Landmark' were inoculated by leaf grafting (Frazier, 1974) from each of the three virus cultures individually and in all possible combinations to establish plants with single, double, and triple virus infections. Inoculated 'Malling Landmark' plants were assayed by ELISA several months after inoculation, and again during subsequent growing seasons. Single plants were selected, based on ELISA results, which were infected with individual viruses as well as with multiple infections (Table 3-1). An uninoculated 'Malling Landmark' plant was used as a healthy control.

TABLE 3-1

VIRUS COMBINATIONS AND NUMBER OF REPLICATES USED FOR 'MALLING LANDMARK' RASPBERRY *IN VITRO* MULTIPLICATION TESTS

| Infecting virus (treatment) | No. of replicates[a] (culture period) | | | |
|---|---|---|---|---|
| | 1 (5 – 7 mo.) | 2 (9 – 11 mo.) | 3 (13 – 15 mo.) | 4 (17 – 19 mo.) |
| None (healthy plant as a control) | 3 | 3 | 2 | 3 |
| TomRSV (tomato ringspot virus) | - | - | 2 | 3 |
| RBDV (raspberry bushy dwarf virus) | 1 | 3 | 3 | 3 |
| TSV (tobacco streak virus) | 3 | 3 | 3 | 3 |
| TomRSV + RBDV | 1 | 3 | 3 | 3 |
| RBDV + TSV | 1 | 3 | 3 | 3 |
| TomRSV + RBDV + TSV | 1 | 3 | 3 | 3 |

[a] Ten plantlets per replicate.

*Establishment of in vitro cultures*

Nodal cuttings (≈ 3.0 cm) of virus-free and infected 'Malling Landmark' were collected in May, 1997, from greenhouse-grown plants. All but newly forming leaves were removed; explants were washed under running water for 5 minutes, surface-disinfected in a 10% bleach solution (0.525% sodium hypochlorite) with 0.1 ml per L of Tween 20 (Sigma) on a rotary shaker for 10 minutes, and rinsed twice in sterile water (five minutes each).

All materials were initiated in 16 X 100-mm tubes with 5 ml of half-strength MS (Murashige and Skoog, 1962) liquid medium (pH 6.9) without growth regulators for seven days for contaminant detection. Shoots were then transferred to Anderson's medium (Anderson, 1980) with 0.49 μM IBA (Indole-3-butyric acid), 0.29 μM $GA_3$ (Gibberellic acid $A_3$), 8.89 μM BA ($N^6$-benzyladenine). Medium was solidified with a combination of 0.35% agar (Difco granulated agar, Detroit, MI) plus 0.145% Gelrite (Schweitzer-Hall, South Plainfield, NJ) and adjusted to pH 5.7 before autoclaving. Growth-room conditions were a 16-hour photoperiod (cool white fluorescent illumination, 25 $\mu mol \bullet m^{-2} \bullet s^{-1}$) at 25 $^0$C. Plantlets were multiplied in GA7 Magenta boxes (Magenta Corp., Chicago, IL) and maintained with a three-week (21 days) transfer cycle.

*Virus indexing of in vitro cultures*

Prior to the multiplication experiment at the fifth month in culture, two plantlets from each box (three boxes for each culture) and two young leaves from greenhouse stocks (one pot each) were randomly chosen for virus indexing. ELISA tests were repeated to confirm viral infections of experimental plant materials at the $9^{th}$ and $17^{th}$ month in culture.

*Multiplication test*

Multiplication tests started after the fifth month in culture and were repeated four times. Each repeat had an uneven number of replicates, due to differences in the availability of plant materials (Table 3-1). Ten plantlets (10 mm) were placed in each box for the seven treatments. Boxes (replicates) were arranged in three blocks. All

boxes were randomly placed in three plastic trays (blocks) side by side on the same shelf. Microshoots were multiplied and shoots 5 mm or larger were divided and transferred on a three-week cycle during each 12-week experiment. The number and the length (mm) of shoots were recorded at the 21$^{st}$, 42$^{nd}$, 63$^{rd}$, and 84$^{th}$ day.

*Experimental design and statistical analysis*

*In vitro* multiplication was analyzed for the number of microshoots produced during four 12-week culture periods. Randomized block design was used with uneven replicates (Magenta boxes) for each treatment within the four culture period repetitions. All data analysis followed the general linear model (GLM) procedure using SAS programming for the analysis of variance (ANOVA) (SAS 6.12, 1989-1996; SAS Institute Inc., Cary, N.C.). The results of statistical analysis were considered significant at $P \leq 0.05$.

## RESULTS AND DISCUSSION

The virus-infected greenhouse-grown 'Malling Landmark' plants were generally symptomless and were indistinguishable from the controls, but the plant infected with RBDV + TomRSV + TSV was stunted and had 'yellows' symptoms (data not shown). This is similar to growth under field conditions where virus symptom development is dependent on the cultivar, virus, and environmental conditions (Daubeny *et al.*, 1982; Diekmann *et al.*, 1994). Virus infected raspberries often grow best in early spring and also exhibit symptom development. RBDV, TomRSV, and TSV infected plants often show low fruit production and slow growth in the field (Murant, 1987; Stace-Smith, 1987; Stace-Smith and Converse, 1987).

ELISA tests confirmed that all *in vitro* plants remained infected with the same viruses from the beginning to the end of the multiplication experiments (data not shown). All cultures multiplied at a low rate for the first six months then increased greatly after one year, during the third experimental period (Fig. 3-1). The number of microshoots

Fig. 3-1. Multiplication of virus-infected 'Malling Landmark' raspberry shoot cultures at four culture periods. Explants were cultured for 12 weeks on Anderson's medium with 8.89 μ M BA in Magenta GA 7 and transferred at three-week-intervals. Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom).

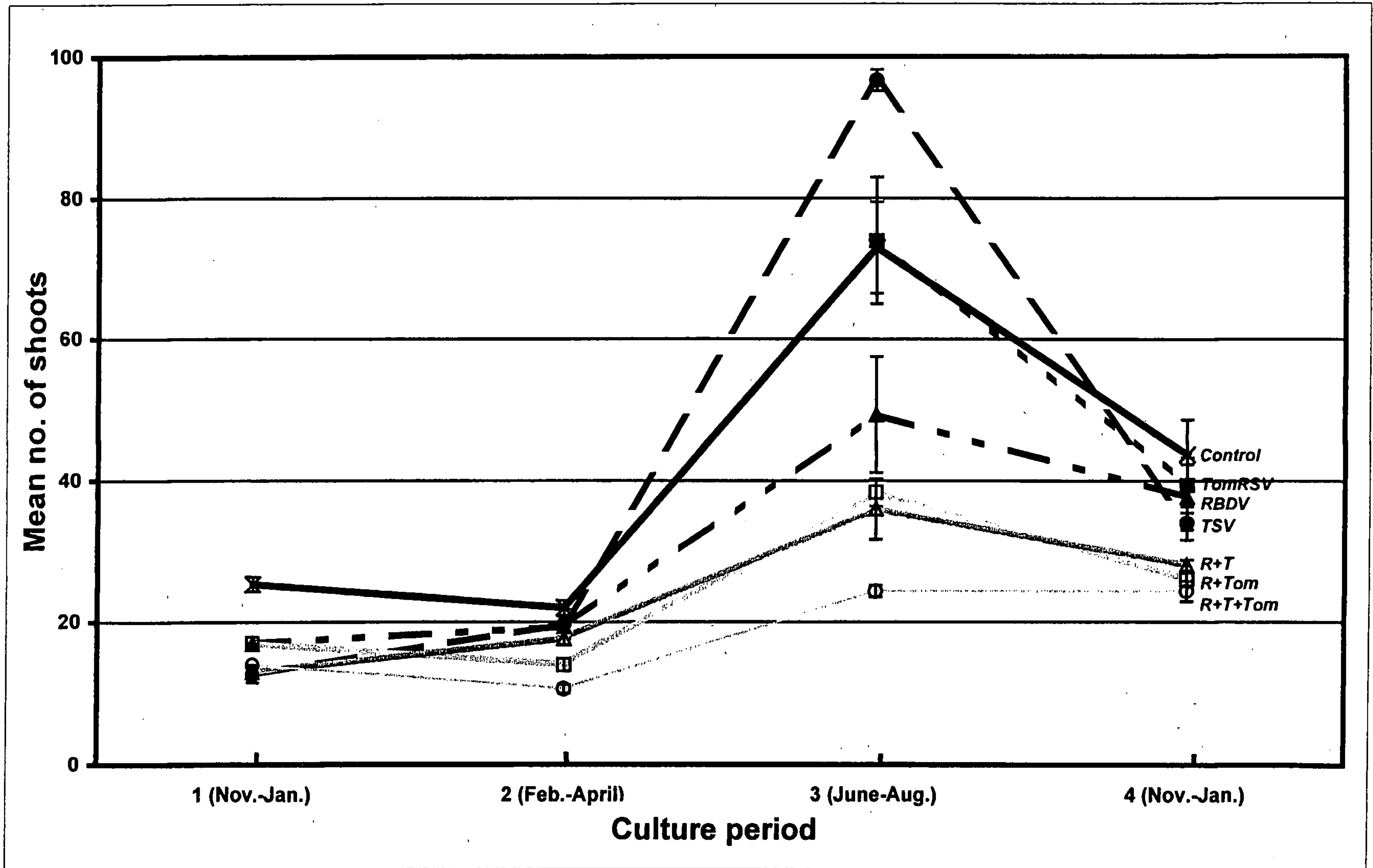


Fig. 3-1

produced during the first culture period (5 - 7 months) from November to January was significantly less than for the same season during the fourth period (17 - 19 months) (Fig. 3-1). Multiplication for *Rubus* cultures may vary greatly from year to year even when initiated at the same time (Jin *et al.*, 1992). Rapid multiplication after the third subculture occurred in all treatments during one 12-week culture period (data not shown). In the third period (13 – 15 months), four of the virus-infected cultures produced significantly fewer shoots than the control, while the TSV-infected culture produced significantly more. The fourth experiment had lower multiplication for one two-virus culture (RBDV + TomRSV) and for the three-virus culture (RBDV + TomRSV + TSV) compared to the uninfected control, but the remaining treatments were not significantly different from the control or from two virus culture (RBDV + TSV) (Table 3-2). There was a significant interaction between virus infection (treatment) and the date (culture period) (Table 3-3). The TomRSV-infected culture was initially very slow to multiply and was not included in the first two experiments due to a shortage of plant material. The mean multiplication of the TomRSV-infected culture was high for the last two culture periods and was not significantly different from the control (data not shown).

