

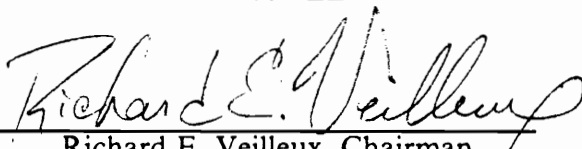
De Novo Morphogenesis on Tomato Thin Cell Layers and Variation for Genetic
Recombination Among Plantlets Regenerated from Tissue Culture

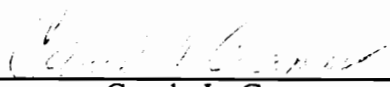
by

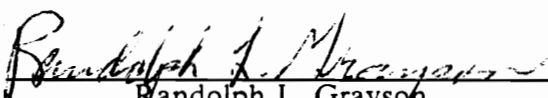
Michael E. Compton

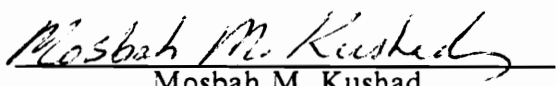
Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Horticulture

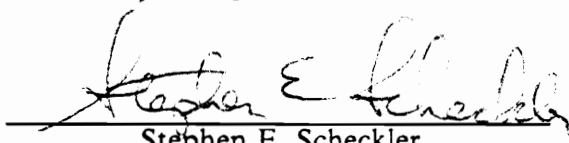
APPROVED:


Richard E. Veilleux, Chairman


Carole L. Cramer


Randolph L. Grayson


Mosbah M. Kushad


Stephen E. Scheckler

May, 1990

Blacksburg, Virginia

De Novo Morphogenesis on Tomato Thin Cell Layers and Variation for Genetic Recombination Among Plantlets Regenerated from Tissue Culture

by

Michael E. Compton

Richard E. Veilleux, Chairman

Horticulture

(ABSTRACT)

De novo shoots, roots, and flower buds were regenerated on thin cell layer explants excised from pedicel tissue of tomato. Direct shoot organogenesis was greatest when media contained 10 μ M kinetin and 0.001 μ M indole-3-acetic acid (IAA); however, shoot regeneration was increased in subsequent experiments by substituting 10 μ M zeatin or 10 μ M benzyladenine for kinetin. Root formation occurred when media contained higher (0.1 and 10 μ M) auxin concentrations. Flowers were formed on elongated shoots with several leaves when media contained 10 μ M IAA and 0.1 μ M kinetin.

Competence for *de novo* shoot morphogenesis was tested on thin cell layers of eleven tomato cultivars. All tomato cultivars formed shoots directly on thin cell layer explants at varying frequencies (29%-63%). The mean number of shoots per explant was greatest for 'Large Red Cherry', 'Ohio 7814' and 'BL 6807', and poorest for 'Campbell 1327' and 'Red Alert'. Active cell divisions were observed in subepidermal cells during the first week of culture, and meristematic centers of dividing cells were evident after 2 weeks. Well developed shoot apices were observed on 50% of the explants 4 weeks after culture initiation.

Shoot morphogenesis was compared among tomato plants placed into micropropagation, callus, and thin cell layer tissue culture systems. More shoots were produced on thin cell layer explants than on cotyledon calli, or micropropagated shoot tips. Genetic recombination rates and map distances were compared among hybrid plants grown in the greenhouse and regenerated from the aforementioned tissue culture systems. Increased recombination rates and map distances were detected between the *sunny* (*sy*) and *baby leaf syndrome* (*bls*) genes on chromosome 3, and between the *white virescence* (*wv*) and *anthocyanin reduced* (*are*) genes on chromosome 2. The percent change in the former ranged from 4.5%-5.9% for micropropagated shoot tips, 3.7%-8.5% for plants from cotyledon calli and 2.8%-5.9% for plants from thin cell layers. The percent change between the *wv* and *are* loci ranged from 4.5%-6.1% for micropropagated shoot tips, and 3.2%-5.0% and 3.9%-5.7% for plants from cotyledon calli and thin cell layers, respectively. Conversely, a decreased map distance was observed between *bls* and the *solanifolia* (*sf*) locus which is more distal to the centromere on the same arm of chromosome 3 as *bls*. Changes in recombination rates among plants regenerated from tissue culture may result from an influence of the tissue culture process on meiosis of regenerated plants.