The length of 'Malling Landmark' microshoots did not vary with the virus treatments (Table 3-4). The mean height of shoots varied within each 12-week culture period, because only shoots longer than 5 mm were recorded. The third data set (9 week) of each clture period produced more shoots, resulting in significantly shorter shoots in average. The initial and final shoot lengths among virus treatments and one virus-free culture were not significantly different in this study. Although *in vitro* cultured potato cv. Igor infected with potato virus $Y^{NTN}$ accumulated jasmonic acid in the roots and roots were significantly shorter, there was no significant difference in the average length of infected and healthy potato plantlets (Petrovic *et al.*, 1997). For the field-grown 'Lloyd George' raspberry infected with RBDV, Jones (1979) found no significant difference in plant heights between 1975 and 1976. Fresh and dry weights of infected shoots and roots were reduced but no *in vitro* shoot symptoms appeared, such as the stunting, necrosis, and mosaic patterns, normally observed on field plants (Petrovic *et al.*, 1997; De Vries-Paterson *et al.*, 1992). Greno *et al.* (1988) indicated that virus and virus-like agent infections affected the number and size of shoots produced from nodal citrus-stem

TABLE 3-2

MEAN MULTIPLICATION OF VIRUS-INFECTED 'MALLING LANDMARK' RASPBERRY CULTURES AT FOUR CULTURE PERIODS

| Treatment[d] | Mean no. of microshoots at four culture periods[a, b] | | | | Treatment |
|---|---|---|---|---|---|
| | 1 (5 – 7 mo.) | 2 (9 – 11 mo.) | 3 (13 - 15 mo.) | 4 (17 - 19 mo.) | means[c] |
| Control | Z 25.33 x | Z 22.00 x | Y 73.00 z | Z 43.67 y | Z 41.00 |
| TSV | Z 12.67 x | Z 19.67 xy | Z 96.67 z | YZ 34.00 y | Z 40.75 |
| RBDV | Z 17.00 y | Z 19.33 y | X 49.33 z | YZ 37.67 yz | YZ 30.83 |
| RBDV + TomRSV | Z 17.00 yz | Z 14.00 y | WX 38.33 z | Y 26.00 yz | Y 23.83 |
| RBDV + TSV | Z 13.00 y | Z 17.67 y | WX 36.00 z | YZ 28.00 yz | Y 23.67 |
| RBDV + TomRSV + TSV | Z 14.00 z | Z 10.67 z | W 24.33 z | Y 24.33 z | Y 18.33 |
| Culture period means | 16.50 x | 17.22 x | 52.94 z | 32.28 y | |

[a] Means with different capital letters in a column are significantly different within six treatments at $P \leq 0.05$.

[b] Means with different small letters in a row are significant different within four culture-period-repetitions at $P \leq 0.05$.

[c] Treatment means and culture period means are the average of least square means for each treatment and culture period.

[d] Virus treatment, TomRSV was not included in this analysis, because there was not enough TomRSV infected *in vitro* cultures for the first and second experiments.

TABLE 3-3

ANALYSIS OF VARIANCE OF MEAN MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY CULTURES FOR CULTURE PERIODS AND VIRUS INFECTION

| Source of variation | DF | Mean square |
|---|---|---|
| Treatment | 5 | 866.37 *** |
| Culture period | 3 | 4352.24 *** |
| Treatment X Culture period | 15 | 431.56 *** |
| Error | 39 | 106.58 |

*** Significant at $P \leq 0.001$.

TABLE 3-4

ANALYSIS OF VARIANCE OF MEAN HEIGHT OF VIRUS INFECTED 'MALLING LANDMARK' SHOOT CULTURES DURING A 12 WEEK-MULTIPLICATION CYCLE

| Source of variation | DF | Mean height of microshoots (cm)[a] / no. shoots produced | Mean square |
|---|---|---|---|
| Treatment | 6 | | 0.0078 [NS] |
| Time | 3 | | 0.0858 *** |
| 3 wk | | 1.3064 z | |
| 6 wk | | 1.2457 z | |
| 9 wk | | 1.1571 y | |
| 12 wk | | 1.2945 z | |
| Treatment X time | 18 | | 0.0196 [NS] |
| Error | 50 | | 0.0174 |

[a] Means with different letters are significant different at $P \leq 0.05$.

[NS], *** Nonsignificant or significant at $P \leq 0.001$.

segments. Citrus shoot development is plant host, virus, and culture environment dependent. Duran-Vila *et al.* (1989) found that citrus tristeza virus (CTV) and citrus infectious variegation virus (CIVV) inhibited *in vitro* root formation of various citrus species, but *in vitro* morphogenesis depended on the virus strain and the host. Callus induction by CTV-infected tissue was not different from healthy controls, but significantly less primary callus was formed on CIVV-infected cultures.

Growers and researchers working with blackberry and raspberry cultures should be aware of the problems involved with using virus-contaminated plants as stocks. Viruses contaminating *in vitro* cultures may have significant impacts on multiplication and elongation. This study showed the effects of three common raspberry viruses on 'Malling Landmark' shoot cultures. These viruses are sometimes symptomless on *Rubus*, but other plants or other viruses may produce definite *in vitro* symptoms, such as streak symptoms on MSV-infected *in vitro* sugarcane (Peros *et al.*, 1990). Although greenhouse stock plants infected with the viruses showed little apparent growth inhibition, *in vitro* cultures with two or three viruses multiplied more slowly than controls or singly infected cultures. Shoot elongation was not impacted in this study. The elimination of viruses from stock plants can make a significant difference in experimental results and in yield from commercial micropropagation. We recommend the use of virus-free explants for *in vitro* culture experiments and micropropagation.

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REFERENCES

Anderson, W. C. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *Rubus occidentalis*. Acta Hort. 112:13-20; 1980.

Clark, M. F.; Adams, A. N. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. of General Virol. 34:475-483; 1977.

Converse, R. H. Influence of heat-labile components of the raspberry mosaic complex on growth and yield of red raspberries. Phytopathology 53:1251-1254; 1963.

Converse, R. H., ed. Virus Diseases of Small Fruits. USDA Agriculture Handbook No. 631. 277 pp; 1987.

Daubeny, H. A.; Freeman, J. A.; Stace-Smith, R. Effects of raspberry bushy dwarf virus on yield and cane growth in susceptible red raspberry cultivars. HortScience 17:645-647; 1982.

De Vries-Paterson, R. M.; Evans, T. A.; Stephens, C. T. The effect of asparagus virus infection on asparagus tissue culture. Plant Cell Tiss. Org. Cult. 31:31-35; 1992.

Diekmann, M.; Frison, E. A.; Putter, T. FAO/IPGRI technical guidelines for the safe movement of small fruit germplasm. Food and Agriculture Organization of the United States, Rome/International Plant Genetic Resources Institute, Rome; 1994.

Duran-Vila N.; Cambra, M.; Medina, V.; Ortega, C.; Navarro, L. Growth and morphogenesis of citrus tissue cultures infected with citrus tristeza virus and citrus infectious variegation virus. Phytopathology 79:820-826; 1989.

Frazier, N. W., ed. Virus Diseases of Small Fruits and Grapevines. Univ. of Calif. Div. of Agric. Sci., Berkeley, Calif. 290 pp; 1970.

Frazier, N. W. Detection of graft-transmissible diseases in strawberry by a modified leaf grafting technique. Plant Disease Reporter 58:28-31; 1974.

Freeman, J. A.; Stace-Smith R. Effect of tomato ringspot virus on plant and fruit development of raspberries. Canadian J. of Plant Science 48:25-29; 1968.

Greno, V.; Navarro, L.; Duran-Vila, N. Influence of virus and virus-like agents on the development of citrus buds cultured *in vitro*. Plant Cell Tiss. Org. Cult. 15:113-124; 1988.

Jin, W.; Gu, Y.; Zhen, S. Z. *In vitro* propagation of *Rubus* species. Scientia Horticulturae 49:335-340; 1992.

Jones, A. T. The effects of black raspberry necrosis and raspberry bushy dwarf viruses in Lloyd George raspberry and their involvement in raspberry bushy dwarf disease. J. Hortic. Sci. 54:267-272; 1979.

Jones, A. T.; Murant, A. F.; Jennings, D. L.; Wood, G. A. Association of raspberry bushy dwarf virus with raspberry yellows disease; reaction of *Rubus* species and cultivars, and the inheritance of resistance. Annals of Applied Biology 100:135-147; 1982.

Murant, A. F. Raspberry bushy dwarf. pp. 229-234. In: Virus Diseases of Small Fruits. ed. Converse, R.H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol. Plant 15:473-479; 1962.

Peros, J. P.; Bonnel, E.; Reynaud, B. *In vitro* culture of sugarcane infected with maize streak virus (MSV). Plant Cell Tiss. Org. Cult. 23:145-149; 1990.

Petrovic, N.; Miersch, O.; Ravnikar, M.; Kovac, M. Potato virus $Y^{NTN}$ alters the distribution and concentration of endogenous jasmonic acid in potato plants grown *in vitro*. Physiological and Molecular Plant Pathology 50:237-244; 1997.

Postman, J. D. Temperate fruit germplasm borne pathogen collections at NCGR-Corvallis. Acta Hort. 471:111-114; 1998.

Pyott, J. L.; Converse, R. H. *In vitro* propagation of heat-treated red raspberry clones. HortScience 3:308-309; 1981.

Stace-Smith, R. Tobacco streak virus in *Rubus*. pp. 235-239. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Stace-Smith, R.; Converse, R. H. Tomato ringspot virus in *Rubus*. pp. 223-227. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

# CHAPTER 4. CONCLUSIONS

Of the more than one dozen *Rubus* regeneration protocols, none works for most genotypes and most are very genotype specific. The research reported here screened a wide range of *Rubus* genotypes to determine a method suitable for regenerating shoots from diverse germplasm sources.

Screening of 13 commercially important *Rubus* cultivars indicated that regeneration medium (RM) with 5 or 10 μM BA or 1 μM TDZ would induce shoots from intact whole-leaf explants of *in vitro* plantlets. Adding the iron chelate sequestrene to the second RM increased the number of regenerating explants by 60% for the two genotypes tested. Varied gelling agents and silver nitrate in the RM did not affect regeneration and there were also no effects from berry type or ploidy level. Future study should include screening cultivars on RM with 200 mg per l sequestrene and the same range of plant growth regulators.

Micropropagation of 'Malling Landmark' raspberry with and without virus infections was also investigated. *In vitro* multiplication was significantly reduced with multiple infections (RBDV + TSV, RBDV + TomRSV, and RBDV + TSV + TomRSV) compared to virus-free cultures. Multiplication was reduced by 2/3 for the three virus infection. There were no obvious physical symptoms *in vitro* and shoot height was not significantly different for infected and virus-free plantlets. We recommend using virus-free plants as explant sources for *in vitro* experiments and micropropagation purposes.

# BIBLIOGHAPHY


Abu-Qaoud, H.; Skirvin, R. M.; Below, F. E. Influence of nitrogen from and $NH^{4+}/NO^{3-}$ ratios on adventitious shoot formation from pear (*Pyrus communis*) leaf explants *in vitro*. Plant Cell Tiss. Org. Cult. 27:315-319; 1991.

Anderson, W. C. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *Rubus occidentalis*. Acta Hort. 112:13-20; 1980.

Arisumi, T.; Frazier, C. C. Cytological and morphological evidence for the single-cell origin or vegetively propagated shoots in thirteen species of *Saintpaulia* treated with colchicine. Proc. Amer. Soc. Hort. Sci. 93:679-685; 1968.

Asker, S. E.; Jerling, L. Apomixis in Plants. Boca Raton Fla: CRC Press; 1992.

Barton, K. A.; Binns, A. J.; Matzke, A. J. M.; Chilton, M. D. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32:1033-1043; 1983.

Bilkey, P. C.; Cocking, E. C. Increased plant vigor by *in vitro* propagation of *Saintpaulia ionantha* Wendl. from sub-epidermal tissue. HortScience 16:643-644; 1981.

Borgman, C. A.; Mudge, K. W. Factors affecting the establishment and maintenance of 'Titan' red raspberry root organ cultures. Plant Cell Tiss. Org. Cult. 6:127-137; 1986.

Boxtel, J. V.; Berthouly, M. High frequency somatic embryogenesis from coffee leaves. Plant Cell Tiss. Org. Cult. 44:7-17; 1996.

Brand, M. H.; Lineberger, R. D. The effect of leaf source and development stage on shoot organogenic potential of sweet gum (*Liquidambar styraciflua* L.) leaf explants. Plant Cell Tiss. Org. Cult. 24:1-7; 1991.

Britton, D. M.; Hull, J. W. Miotic instability in blackberry seedlings from progenies of Boysen and of Young. J. Hered. 48, 11-20; 1956.

Britton, D. M.; Hull, J. W. Miotic instability in *Rubus*. J. Hered. 48:11-20; 1957.

Broertjes, C. B.; Keen, A. Adventitious shoots: Do they develop from one cell? Euphytica 29:73-87; 1980.

Broome, O. C.; Zimmerman, R. H. *In vitro* propagation of blackberry. HortScience 13:151-153; 1978.

Calvo, M. C.; Segura, J. Plant regeneration from cultured leaves of *Lavandula latifolia* Mediaes: Influence of growth regulators and illumination conditions. Plant Cell Tiss. Org. Cult. 16:75-87; 1989.

Cantoni, G.; Berardi, G.; Rosati, P. Organogenesis and somatic embryogenesis from immature embryos of blackberry. Acta Hort. 352:37-42; 1993.

Carrillo, C. G.; Mendoza, J. L. Effect of natural products on *in vitro* development of *Rubus* spp. buds. Turrialba 29:299-303; 1979.

Castillo, B.; Smith, M. A. L.; Madhavi, D. L. Interactions of irradiance level and iron chelate source during shoot tip culture of *Carica papaya* L. HortScience 32:1120-1123; 1997.

Chaudhury, A. M.; Signer, E. R. Relative regeneration proficiency of *Arabidopsis thaliana* ecotypes. Plant Cell Rep. 8:368-369; 1989.

Chevreau, E.; Mourgues, F.; Neveu, M.; Chevalier, M. Effect of gelling agents and antibiotics on adventitious bud regeneration from *in vitro* leaves of pear. *In vitro* Cell. Dev. Biol. – Plant 33:173-179; 1997.

Chevreau, E.; Skirvin, R. M.; Abu-Qaoud, H. A.; Korban, S. S.; Sullivan, J. G. Adventitious shoot regeneration from leaf tissue of three pear (*Pyrus* sp.) cultivars *in vitro*. Plant Cell Rep. 7:688-691; 1989.

Clark, M. F.; Adams, A. N. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. of General Virol. 34:475-483; 1977.

Compton, M. E.; Gray, D. J. Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid, and tetraploid watermelon. J. Amer. Soc. Hort. Sci. 118:151-157; 1993.

Converse, R. H. Influence of heat-labile components of the raspberry mosaic complex on growth and yield of red raspberries. Phytopathology 53:1251-1254; 1963.

Converse, R. H., ed. Virus Diseases of Small Fruits. USDA Agriculture Handbook No. 631. 277 pp; 1987.

Cousineau, J. C.; Donnely, D. J. Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss. Org. Cult. 27:249-255; 1991.

Crane, M. B.; Thomas, P. T. Reproductive versatility in *Rubus*. III Raspberry-blackberry hybrids. Heridity 3:99-107; 1949.

Daminano, C.; Ascarelli, A.; Frrattarelli, A.; Lauri, P. Adventitious regeneration and genetic variability in strawberry. Acta Hort. 392:107-114; 1995.

Daubeny, H. A.; Freeman, J. A.; Stace-Smith, R. Effects of raspberry bushy dwarf virus on yield and cane growth in susceptible red raspberry cultivars. HortScience 17:645-647; 1982.

Daubeny, H. A.; Stace-Smith, R., Freeman, J. A. The occurrence and some effects of raspberry bushy dwarf virus in red raspberry. J. Amer. Soc. Hort. Sci. 103:519-522; 1978.

De Vries-Paterson, R. M.; Evans, T. A.; Stephens, C. T. The effect of asparagus virus infection on asparagus tissue culture. Plant Cell Tiss. Org. Cult. 31:31-35; 1992.

Debergh, P. C. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant 59:270-276; 1983.

Dhir, S. K.; Dhir, S.; Widholm, J. M. Regeneration of fertile plants from protoplasts of soybean (*Glycine max* L. Merr.): genotypic differences in culture response. Plant Cell Rep. 11:285-289; 1992.

Diekmann, M.; Frison, E. A.; Putter, T. FAO/IPGRI technical guidelines for the safe movement of small fruit germplasm. Food and Agriculture Organization of the United States, Rome/International Plant Genetic Resources Institute, Rome; 1994.

Duran-Vila N.; Cambra, M.; Medina, V.; Ortega, C.; Navarro, L. Growth and morphogenesis of citrus tissue cultures infected with citrus tristeza virus and citrus infectious variegation virus. Phytopathology 79:820-826; 1989.

Einset, J. Apomixis in American polyploid blackberries. Am. J. Bot. 38:768-772; 1951.

Fasolo, F.; Zimmerman, R. H.; Fordham, I. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16:75-87; 1989.

Fillatti, J. A. J.; Sellmer, J.; McCown, B., Haissig, B., Comai, L. *Agrobacterium* mediated transformation and regeneration of *Populus*. Molecular General Genetics 206:192-199; 1987.

Fiola, J. A.; Hassan, M. A.; Swartz, H. J.; Bors, R. H.; McNicol, R. J. Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss. Org. Cult. 20:223-228; 1990.

Fiola, J. A.; Swartz, H. J. Somatic embryogenesis, organogenesis and proliferation *in vitro* from *Rubus* embryos. Acta Hort. 183:91-98; 1986.

Focke, W. O. Species Ruborum: Monographiae generis Rubi Podromus, part I. Biblioth. Bot. 17:1-120; 1910.

Fralay, R. T.; Rogers, S. G.; Horsch, R. B.; Sanders, P. R.; Flock, J. S.; Adams, S. P.; Bittner, M. L.; Brand, L. A.; Fink, L. L.; Fry, J. S.; Galluppi, G. R.; Goldberg, S. B.; Hoffmann, N. L.; Woo, S. C. Expression of bacterial genes in plant cells. Proceedings National Academy Science USA 80:4803-4807; 1983.

Frazier, N. W. Detection of graft-transmissible diseases in strawberry by a modified leaf grafting technique. Plant Disease Reporter 58:28-31; 1974.

Frazier, N. W., ed. Virus Diseases of Small Fruits and Grapevines. Univ. of Calif. Div. of Agric. Sci., Berkeley, Calif. 290 pp; 1970.

Freeman, J. A.; Stace-Smith R. Effect of tomato ringspot virus on plant and fruit development of raspberries. Canadian J. of Plant Science 48:25-29; 1968.

Gamborg, O. C.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50:148-151; 1968.

Gelvin, S. B. Crown gall disease and hairy root disease. Plant Physiol. 92:281-285; 1990.

Gingas, V. M.; Stokes, B. D. *Rubus* plant regeneration via asexual embryogenesis. HortScience 28:58; 1993.

Graham, J. The development and application of methods for using *Agrobacteium* spp. as DNA vectors in soft fruit. Ph.D. Thesis, St. Andrews University; 1990.

Graham, J.; Iasi, L.; Millam, S. Genotype-specific regeneration from a number of *Rubus* cultivars. Plant Cell Tiss. Org. Cult. 48:167-173; 1997.

Graham, J.; McNicol, R. J. Plant regeneration and genetic transformation in soft fruit species. Acta Hort. 280:517-522; 1990.

Graham, J.; McNicol, R. J.; Greig, K.; Van de Ven W. T. G. Identification of red raspberry cultivars and an assessment of their relatedness using fingerprints produced by random primers. J. Hort. Sci. 69:123-130; 1994.

Graham, J.; McNicol, R. J.; Kumar, A. *Agrobacterium*-mediated transformation of soft fruit *Rubus*, *Ribes*, and *Fragaria*. Methods in Molecular Biology. v. 44, pp.129-133; 1995.

Graham, J.; McNicol, R. J.; Kumar, A. Use of the GUS gene as a selectable marker for *Agrobacterium*-mediated transformation of *Rubus*. Plant Cell Tiss. Org. Cult. 20:35-39; 1990.

Greno, V.; Navarro, L.; Duran-Vila, N. Influence of virus and virus-like agents on the development of citrus buds cultured *in vitro*. Plant Cell Tiss. Org. Cult. 15:113-124; 1988.

Gu, Y.; Zhao, C. M.; Jun, W. O. *Rubus* resources in Hunan and Fujian provinces. XXIII Intl. Hort. Congr. 4014; 1990 (abst.).

Hall, H. K.; Quazi, M. H.; Skirvin, R. M. Isolation of a pure thornless loganberry by meristem tip culture. Euphytica. 35:1039-1044; 1986.

Harper, P. C. Tissue culture propagation of blackberry and tayberry. Hort. Res. 18:141-143; 1978.

Haskell, G.; Tun, N. N. Developmental sequence of chromosome number in a cytologically unstable *Rubus* hybrid. Genet. Res. 2:10-24; 1961.

Hassan, M. A.; Swartz H. J.; Inamine G.; Mullineaux, P. *Agrobacterium tumefaciens*-mediated transformation of several *Rubus* genotypes and recovery of transformed plants. Plant Cell Tiss. Org. Cult. 33:9-17; 1993.

Heit, C. E.; Slate, G. L. Treatment of blackberry seed to secure first year germination. Proc. Amer. Soc. Hort. Sci. 55: 297; 1950.

Henry, Y.; Vain, P.; Buyser, J. D. Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. Euphytica 79:45-58; 1994.

Hoepfner, A. S. *In vitro* propagation of red raspberry. Acta Hort. 262:285-288; 1989.

Hoepfner, A. S.; Nestby, R.; Nybom, H. Genetic deviation initiated by adventitious shoot regeneration from tissue cultured red raspberry. J. of Hort. Sci. 71:71-79; 1996.

Huang, L. C.; Chi, D. L. Pivotal roles of picloram and gelrite in banana callus culture. Environmental and Experimental Botany 28:249-258; 1988.