Acknowledgements

First of all, I would like to express my deepest appreciation to Drs. John E. Preece and Richard E. Veilleux for their generous support and friendly advice during my years at Southern Illinois University and Virginia Tech, respectively. I would also like to express my gratitude to Richard Veilleux for allowing me the freedom to conduct a research project of my own interest that was not directly in line with his own research objectives. These men are truly the best advisors that a person could possibly have.

I would also like to thank Drs. Carole Cramer, Randolph Grayson, Mosbah Kushad and Stephen Scheckler for their contributions and for serving on my graduate committee.

A special thanks to Suzanne Piovano for her help in the laboratory and greenhouse. She spent endless hours helping me wash culture tubes, and was always willing to give me a hand with any task. In addition, I would like to thank Jody Booze-Daniels, Kathy Bourke and Amelia Walton for their technical assistance in tissue sectioning and staining experiments.

I would also like to thank my parents, Jim and Carol Compton, for their support and encouragement. Mom, I cannot thank you enough for your patience.

Finally, I would like to dedicate this work in memory of my good friend Mitch Kinney who lost his battle with Leukemia in 1987. Mitch, your courage has helped inspire me to do my best.

Table of Contents

Chapter One: Introduction 1

Literature Cited 4

Chapter Two: Factors that Influence Thin Cell Layer Morphogenesis 6

Introduction 6

Literature Review 8

De Novo Floral Bud Production 9

 Explant Source 9

 Explant Structure 12

 Auxin 13

 Anti-auxins 15

 Cytokinin 15

 Anti-cytokinins 17

 Gibberellins 17

 Polyamines 18

 Other Growth Promoting Substances 20

Carbon Source	20
Physical State of the Medium	21
Light	21
Genotypic Influences	21
De Novo Shoot Production	24
Summary	26
Literature Cited	27
 Chapter Three: Shoot, Root and Flower Morphogenesis on Tomato Thin Cell Layers	36
Abstract	36
Introduction	37
Material and Methods	40
Effects of auxin on thin cell layer morphogenesis	40
Surface-sterilization	41
Effects of zeatin, benzyladenine, kinetin and 2iP on shoot morphogenesis	42
Shoot morphogenic competence among tomato cultivars	43
Histological development of tomato thin cell layers	44
Results and Discussion	45
Effects of auxin on thin cell layer morphogenesis	45
Surface-sterilization	46
Effects of zeatin, BA, kinetin and 2iP on shoot morphogenesis	47
Shoot morphogenic competence among tomato cultivars	47
Histological development of tomato thin cell layers	48
Summary	50
Literature Cited	52
 Chapter Four: Variation for Genetic Recombination Among Tomato Plants Regenerated from Three Tissue Culture Systems	72

Abstract	72
Introduction	73
Materials and Methods	77
Plant Material	77
Micropropagation	78
Callus culture	79
Thin cell layers	80
Seed set in F ₁ hybrids backcrossed to mutant parents	81
Mitotic Recombination	82
Map Distance Between Selected Marker Loci	82
Results and Discussion	83
Tissue Culture Treatments	83
Micropropagation	83
Callus culture	83
Thin cell layers	84
Seed set for F ₁ hybrids backcrossed to mutant parents	85
Mitotic recombination	86
Map Distance Between Selected Marker Loci.	87
Summary	91
Literature Cited	94

Appendix A. Shoot regeneration from cotyledon calli of Large Red Cherry and Red Alert on two different media	114
---	------------