Huetteman, C. A.; Preece, J. E. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33:105-119; 1993.

Hummer, K. E. *Rubus* diversity. HortScience 31:182-183; 1996.

Huy, B. P.; Landi, L.; Taruschio, L.; Mott, D.; Mezzetti, B.; Rosati, P. Protoplast isolation, culture, and cell differentiation in raspberry and blackberry cultivars *(Rubus* spp.). J. Applied Botany 71:131-137; 1997.

Hyde, C. L.; Phillips, G. C. Silver nitrate promotes shoot development and plant regeneration of chili pepper (*Capsium annuum* L.) via organogenesis. *In vitro* Cell. Dev. Biol.- Plant 32:72-80; 1996.

Infante, R. Long-term cell suspension culture and regeneration of the single-leafed strawberry *Fragaria vesca* monophylla. J. Sci. Food Agric. 72:196-200; 1996.

Infante, R.; Rosati, P. *Fragaria vesca* L. 'Alpine' protoplast culture and regeneration. Acta Hort. 348:432-434; 1993.

James, D. J.; Knight, V. H.; Thurbon, I. J. Micropropagation of red raspberry and the influence of phloroglucinol. Sci. Hort. 12:313-319; 1980.

Jefferson, R. A. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter 8:387-405; 1987.

Jennings, D. L. Raspberries and blackberries: Their Breeding, Diseases and Growth. Academic Press, London; 1988.

Jennings, D. L.; Daubeny, H. A.; Moore, J. N. Blackberries and raspberries (*Rubus*). Acta Hort. 290:331-389; 1990.

Jennings, D. L.; Tulloch, B. M. M. Studies on factors which promote germination of raspberry seeds. J. Exp. Bot. 16:329-340; 1965.

Jin, W.; Gu, Y.; Zhen, S. Z. *In vitro* propagation of *Rubus* species. Scientia Horticulturae 49:335-340; 1992.

Jones, A. T. Advances in the study, detection and control of viruses and viruse diseases of *Rubus* with a particular reference to the United Kingdom. Crop Res. 26:127-171; 1986.

Jones, A. T. The effects of black raspberry necrosis and raspberry bushy dwarf viruses in Lloyd George raspberry and their involvement in raspberry bushy dwarf disease. J. Hortic. Sci. 54:267-272; 1979.

Jones, A. T.; Murant, A. F.; Jennings, D. L.; Wood, G. A. Association of raspberry bushy dwarf virus with raspberry yellows disease; reaction of *Rubus* species and cultivars, and the inheritance of resistance. Annals of Applied Biology 100:135-147; 1982.

Jourdan, P. S.; Earle, E. D. Genotypic variability in the frequency of plant regeneration from leaf protoplasts of four *Brassica* spp. and of *Raphanus sativus*. J. Amer. Soc. Hort. Sci. 114:343-349; 1989.

Kaul, V.; Miller, R. M.; Hutchinson, J. F.; Richards, D. Shoot regeneration from stem and leaf explants of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell Tiss. Org. Cult. 21:21-30; 1990.

Kavi Kishor, P. B.; Reddy, G. M. Retention and revival of regenerating ability by osmotic adjustment in long-term culture of four varieties of rice. J. Plant Phys. 126:49-54; 1986.

Ke, S. Q.; Skirvin, R. M.; McPheeters, K. D.; Otterbacher, A. G.; Galletta, G. *In vitro* germination and growth of *Rubus* seeds and embryos. HortScience 20:1047-1049; 1985.

Keep, E. Incompatibility in *Rubus* with special reference to *R. idaeus* L. Can. J. Genet. Crytol. 10:253-262; 1968.

Kiss, F.; Zatykó, J. Vegetative propagation of *Rubus* species *in vitro*. Bot. Koxlem 65:65-68; 1978.

Kouider, M.; Korban, S. S.; Skirvin, R. M.; Chu, M. C. Influence of embryonic dominance and polarity on adventitious shoot formation from apple cotyledons *in vitro*. J. Amer. Soc. Hort. Sci. 109:381-385; 1984.

Kouider, M.; Korban, S. S.; Skirvin, R. M.; Joung, H. The relationship of apple embryos and their cotyledons to maturity, dormancy, and the potential to form shoots *in vitro*. J. Amer. Soc. Hort. Sci. 110:93-96; 1985.

Kurppa, A.; Martin, R. R. Use of double-stranded RNA for detection and identification of virus diseases of *Rubus* species. Acta Hort. 186:51-62; 1986.

Lashecn, A. M.; Blackhurst, H. T. Biochemical changes associated with dormancy and after-ripening of blackberry seeds. Proc. Amer. Soc. Hort. Sci. 67:331-340; 1956.

Linsmaier, E. M.; Skoog, F. Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum 18:100-127; 1965.

Lis, E. K. Strawberry plant regeneration by organogenesis from peduncle and stolon segments. Acta Hort. 348:435-438; 1993.

Liu, J. R.; Sink, K. C.; Dennis, F. G. Plant regeneration from apple seedling explants and callus culture. Plant Cell Tiss. Org. Cult. 2:293-304; 1983.

Liu, Z. R.; Sanford, J. C. Plant regeneration by organogenesis from strawberry leaf and runner tissue. HortScience 23:1057-1059; 1988.

Mathews, H.; Wagoner, W.; Cohen, C.; Kellogg, J., Bestwick, R. Efficient genetic transformation of red raspberry, *Rubus idaeus* L. Plant Cell Rep. 14:471-476; 1995.

McNicol, R. J.; Graham, J. Genetic manipulation in *Rubus* and *Ribes*. Acta Hort. 262:41-46; 1989.

McNicol, R. J.; Graham, J. *In vitro* regeneration of *Rubus* from leaf and stem segments. Plant Cell Tiss. Org. Cult. 21:45-50; 1990.

Mezzetti, B.; Savini, G.; Carnevali, F.; Mott, D. Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biologia-Plantarum 39:139-150; 1997.

Millan-Mendoza, B. Regeneration of *Rubus in vitro* using forchlorfenuron (CPPU). Rev. Fac. Agron. 15:242-248; 1988.

Miller, A. R. Plant regeneration from excised cotyledons of mature strawberry achenes. HortScience 25:569-571; 1990.

Mok, M. C.; Mok, D. W. S.; Dixon, K. W.; Armonstrong, D. J.; Shaw, G. Cytokinin structure-activity relationships and the metabolism of N6-(Delta 2-Isopentenyl) Adensine-8-14C in callus tissues. Plant Physiol. 70:173-178; 1982.

Monacelli, B.; Altamura, M. M.; Pasqua, G. The histogenesis of somaclones from tomato (*Lycopersicon esculentum* Mill.) cotyledons. Protoplasma 142:156-163; 1988.

Morrish, F. M.; Vasil, I. K. DNA methylation and embryogenic competence in leaves and callus of napiergrass (*Pennisetum purpureum* Schum.). Plant Physiol. 90:37-40; 1988.

Murant, A. F. Raspberry bushy dwarf. pp. 229-234. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiol. Plant 15:473-479; 1962.

Nehra, N. S.; Stushnoff, C.; Kartha, K. K. Direct shoot regeneration from strawberry leaf disks. J. Amer. Soc. Hort. Sci. 114:1014-1018; 1989.

Niederwieser, J. G.; Van Staden, J. The relationship between genotype tissue age and endogenous cytokinin levels on adventitious bud formation on leaves of *Lachenalia*. Plant Cell Tiss. Org. Cult. 22:223-228; 1990.

Norris, R.; Smith, R. H.; Vaughn, K. C. Plant chimeras used to establish *de novo* origin of shoots. Science 220:75-76; 1983.

Norton, M. A. Regeneration and evaluation of first and second cycle somaclones of 'Thornless Evergreen' blackberry (*Rubus laciniatus* Willd.). Ph. D. Thesis, University of Illinois at Urbana-Champaign; 1994.

Nybom, H. Chromosome numbers in *Rubus* species from Sri Lanka. Bot. Notiser 133:47-48; 1980.

Ourecky, D. K. Brambles. In: Advances in Fruit Breeding. eds. Janick J. and Moore N. Purdue University Press, West Lafayette, Indiana, USA; 1975.

Owens y de N, C. Empirical evaluation of *in vitro* media components for cell growth and shoot regeneration from *Rubus* explants. New Zealand Natural Sciences 19:79-86; 1992.

Owens y de N, C.; Conner, A. J. Comparison of *in vitro* shoot regeneration protocols from *Rubus* leaf explants. New Zealand Journal of Crop and Horticultural Science 20:471-473; 1992.

Peros, J. P.; Bonnel, E.; Reynaud, B. *In vitro* culture of sugarcane infected with maize streak virus (MSV). Plant Cell Tiss. Org. Cult. 23:145-149; 1990.

Petrovic, N.; Miersch, O.; Ravnikar, M.; Kovac, M. Potato virus $Y^{NTN}$ alters the distribution and concentration of endogenous jasmonic acid in potato plants grown *in vitro*. Physiological and Molecular Plant Pathology 50:237-244; 1997.

Postman, J. D. Incidence of viruses in the U.S. National Clonal Germplasm Repository *Ribes* and *Rubus* collections. Acta Hort. 262:217-222; 1989.

Postman, J. D. Temperate fruit germplasm borne pathogen collections at NCGR-Corvallis. Acta Hort. 471:111-114; 1998.

Pritts, M.; Handley, D. Bramble Production Guide. Northeast Regional Agricultural Engineering Service. New York; 1994.

Pyott, J. L.; Converse, R. H. *In vitro* propagation of heat-treated red raspberry clones. HortScience 16:308-309; 1981.

Reed, B. M. Multiplication of *Rubus* germplasm *in vitro*: a screen of 256 accessions. Fruit Var. J. 44:141-148; 1990.

Reed, B. M.; Buckley, P. M.; DeWilde, T. N. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In vitro* Cell. Dev. Biol. –Plant 31:53-57; 1995.

Rowland, L. J.; Ogden, E. L. Use of a cytokinin conjugate for efficient shoot regeneration from leaf sections of high bush blueberry. HortScience 27:1127-1129; 1992.

SAS Institute, Inc. SAS User's Guide. version 6, 2nd ed. SAS Institute, Inc., Cary, N.C.; 1993.

Schopmeyer, C. S. Seeds of Woody Plants in the United States. Agric. Handbook No. 450:738-742; 1974.

Scott, D. H.; Draper, A. O. Light in relation to seed germination of blueberry, strawberry, and *Rubus*. HortScience 2:107-108; 1967.

Shchelkunova, S. E.; Popov, Y. G. Effect of growth regulator differentiation of isolated stem apexes of raspberry plants. Fiziol. Rast. 17:618-622; 1970.

Skene, K. G. M.; Barlass, M. Studies on the fragmented shoot apex of grapevine IV. Separation of phenotypes in a periclinal chimera *in vitro*. J. Exp. Bot. 34:1271-1280; 1983.

Skirvin, R. M.; Chu, M. C.; Gomez, E. *In vitro* propagation of thornless trailing blackberries. HortScience 16:310-312; 1981.