Appendix B. Chi-square analysis of recombination rates among tomato hybrids regenerated from tissue culture	116
--	------------

Appendix C. Chemical names and abbreviations	127
Vita	129

List of Illustrations

Figure 1.	Floral morphogenesis on tomato ('Red Alert') thin cell layer explants from pedicel tissue	61
Figure 2.	Longitudinal section of a freshly isolated tomato 'Ohio 7814' thin cell layer explant derived from pedicel tissue	62
Figure 3.	Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 7 days after culture initiation	63
Figure 4.	Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 7 days after culture initiation	64
Figure 5.	Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation	65
Figure 6.	Longitudinal section through a meristematic center of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation	66
Figure 7.	Longitudinal section through a meristematic center of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation	67
Figure 8.	Transverse section of a meristematic region of a tomato 'Ohio 7814' thin cell layer explant 21 days after culture initiation	68
Figure 9.	Longitudinal section through a shoot tip present on a tomato 'Ohio 7814' thin cell layer explant 28 days after culture initiation	69
Figure 10.	Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 28 days after culture iniation	70
Figure 11.	Shoot morphogenesis on a tomato 'Ohio 7814' thin cell layer explant 8 wks after culture initiation (20×).	71
Figure 12.	Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of Red Alert, LA 1430, LA 1526 and their reciprocal F ₁ hybrids.	108

Figure 13. Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of Large Red Cherry, LA 1430, LA 1526 and their reciprocal F_1 hybrids.	109
Figure 14. Map distance between marker loci of LA 1430 F_1 hybrid plants grown from seed and regenerated from micropropagation, cotyledon calli and thin cell layers	110
Figure 15. Map distance between marker loci of Red Alert $\times$ LA 1430 F_1 hybrid plants grown from seed and regenerated from cotyledon calli	111
Figure 16. Map distance between selected marker loci of LA 1430 $\times$ LRC F_1 hybrid plants grown from seed and regenerated from cotyledon calli	112
Figure 17. Map distance between marker loci of LA 1526 F_1 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers	113

List of Tables

Table 1.	Response of tobacco thin cell layer explants to media containing different auxins, cytokinins, gibberellin and polyamines.	32
Table 2.	Nicotiana genotypes capable of flower formation on thin cell layer explants.	34
Table 3.	Names and sources of tomato cultivars used in thin cell layer shoot morphogenic competence experiments.	55
Table 4.	Morphogenic response of tomato thin cell layers from pedicel and peduncle tissue to media containing different levels of kinetin, IAA, IBA and NAA.	56
Table 5.	Shoot and root formation on tomato thin cell layer explants from pedicel tissue as influenced by different concentrations of kinetin, IAA, IBA and NAA.	57
Table 6.	Flower production and explant quality ratings for tomato thin cell layer explants from pedicel and peduncle tissue as influenced by eight different surface-sterilization treatments.	58
Table 7.	Shoot organogenesis on thin cell layer explants from tomato pedicel tissue on media containing 1nM IAA and 10 μ M of either zeatin, BA, kinetin or 2iP.	59
Table 8.	Mean shoot number and the frequency of responding explants among thin cell layers explants from pedicel tissue of 11 tomato cultivars.	60
Table 9.	Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of 12 tomato genetic lines.	98
Table 10.	Seed set for F ₁ hybrids derived by four propagation methods and used as female parents in backcrosses with LA 1430 and LA 1526.	99
Table 11.	Recombination between marker loci of Red Alert $\times$ LA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	100