Slivinski, J. A.; Preece, J. E.; Myers, O. *In vitro* micropropagation of thornless blackberries utilizing single node explants. Plant Propagator 30:4-5; 1983.

Snir, I. Micropropagation of red raspberry. Sci. Hort. 14:139-143; 1981.

Soolova, V. A.; Kichira, V. V. Increasing the germination rate of raspberry seeds. Vestnik Sel Skok Hozyaistvennol Nanl. 16:87-90; 1971.

Sorvari, S.; Ulvinen, S.; Hietaranta, T.; Hiirsalmi, H. Preculture medium promotes direct shoot regeneration from micropropagated strawberry leaf disks. HortScience 28:55-57; 1993.

Sriskandarajah, S.; Skirvin, R. M.; Abu-Qaoud, H.; Korban, S. S. Factors involved in shoot elongation and growth of adventitious and axillary shoots of three apple scion cultivars *in vitro*. J. Hort. Sci. 65:113-121; 1990.

Stace-Smith, R. Tobacco streak virus in *Rubus*. pp. 235-239. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Stace-Smith, R.; Converse, R. H. Tomato ringspot virus in *Rubus*. pp. 223-227. In: Virus Diseases of Small Fruits. ed. Converse, R. H. United States Department of Agriculture, Agricultural Handbook No. 631, Washington D.C.; 1987.

Swartz, H. J.; Bors, R.; Mohamed, F.; Naess, S. K. The effect of pretreatment on subsequent shoot organogenesis from *Rubus* and *Malus* leaves. Plant Cell Tiss. Org. Cult. 21:179-184; 1990.

Tammisela, J.; Ryxjnänen, A. Incompatibility in *Rubus arctius* L.. Hereditas 66:269-278; 1970.

Taylor, P. W. J.; Ko, H. L.; Fraser, T. A.; Masel, N.; Adkins, S. W. Effect of silver nitrate on sugarcane cell suspension growth, protoplast isolation, ehylene production and shoot regeneration from cell suspension cultures. J. of Experimental Botany 45:1163-1168; 1994.

Thompson, M. M. Chromosome numbers of *Rubus* species at the National Clonal Germplasm Repository. HortScience 30:1447-1452; 1995a.

Thompson, M. M. Chromosome numbers of *Rubus* cultivars at the National Clonal Germplasm Repository. HortScience 30:1453-1456; 1995b.

Thompson, M. M. Survey of Chromosome numbers in *Rubus*. Annals of the Missouri Botanical Garden 84:128-165; 1997.

Tomanova, R. N. The effect of potassium iodide on raspberry seed germination and on physiological process in the seedlings. ObraZovania 42:169-171; 1971.

Turk, B. A.; Swartz, H. J.; Zimmerman, R. H. Adventitious shoot regeneration from *in vitro*-cultured leaves of *Rubus* genotypes. Plant Cell Tiss. Org. Cult. 38:11-17; 1994 (abst.).

Van der Salm, T. P. M.; Van der Toorn, C. J. G.; Hanisch ten Cate, C. H. Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tiss. Org. Cult. 37:73-77; 1994.

Van Nieuwkerk, J. P.; Zimmerman R. H.; Fordham, I. Thidiazuron stimulation of apple shoot proliferation *in vitro*. HortScience 21:516-518; 1986.

Walden, R.; Wingender, R. Gene transfer and plant regeneration techniques. Trends in biotechnology 13:324-331; 1995.

Wang, D.; Wergin, W. P.; Zimmerman, R. H. Somatic embryogenesis and plant regeneration from immature embryos of strawberry. HortScience 19:71-72; 1984.

Wang, G. Y.; Wang, G. H. Regeneration of blackberry plantlets from leaves and stem segments of *in vitro* plants. Acta Agriculturae Universitatis Pekinensis 21:12-16; 1995.

Zielinski, Q. B.; Thompson, M. M. Pollen germination in *Pyrus* species and species hybrids. Euphytica 15:195-198; 1966.

Zimmerman, R. H.; Bhardwaj, S. V.; Fordham, I. M. Use of starched-gelled medium for tissue culture of some fruit crops. Plant Cell Tiss. Org. Cult. 43:207-213; 1995.

Zimmerman, R. H.; Galletta, G. J.; Broome, O. C. Propagation of thornless blackberries by one-node cuttings. J. Amer. Soc. Hort. Sci. 105:405-407; 1980.

# APPENDICES

# APPENDIX A.

## IRON CHELATE SEQUESTRENE IMPROVES ADVENTITIOUS SHOOT PRODUCTION FROM WHOLE-LEAF EXPLANTS OF *RUBUS* CULTIVARS

## APPENDIX A-1

### PLOIDY LEVEL, CANE CHARACTERISTICS, AND ORIGIN OF THIRTEEN COMMERCIALLY IMPORTANT BLACKBERRY AND RASPBERRY CULTIVARS USED FOR LEAF REGENERATION TRIALS

| Cultivar name (accession)[a] | Species | Ploidy level | Cane characteristics | Origin |
|---|---|---|---|---|
| Cherokee (67.001) | *Rubus* hybrid | 4X | Erect, thorny | Arkansas |
| Chester Thornless (839.001) | *Rubus* hybrid | 4X | Semi-erect, thornless | Maryland |
| Hull Thornless (389.001) | *Rubus* hybrid | 4X | Semi-erect, thornless | Maryland |
| Kotata (992.001) | *Rubus* hybrid | 7X | Trailing, thorny | Oregon |
| Marion (385.001) | *Rubus* hybrid | 6X | Trailing, thorny | Oregon |
| Navaho (1115.001) | *Rubus* hybrid | 4X | Erect, thornless | Arkansas |
| Shawnee (836.001) | *Rubus* hybrid | 4X | Erect, thorny | Arkansas |
| Thornless Evergreen (991.001) | *R. laciniatus* Willd. | 4X | Trailing, thornless (L1 chimera) | Oregon |
| Waldo (1395.001) | *Rubus* hybrid | 6X | Trailing, thornless | Oregon |
| Autumn Bliss (856.001) | *R. idaeus* L. | 2X | Erect, thorny | New York |
| Heritage (990.001) | *R. idaeus* L. | 2X | Erect, thorny | New York |
| Latham (1200.001) | *R. idaeus* L. | 2X | Erect, thorny | Minnesota |
| Watson (Ruby™) (1129.001) | *R. idaeus* L. | 2X | Erect, thorny | New York |

[a] The number in parenthesis is the accession number of each cultivar at the National Clonal Germplasm Repository (NCGR) in Corvallis, OR.

APPENDIX A-2

ANALYSIS OF VARIANCE OF NUMBER OF REGENERANTS FROM HALF-LEAF EXPLANTS OF EIGHT BLACKBERRY CULTIVARS

| Source of variation | Half-leaf explant | |
|---|---|---|
| | D.F. | Mean square |
| Ploidy level | 2 | 10.17 *** |
| Cultivar (Ploidy) | 5 | 0.86 ns |
| IBA concentration | 2 | 6.91 *** |
| Cytokinin concentration | 7 | 4.02 *** |
| Ploidy X IBA | 4 | 1.16 ns |
| Ploidy X cytokinin | 14 | 2.26 *** |
| Cultivar (Ploidy) X IBA | 10 | 2.03 ** |
| Cultivar (Ploidy) X cytokinin | 35 | 0.50 ns |
| IBA X cytokinin | 14 | 0.55 ns |
| Error[a] | 98 | 0.66 |

ns, **, *** Non-significant or significant at $P \leq 0.01$ and 0.001.

[a] The three-interaction term was used as the error term for the analysis.

APPENDIX A-3

ANALYSIS OF VARIANCE OF COMPARING THE NUMBER OF REGENERANTS FROM HALF- AND WHOLE-LEAF EXPLANTS OF SEVEN BLACKBERRY CULTIVARS

| Source of variation | D.F. | Mean square |
|---|---|---|
| Cultivar | 6 | 6.79 *** |
| Explant type | 1 | 8.14 ** |
| IBA conc. | 2 | 10.84 *** |
| Cytokinin conc. | 7 | 10.48 *** |
| Cultivar X explant | 6 | 0.71 ns |
| Cultivar X IBA | 12 | 1.71 * |
| Cultivar X cytokinin | 42 | 2.30 *** |
| Explant X IBA | 2 | 2.45 ** |
| Explant X cytokinin | 7 | 1.69 ** |
| IBA X cytokinin | 9[a] | 0.74 ns |
| Error[b] | 108 | 0.91 |

ns, *, **, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001.
[a] Five treatments used for half-leaf explants were not tested on whole-leaf explants (D.F. = 9 instead of 14).
[b] The four-variable-interaction term was used as the error term for the analysis.

APPENDIX A-4

ANALYSIS OF VARIANCE OF THE EFFECT OF THREE GELLING AGENTS IN THE REGENERATION MEDIUM[a] ON *RUBUS* WHOLE-LEAF EXPLANTS

| Source of variation | D.F. | Mean square[b] | | |
|---|---|---|---|---|
| | | Regeneration (%) | Shoots per explant | Callus index[c] |
| Cultivar | 2 | 1058.33 *** | 34.62 * | 23.29 *** |
| Agent | 2 | 2.78 ns | 2.25 ns | 0.13 ns |
| Treatment | 3 | 343.21 * | 23.99 ns | 25.25 *** |
| Cultivar X agent | 4 | 52.78 ns | 11.44 ns | 3.05 *** |
| Cultivar X treatment | 6 | 90.43 ns | 14.69 ns | 3.26 *** |
| Agent X treatment | 6 | 238.58 * | 27.45 * | 1.67 *** |
| Cultivar X agent X treatment | 12 | 183.02 ns | 17.02 ns | 1.54 *** |
| Error | 72 | 101.85 | 9.86 | 0.22 |

[a] The NCGR-RUB medium with 2.9 µM $GA_3$, 4.45 µM BA, and 0.49 µM IBA.
[b] ns, *, *** Non-significant or significant at $P \leq 0.05$ and 0.001.
[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

APPENDIX A-5

ANALYSIS OF VARIANCE OF THE EFFECT OF SEQUESTRENE IN THE REGENERATION MEDIUM[a] ON *RUBUS* WHOLE-LEAF EXPLANTS

| Source of variation | D.F. | Mean square[b] | | |
|---|---|---|---|---|
| | | Regeneration (%) | Shoots per explant | Callus index[c] |
| Cultivar | 1 | 168.75 ns | 11.07 * | 1.30 ns |
| IBA conc. | 1 | 2552.08 *** | 27.68 ** | 38.34 *** |
| Sequestrene | 3 | 246.53 ns | 1.67 ns | 0.42 ns |
| Cultivar X IBA conc. | 1 | 168.75 ns | 0.38 ns | 0.01 ns |
| Cultivar X sequestrene | 3 | 146.53 ns | 4.01 ns | 0.51 ns |
| IBA conc. X sequestrene | 3 | 240.97 ns | 2.61 ns | 0.45 ns |
| Cultivar X IBA conc. X sequestrene | 3 | 368.75 ns | 1.14 ns | 0.33 ns |
| Error | 32 | 129.17 | 2.53 | 0.43 |

[a] The NCGR-RUB medium with 2.9 µM $GA_3$, BA, IBA, and solidified by o.19% Gelrite.

[b] ns, *, **, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001.

[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

APPENDIX A-6

CONTRASTS OF THE EFFECT OF SEQUESTRENE TREATMENTS ON THE OF REGENERATION RESPONSE OF *RUBUS* WHOLE-LEAF EXPLANTS

| Contrast of sequestrene treatment[b] | D.F. | Mean square[a] | | |
|---|---|---|---|---|
| | | Regeneration (%) | Shoots per explant | Callus index[c] |
| oo-os | 1 | 704.17 * | 0.76 ns | 0.02 ns |
| oo-so | 1 | 150.00 ns | 4.52 ns | 0.02 ns |
| oo-ss | 1 | 66.67 ns | 0.20 ns | 1.00 ns |
| os-so | 1 | 204.17 ns | 1.58 ns | 0 ns |
| os-ss | 1 | 337.50 ns | 0.19 ns | 0.74 ns |
| so-ss | 1 | 16.67 ns | 2.81 ns | 0.74 ns |

[a] ns, * Non-significant or significant at $P \leq 0.05$.

[b] Addition of sequestrene [with (s) or without (o)] in the regeneration medium (RM). oo = no sequestrene in either RM and ss = sequestrene in both RM.

[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

## APPENDIX A-7

RESPONSE OF 'KOTATA' AND 'MARION' BLACKBERRIES ON REGENERATION MEDIUM[a] WITH IBA AND WITH OR WITHOUT SEQUESTRENE

| IBA conc. (µM) | Least square means[b] | | | | | |
|---|---|---|---|---|---|---|
| | Regeneration (%) | | Shoots per explant | | Callus index[c] | |
| | Kotata | Marion | Kotata | Marion | Kotata | Marion |
| 0 | 26.67 a | 34.17 a | 3.51 a | 2.73 b | 2.93 b | 2.58 b |
| 0.5 | 15.83 b | 15.83 b | 2.17 bc | 1.03 c | 4.70 a | 4.39 a |

[a] The NCGR-RUB medium with 2.9 µM $GA_3$, BA, IBA, and solidified by o.19% Gelrite.

[b] Means with the same letter are not significantly different at $P \leq 0.05$.

[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

APPENDIX A-8

A COMPARISON OF THE PERCENTAGE OF REGENERATION FROM THREE REGENERATION EXPERIMENTS WITH 'KOTATA' AND 'MARION' BLACKBERRIES

| Experiment | Regeneration (%)[a] | | |
|---|---|---|---|
| | All (n)[b] | Kotata | Marion |
| Leaf regeneration screening | | | |
| Half leaf | 3.33 (8) | 8.33 | 0 |
| Whole leaf | 8.13 (12) | 26.67 | 6.67 |
| Gelling agent test | | | |
| Agar-Gelrite combined medium | 14.44 (3) | 10.00 | 30.00 |
| Gelrite medium | 14.44 (3) | 10.00 | 26.67 |
| Sequestrene test | | | |
| oo (no sequestrene as a control) | 26.67 (2) | 23.33 | 30.00 |
| os (with sequestrene in the RM 2) | 41.67 (2) | 43.33 | 40.00 |

[a] The percentage of regeneration means the number of explant producing shoots divided by the number of explants.
[b] (n) total number of cultivars tested.

APPENDIX A-9

ANALYSIS OF VARIANCE OF THE EFFECT OF SILVER NITRATE IN THE REGENERATION MEDIUM[a] ON *RUBUS* WHOLE-LEAF EXPLANTS

| Source of variation | D.F. | Mean square[b] | | |
|---|---|---|---|---|
| | | Regeneration (%) | Shoots per explant | Callus index[c] |
| Cultivar | 3 | 2568.06 *** | 40.62 *** | 3.97 *** |
| IBA conc. | 1 | 16.67 ns | 8.27 ns | 68.85 *** |
| Silver nitrate | 3 | 181.94 ns | 11.17 ns | 2.38 *** |
| Cultivar X IBA conc. | 3 | 119.44 ns | 7.94 ns | 4.35 *** |
| Cultivar X silver nitrate | 9 | 64.35 ns | 5.56 ns | 0.42 ns |
| IBA conc. X silver nitrate | 3 | 11.11 ns | 4.02 ns | 0.21 ns |
| Cultivar X IBA conc. X silver nitrate | 9 | 54.63 ns | 3.24 ns | 0.87 *** |
| Error | 64 | 110.42 | 4.16 | 0.23 |

[a] The NCGR-RUB medium with 2.9 µM $GA_3$, BA, IBA, and solidified by 0.19% Gelrite.
[b] ns, *** Non-significant or significant at $P \leq 0.001$.
[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

APPENDIX A-10

THE EFFECT OF IBA CONCENTRATION ON THE REGENERATION OF FOUR BLACKBERRY CULTIVARS ON REGENERATION MEDIUM[a] WITH SILVER NITRATE

| Cultivar | % Regeneration[b] | | Shoots per explant[c] | | Callus index[d] | |
|---|---|---|---|---|---|---|
| | IBA 0 µM | IBA 0.5 µM | IBA 0 µM | IBA 0.5 µM | IBA 0 µM | IBA 0.5 µM |
| Chester Thornless | 1.67 y | 8.33 y | 0.33 w | 1.46 wxy | 2.18 v | 3.35 x |
| Kotata | 25.00 z | 25.00 z | 4.00 z | 2.87 yz | 2.78 w | 4.40 z |
| Marion | 6.67 y | 7.50 y | 2.22 xy | 0.88 wx | 1.28 u | 4.20 yz |
| Navaho | 4.17 y | 0 y | 1.00 wx | 0 w | 2.30 v | 3.36 x |

[a] The NCGR-RUB medium with 2.9 µM $GA_3$, 10 µM BA, and solidified by 0.19% Gelrite.

[b] n = 30 whole-leaf explants. % regeneration means with the same letter are not significantly different at $P \leq 0.05$.

[c] Shoots per explant means with the same letter are not significantly different at $P \leq 0.05$.

[d] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface). Callus index means with the same letter are not significantly different at $P \leq 0.05$.

APPENDIX A-11

RESPONSE OF FOUR BLACKBERRY CULTIVARS TO SILVER NITRATE IN THE REGENERATION MEDIUM[a]

| Cultivar | Means[b] | | |
|---|---|---|---|
| | Regeneration (%) | No. of shoots / regenerating explant | Callus index[c] |
| Chester Thornless | 5.00 y | 0.90 y | 2.76 y |
| Kotata | 25.00 z | 3.44 z | 3.59 z |
| Marion | 7.08 y | 1.55 y | 2.74 y |
| Navaho | 2.08 y | 0.50 y | 2.83 y |

[a] The NCGR-RUB medium with silver nitrate in either the first or second, or both media was solidified by 0.19% Gelrite.
[b] n = 30 whole leaf explants. Means with the same letter in a column are not significantly different at $P \leq 0.05$.
[c] Callus index ranged from 0 (no callus) to 5 (81 to 100% callus on the leaf surface).

APPENDIX A-12

RESPONSE OF WHOLE BLACKBERRY-LEAF EXPLANTS TO SILVER NITRATE IN THE REGENERATION MEDIUM (RM)

| RM[a] | | | Means[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | Regeneration (%) | | | | Callus index[c] |
| IBA conc. (μM) | RM 1 | RM 2 | Chester Thornless | Kotata | Marion | Navaho | |
| 0 | + | + | 3.33 | 20.00 | 3.33 | 3.33 | 1.89 v[c] |
| | + | - | 0 | 26.67 | 3.33 | 0 | 1.98 v |
| | - | + | 3.33 | 26.67 | 13.33 | 6.67 | 2.20 vw |
| | - | - | 0 | 26.67 | 6.67 | 6.67 | 2.47 w |
| 0.5 | + | + | 0 | 26.67 | 0 | 0 | 3.32 x |
| | + | - | 6.67 | 26.67 | 0 | 0 | 3.75 y |
| | - | + | 16.67 | 20.00 | 16.67 | 0 | 4.05 yz |
| | - | - | 10.00 | 26.67 | 13.33 | 0 | 4.19 z |
| | | | 5.00 y | 25.00 z | 7.08 y | 2.08 y | |

[a] The NCGR-RUB medium with 2.9 μM $GA_3$, 10 μM BA, and solidified by 0.19% Gelrite. + or – silver nitrate (10 mg per l).
[b] n = 30 whole-leaf explants. Means with the same letter in a row are not significantly different at $P \leq 0.05$.
[c] Callus index means with the same letter in a column are not significant different. Callus index ranged from 0 (no callus) to 5 (81 to 100% callus formed on the leaf surface).

A–13. *Rubus* leaf regeneration treatments on agar-Gelrite regeneration medium with combinations of IBA, and either BA or TDZ.