Table 12. Recombination between marker loci of LA 1430×Red Alert hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	101
Table 13. Recombination between marker loci of LRC×LA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	102
Table 14. Recombination between marker loci of LA 1430×LRC hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	103
Table 15. Recombination between marker loci of cultivars×LA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	104
Table 16. Recombination between marker loci among 5 individual Red Alert×LA 1430 hybrid plants regenerated from cotyledon calli.	105
Table 17. Recombination between marker loci among 3 individual LA 1430×Large Red Cherry plants regenerated from cotyledon calli.	106
Table 18. Recombination between marker loci of tomato cultivars×LA 1526 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	107
Table 19. Shoot regeneration from cotyledon calli of Large Red Cherry and Red Alert on two different media.	115
Table 20. Recombination between marker loci of hybrids between Red Alert and LA 1430 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	117
Table 21. Recombination between marker loci of hybrids between Large Red Cherry and LA 1430 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	118
Table 22. Recombination between marker loci of F ₁ hybrids between cultivars and LA 1430 grown seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	119
Table 23. Recombination between marker loci of Red Alert×LA 1526 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	120
Table 24. Recombination between marker loci of LA 1526×Red Alert hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	121

Table 25. Recombination between marker loci of LRCxLA 1526 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	122
Table 26. Recombination between marker loci of LA 1526xLRC hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	123
Table 27. Recombination between marker loci of Red Alert and LA 1526 hybrids grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	124
Table 28. Recombination between marker loci of Large Red Cherry and LA 1526 hybrids grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	125
Table 29. Recombination between marker loci of hybrids between tomato cultivars and LA 1526 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.	126
Table 30. List of chemical names and abbreviations.	128

Chapter One: Introduction

While discussing my research intentions with friends outside the field of horticulture, one question ineffably arises, "Why tomato?" The first time I encountered this question my response was; "Because tomato is an important horticultural crop." Being completely dissatisfied with this response, I decided to search for more valid answers.

The tomato is indeed an important horticultural crop. In 1986, 158,799 hectares were contracted for tomato production in the US (USDA 1986). The yield for fresh market tomatoes was 1,424,360 metric tons and 7,386,035.07 metric tons for processing. Total crop production was greater for tomato (8,810,395 metric tons) than for sweet corn (2,293,077 metric tons) or peas (375,117 metric tons). The monetary value of the 1986 tomato crop was more than \$1.4 billion. Florida produced 50.4% of the fresh market tomatoes (15,830,000 cwt¹) valued at \$490,730,000, and California produced 88% of the processing tomatoes (5,877,650.24 metric tons) valued at \$403,076,000.

¹ hundred weight

The tomato also has several attributes which make it an exceptional plant for genetic study and tissue culture research (Hille et al. 1989). Tomatoes are basically diploid in nature with few triploid or polyploid species (Stevens and Rick 1986). The cultivated tomato is also a true breeding species which makes it attractive for breeding purposes. Linkage maps of physiological, enzymatic (allozymes), and restriction fragment length polymorphism (RFLP) loci are readily available, and a wide variety of genetic lines (including mutants) can be obtained from the Tomato Genetics Cooperative in Davis, California. Mutant types include variations in leaf morphology and physiology, flower shape and morphology, fruit shape and color (degree of fruit ripening), male-sterility, anthocyanin production, and plant height.

The availability of wild species is also attractive for the incorporation of disease and nematode resistance, increased water use efficiency, increased cold tolerance, and increased soluble solids. However, the ability to incorporate these traits through sexual crosses has been difficult at times because of incompatibility barriers that exist between *Lycopersicon esculentum* and wild *Lycopersicon* species in the *peruvianum* complex (Steven and Rick 1986). The development and use of tissue culture techniques such as embryo culture (Smith 1944) has helped to traverse some of these barriers, but much wild germplasm is still unobtainable because of sexual incompatibility barriers.

The tomato has been actively researched from a tissue culture stand point with successful shoot regeneration occurring from cotyledon (Sibi et al. 1984) and leaf explants (Evans and Sharp 1983; Padmanabhan et al. 1974), and leaf mesophyll protoplasts (Shahin 1984; Neidez et al. 1985; Tan et al. 1987). Tomato cell cultures have also taken a short trip into space (Schutae et al. 1987).