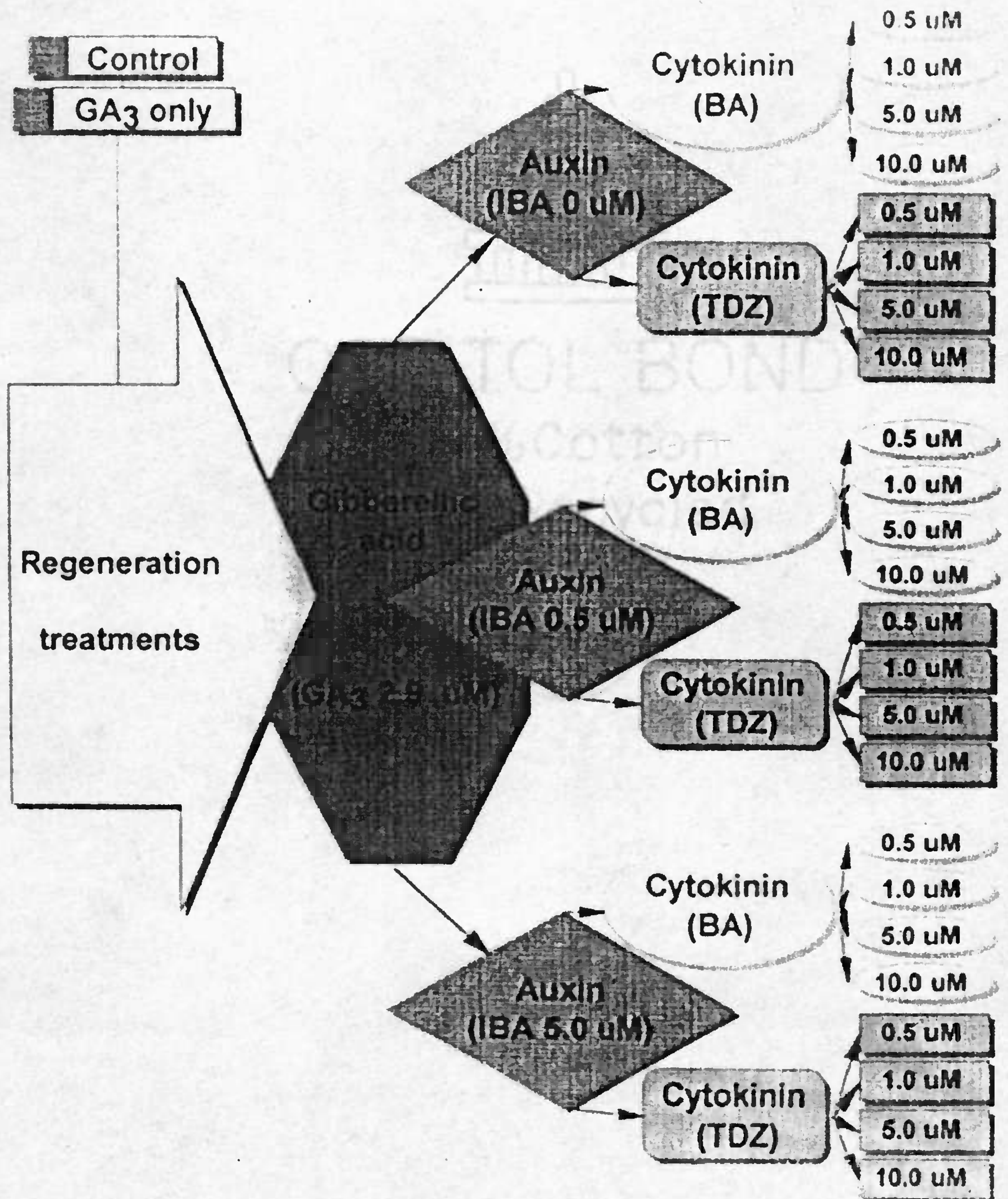
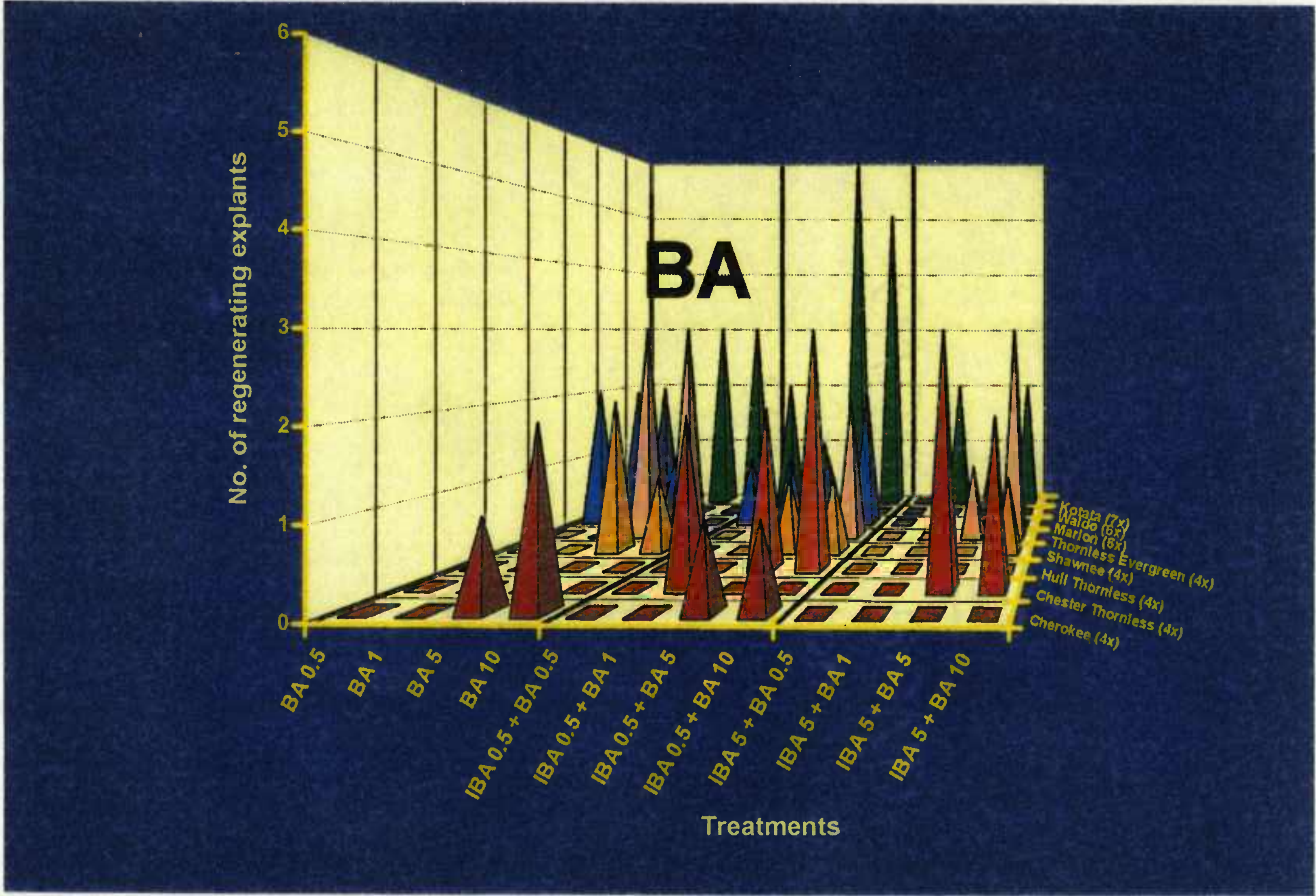



Fig. A-13

A–14. The number of half-leaf explants of eight blackberry cultivars that produced shoots on regeneration medium with growth regulators IBA and either *A*. BA. or *B*. TDZ.

Fig. A-14A.



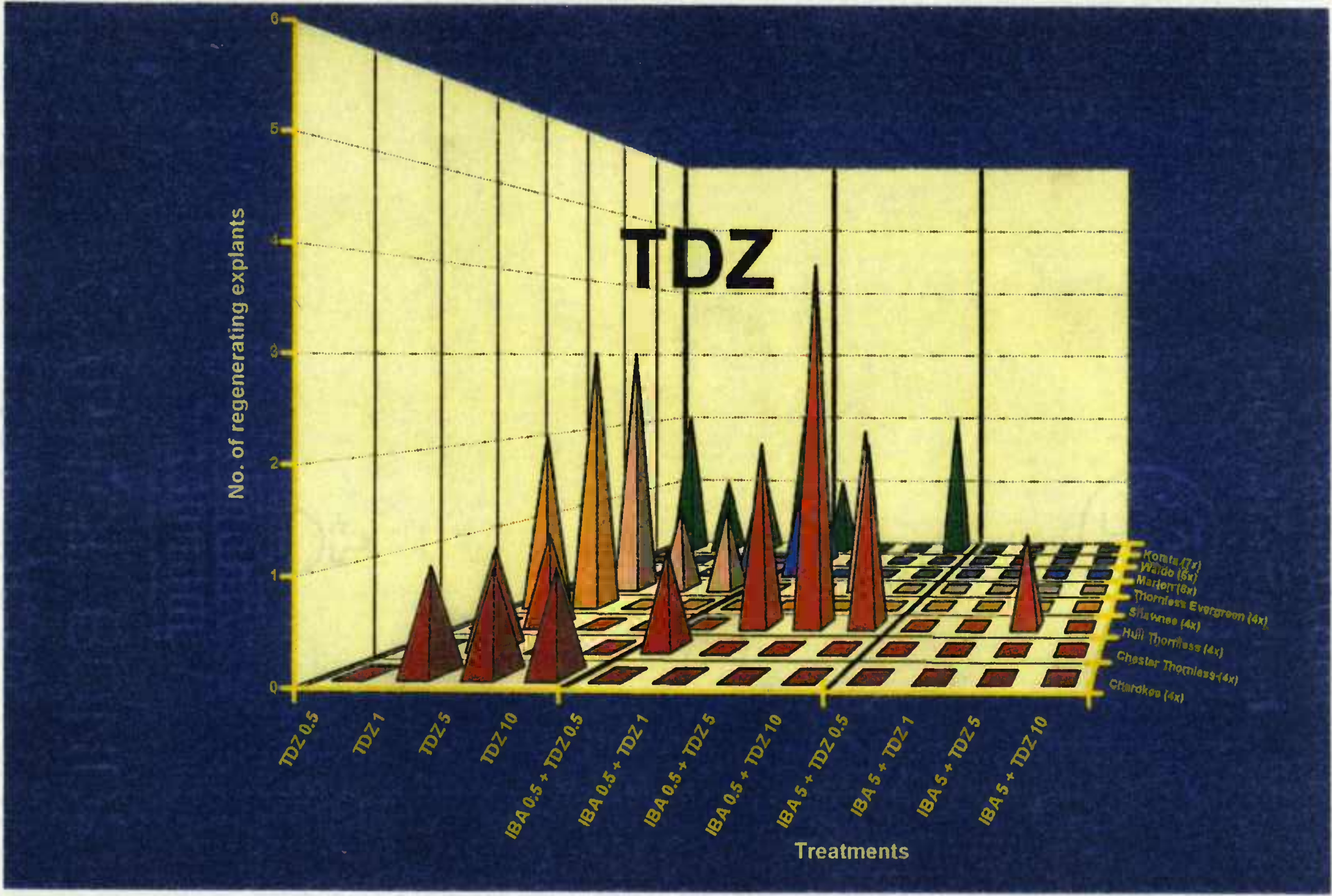



Fig. A-14B.

# APPENDIX B.

## VIRUS INFECTIONS REDUCE *IN VITRO* MULTIPLICATION OF 'MALLING LANDMARK' RASPBERRY

B-1. The mean number of shoots produced on single- or multi-virus infected 'Malling Landmark' raspberry cultures after 17 months in culture (the fourth culture period). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom).