Genetic transformation has been successful in tomato by cocultivation of leaf discs with *Agrobacterium tumefaciens* (Horsch et al. 1985). Tobacco mosaic virus (TMV) resistance has recently been incorporated into tomato in a similar fashion. Nelson et al. (1988) have incorporated a gene which codes for the TMV virus coat protein into the tomato genome via recombination during cocultivation of tomato leaf disc with *Agrobacterium*. The transformed plants expressed the TMV coat protein and were less susceptible (26-35%) to viral infection. The expression of this gene had no influence on tomato growth or yield of plants grown in the greenhouse or field.

Even with this illustrious background, the regeneration of tomato organs from thin cell layers has yet to be reported. By developing a system which would allow *de novo* flower regeneration from tomato thin cell layers, greater insights into tomato flower initiation and development may be achieved. In the same right, developing techniques for *de novo* shoot formation on tomato thin cell layers may offer a more efficient shoot regeneration system for genetic transformation similar to that developed by Charest et al. (1988) for *Brassica napus*. Therefore, the major emphasis of this research was to develop techniques which would promote the regeneration of *de novo* floral buds and shoots on tomato thin cell layers.

Literature Cited

- Charest, P.J., L.A. Holbrook, J. Gabard, V.N. Iyer and B.L. Miki. 1988. *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. Theor. Appl. Genet. 75:438-445.
- Evans, D. and W. Sharp. 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science 221:949-951.
- Hille, J., M. Koornneef, M.S. Ramanna, and P. Zabel. 1989. Tomato: a crop species amenable to improvement by cellular and molecular methods. Euphytica 42:1-23.
- Horsch, R.B., J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers and R.T. Fraley. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.
- Nelson, R.S., S.M. McCormick, X. Delannay, P. Dubé, J. Layton, E.J. Anderson, M. Kaniewska, R.K. Proksch, R.B. Horsch, S.G. Rogers, R.T. Fraley and R.N. Beachy. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Bio/Technology 6:403-409.
- Niedz, R., S. Rutter, L. Handley and K. Sink. 1985. Plant regeneration from leaf protoplasts of six tomato cultivars. Plant Sci. 39:199-204.
- Padmanabhan, V., E. Paddock and W. Sharp. 1974. Plantlet formation from *Lycopersicon esculentum* leaf callus. Can. J. Bot. 52:1429-1432.
- Schutae, R., H. Keike, W. Malz and M. Tairbekov. 1987. Callus and shoot formation in cell cultures of *Lycopersicon esculentum* Mill. after a 7-day space flight in the Soviet Biosatellite "Kosmos 1667". Biochem. Physiol. Pflanzen. 182:367-374.
- Shahin, E.A. 1984. Isolation and culture of protoplasts: tomato. In: Vasil, I.K. (ed.). Cell culture and somatic cell genetics of plants, Vol. 1., Academic Press, Inc., Orlando. pp. 370-380.
- Sibi, M., M. Biglary and Y. Demarly. 1984. Increase in the rate of recombinants in tomato (*Lycopersicon esculentum* L.) after *in vitro* regeneration. Theor. Appl. Genet. 68:317-321.
- Smith, P.G. 1944. Embryo culture of a tomato species hybrid. Proc. Amer. Soc. Hort. Sci. 44:413-416.
- Stevens, M.A. and C.M. Rick. 1986. Genetics and breeding. In: Atherton, J.G. and J. Rudich (eds.). The tomato crop a basis for improvement. Chapman and Hall, Ltd., London. pp. 35-109.

Tan, M., E. Rietveld, G. van Marrewijk and A. Kool. 1987. Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and plant regeneration. Plant Cell Reports 6:172-175.

USDA Agricultural Statistics. 1986. United States Government Printing, Washington, D.C.

Chapter Two: Factors that Influence Thin Cell Layer Morphogenesis

Introduction

One purpose of plant tissue culture has been to propagate or clone large numbers of superior plant species more rapidly than by traditional propagation methods. This is generally done by utilizing meristems, buds or shoot tips as explants. By supplying optimal concentrations of plant growth regulators (PGR), these explants form shoots that can be rooted to form complete plants.

Thin cell layers (TCL) consist of the epidermis plus 1-5 layers of subepidermal tissue (excluding cambium), and are about 2×10 mm in size. Because of their small biomass, TCLs contain lower levels of endogenous plant growth regulators and other regulating substances than larger explants. Therefore, desired morphogenic patterns can be controlled in the TCL system that may not be controllable at the whole plant level.

The flexibility of the TCL system has been demonstrated utilizing tobacco (*Nicotiana* spp.). By manipulation of the auxin-cytokinin ratio, different morphogenic patterns such as floral buds, roots, vegetative buds, and callus can be obtained in a short period of time (Tran Thanh Van 1973b). Some additional features of TCLs include:

1. tissues respond uniformly under inductive conditions;
2. multifactor experiments can be conducted simultaneously;
3. explants are physiologically and genetically homogeneous;
4. exogenously applied PGRs and other morphogens are able to reach the target tissues without interference from other plant organs;
5. information gained could be related to larger explants;
6. results may be comparable to whole plants.

De novo organs develop from single, differentiated parenchyma cells that must dedifferentiate to form meristematic centers, and then differentiate into the desired tissues and organs (Thorpe and Biondi 1981). *De novo* organs can form directly on the explant without prior callus formation, thus reducing the chance of chromosomal aberrations that may occur during a callus phase.

When comparing *de novo* flower production *in vitro* to flowers that arise on whole plants, Tran Thanh Van (1981) observed an average of 20-50 flowers on each tobacco (*N. tabacum* 'Samsun') TCL, whereas 30-60 flowers were produced on whole plants. Assuming that *de novo* flowers mature into normal fruit and that the cost of producing flowers *in vitro* is not prohibitive, a greater amount of fruit and seed may be produced *in vitro* per square meter of growing space.

Studies on *de novo* flower formation on TCLs have led to the confirmation of the floral morphogenic gradient theory (Tran Thanh Van 1973a), the discovery of lower cellular levels of isoperoxidases (Thorpe et al. 1978), increased DNA content in tissues from floral regions of tobacco (Wardell and Skoog 1973; Altamura et al. 1987), the discovery of a specific gene family expressed during *in vitro* flower formation (Meeks-Wagner et al. 1989), and the effects of plant growth regulators, light and sugar sources on flower production *in vitro*. By expanding the number of species competent for *de novo* organ production, greater insight into factors that control morphogenesis *in vitro* may be achieved. This may lead to a greater understanding of morphogenesis in general.

Literature Review

A majority of the thin cell layer literature focuses on *de novo* flower bud formation. However, only *Cichorium*, *Nicotiana*, and *Torenia* have been reported to undergo *de novo* floral bud formation. The following review concentrates mainly on factors that control *de novo* flower bud production on tobacco TCLs and concludes with a segment on *de novo* shoot production.

De Novo Floral Bud Production

Explant Source

A morphogenic gradient has been demonstrated in tobacco plants *in vivo* and *in vitro*. Aghion-Prat (1965) first demonstrated the existence of a floral morphogenic gradient *in vivo* by injecting plants with *Agrobacterium tumefaciens*. She reported that tumors forming near the apex developed flower buds while tumors arising near the base formed vegetative buds.