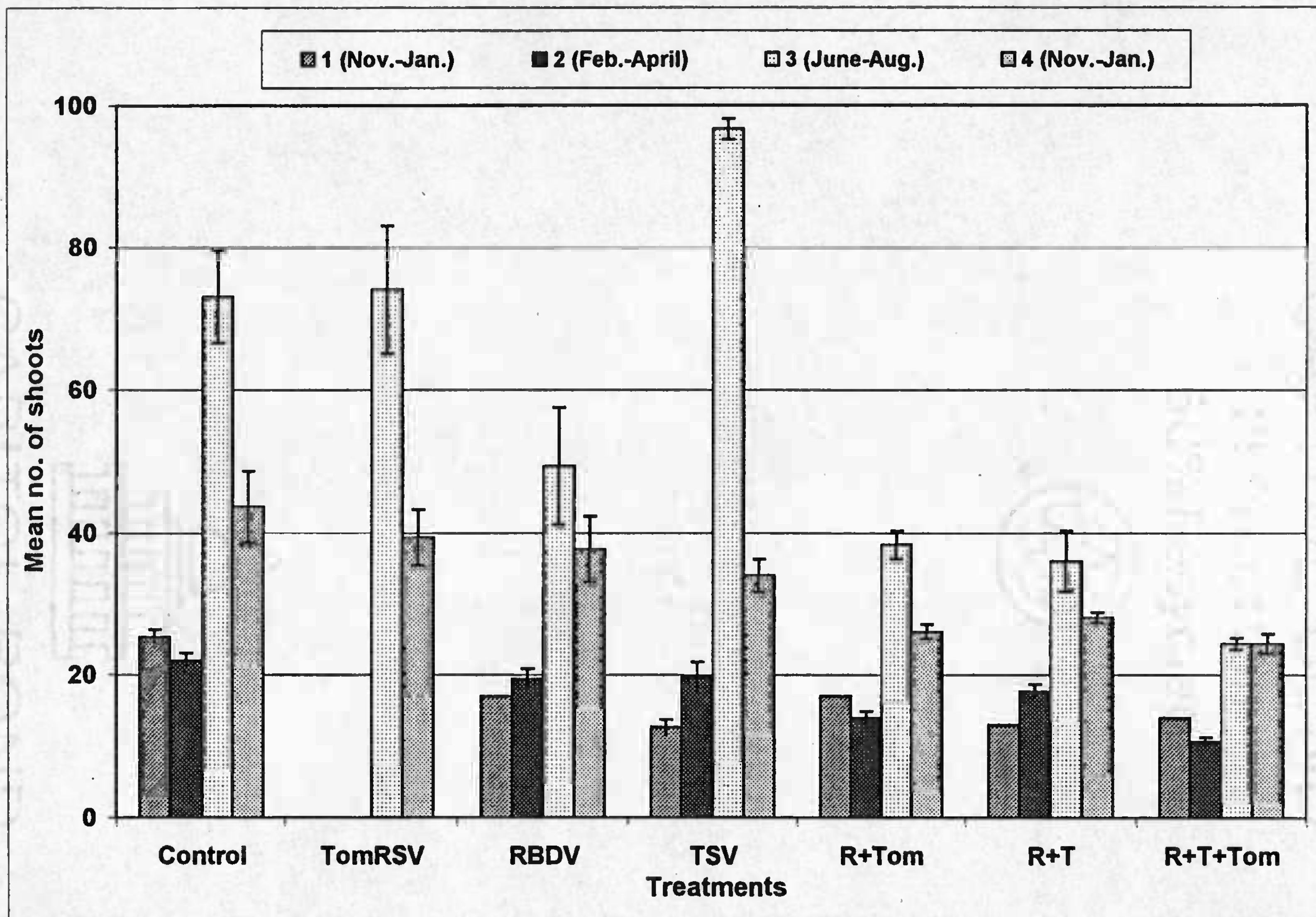



Fig. B-1

B-2. The multiplication of virus-infected 'Malling Landmark' raspberry over four data collection times in the fourth culture period (the 17th to 19th month in culture). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom).

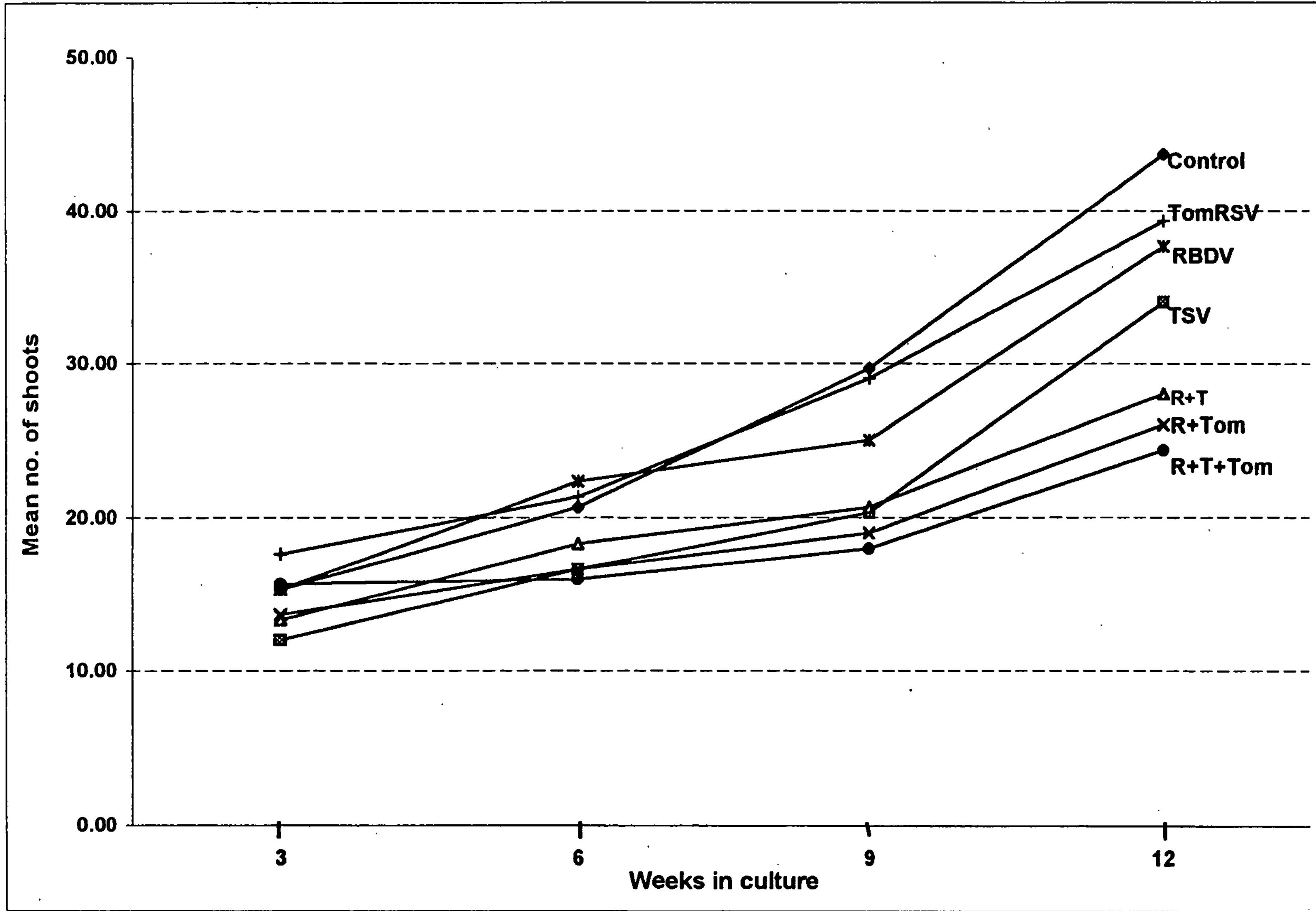



Fig. B-2

B-3. The mean height of virus infected 'Malling Landmark' raspberry at the third culture period (the 13$^{th}$ to 15$^{th}$ month in culture). Raspberry bushy dwarf virus (RBDV), Tomato ringspot virus (TomRSV), and tobacco streak virus (TSV), RBDV + TSV (R + T), RBDV + TomRSV (R + Tom), RBDV + TSV + TomRSV (R + T + Tom).

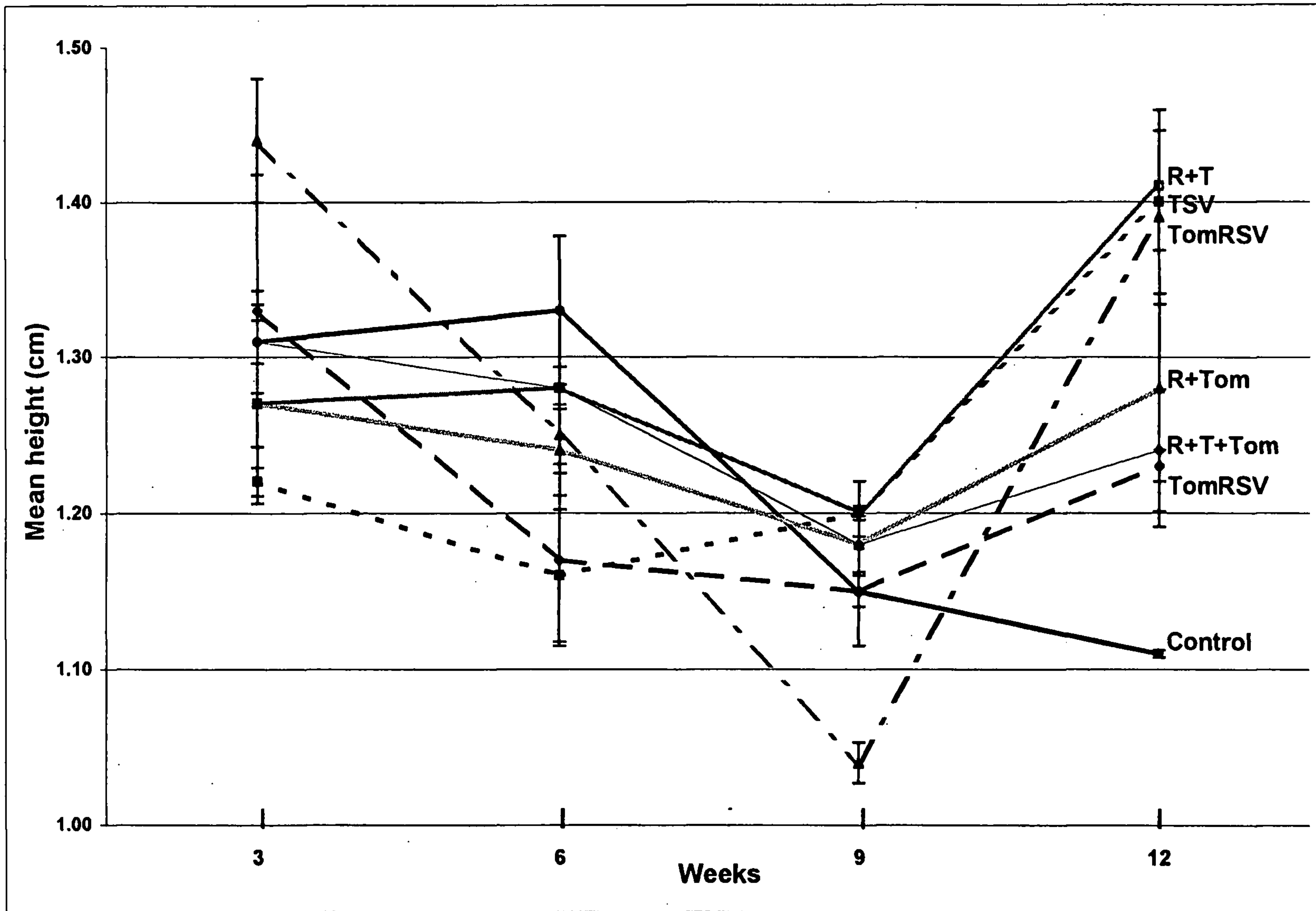



Fig. B-3