Tran Thanh Van (1973a) later illustrated a morphogenic gradient utilizing TCLs. Explants were collected from the base, median zone, subfloral zone and inflorescences of greenhouse grown tobacco (*N. tabacum* L. 'Wisconsin 38') plants. The results were dramatic; 100% vegetative bud formation occurred on basal explants, 75% vegetative and 25% floral buds on TCLs collected from the median zone, 38% vegetative and 62% floral buds from the subfloral zone explants and 100% flowering on inflorescence explants. In the latter, a range of 7-30 floral buds per explant was observed. *De novo* flower bud formation occurred approximately 14-21 days after culture initiation without prior callus formation, and matured to approximately the same size and quality as tobacco flowers found in the greenhouse. In contrast, Bridgen and Veilleux (1988) observed that *in vitro* flower buds on tobacco (*N. tabacum* 'Samsun') TCLs were smaller than *in vivo* flowers, and that *in vitro* flowers varied in anther and pistil number. Therefore, differences in response and the size of *de novo* floral buds may exist between tobacco cultivars.

A floral morphogenic gradient has also been illustrated within the tobacco inflorescence. Croes et al. (1985) observed that flowering on tobacco TCLs was greater in explants obtained from the apical portion of the inflorescence. Flower formation was optimal when explants were collected 1 day before or after anthesis. Mulin and Tran Thanh Van (1989a) observed that shoots arising on the basal end of TCL explants of *Nicotiana plumbaginifolia* and partial somatic hybrids between *N. plumbaginifolia* and *Petunia hybrida* exhibited juvenile leaf morphology, whereas shoots originating from the apical portion of TCL explants exhibited mature leaf morphology similar to flowering plants. Shoots derived from the apical portion of the explants of both genotypes rooted and formed flowers *in vitro* after two to three months.

A polyamine gradient has been illustrated in flowering tobacco plants. Tiburcio et al. (1988) observed an increase in polyamine levels from the main axis to the peduncle in flowering tobacco, but not in the same tissue in fruiting tobacco. Spermidine was the predominant polyamine in all tissue with putrescine being secondary. Spermine was detected only in trace amounts. In addition, Apelbaum et al. (1988) observed that tobacco TCLs formed flowers when 0.5 mM spermidine was added to a medium designed to stimulate vegetative buds.

The presence of a DNA gradient was discovered in tobacco by Wardeli and Skoog (1973). They reported that the amount of DNA per gram fresh weight was greater in stem segments collected from tobacco (*N. tabacum* 'Wisconsin 38') inflorescences than stem segments collected from the base. They concluded that the ability for floral expression of stem segments *in vitro* depended on the quantity and synthesis of DNA in the tissue. Cytophotometric analysis of tobacco (*N. tabacum* 'Samsun') TCL explants by Altamura et al. (1987) confirmed the presence of an acropetal DNA gradient. Indi-

vidual nuclei of TCLs derived from the subfloral zone possessed the 2C DNA content, whereas the DNA content of nuclei collected from TCLs originating from the basal and median zones was reduced by 38.74% and 16.22%, respectively. The DNA content of nuclei derived from floral branches was 27.93% greater than TCLs from the subfloral zone. The increase in DNA content of the latter was attributed to an amplification of medium and highly repeated DNA sequences. They concluded that variations in DNA content corresponded to the capability of TCL explants to differentiate floral buds, and the loss of DNA in the basal and median regions reduced morphogenic competence of those tissues.

Later, Meeks-Wagner et al. (1989) observed the presence and expression of a gene family specific to *de novo* floral bud development. The activity of this gene family was first detected 7 days after culture initiation. A cDNA probe was constructed from mRNA of this gene and hybridized *in situ* with mature and juvenile tobacco plants grown from seed. They found that the gene was expressed in prefloral and floral meristems of mature tobacco plants, but was not expressed in juvenile plant tissue. They deduced that the activity of this gene is not restricted to TCL explants, but also occurred *in vivo* and is involved in flower formation in both instances.

A gradient in isoperoxidase activity has also been identified in tobacco by Thorpe et al. (1978). They observed that isoperoxidase activity in tobacco increased basipetally in both vegetative and flowering plants, and that TCLs from vegetative plants were higher in peroxidase activity than explants from flowering plants. They observed that changes in isoperoxidase activity preceded organogenesis, and that this could be used as a marker to detect differentiation. They recommended that explants from the apical region of flowering plants be chosen because of decreased isoperoxidase activity.

The above examples illustrate that many factors play a decisive role in determining morphogenic competence in whole plants and on thin cell layers. The area from which explants are derived, apex or base, may determine if shoot regeneration or floral organogenesis is to occur. Media formulations alone may not control tissue response, but the genetic competence of the tissue and the concentration of endogenous growth substances such as gibberellins or polyamines may determine the morphogenic response. Therefore, to obtain the desired morphogenic response from TCL explants, it is necessary that the experimenter be aware of these factors, and select the most favorable tissue for inoculation.

Explant Structure

The physical structure of the explant has been demonstrated to be very important in morphogenic competence on TCLs. Chlyah (1974a) compared *Torenia fournieri* L. TCLs to explants that consisted of the epidermis alone, subepidermal parenchyma alone, stem segments minus epidermal tissue, and excised epidermal tissue replaced on the same segment. Only TCLs were capable of direct bud or root morphogenesis without prior callus formation. Excised epidermal tissue replaced on the stem segment did regenerate buds, but only after callus formation. Tissues cultured without the epidermis formed roots. Organ formation was not observed when the epidermis was cultured alone. Therefore, explants (TCLs) which consist of the epidermis plus several layers of subepidermal tissue are necessary for organogenesis *in vitro* without prior callus formation.

Auxin

The response of tobacco TCLs to exogenously applied plant growth regulators is summarized in Table 1. Auxin is required for *in vitro* flower bud formation in *Plumbago indica* L. (Nitsch and Nitsch 1967), *Aquilegia formosa* Fisch. (Bilderback 1972), and tobacco (*Nicotiana tabacum* L.) (Wardell and Skoog 1969a). Auxin concentrations ranging from 0.1-2.2 μ M promote floral bud initiation (Wardell and Skoog 1969a; Tran Thanh Van and Trinh 1978; Cousson and Tran Thanh Van 1981; van den Ende et al. 1984a; Smulders et al. 1988a) on tobacco TCLs and stem segment explants (Table 1). Higher auxin concentrations have led to callus formation and reduced organogenesis. Similar observations have been reported for *Begonia rex* (Chlyah and Tran Thanh Van 1975), *Petunia hybrida* (Mulin and Tran Thanh Van 1989b) and *Torenia* (Chlyah 1973). On examining flower bud initiation and development, van den Ende et al. (1984b) observed that floral bud initiation was greatest when media contained 0.1 μ M naphthaleneacetic acid (NAA), but floral bud maturation progressed faster at 1 μ M NAA. They suggested that the former NAA concentration be used for floral bud initiation, and that when flower buds become visible microscopically, they should be transferred to the latter medium for further development. Generally, the experimenter should test a range of auxin concentrations to identify the optimum for flower formation of each species.

The effects of different auxins on *in vitro* flower bud formation on tobacco TCLs has also been studied. Heylen and Vendrig (1988) observed that flower formation was greatest when media contained NAA or α -(3-chloro-*o*-tolyl)acetic acid (CTA) compared to indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). Flower formation decreased when 2,4-dichlorophenoxyacetic acid (2,4-D) was used. Inflorescences in addition to single flowers were observed when CTA or NAA was the auxin source. Croes et al.

(1985) and Tiburcio et al. (1988) found that NAA increased *de novo* flower bud formation on tobacco TCL explants.

Auxin uptake by tobacco TCL explants occurs very rapidly after culture initiation. Barendse et al. (1987) observed that free NAA concentration in explants increased 16 fold 24 hours after culture initiation, and NAA uptake was greater than BA uptake during the same time period. One possible explanation for this phenomenon may be that auxin is only required during the first four days of culture for tobacco flower formation on TCL explants. Smulders et al. (1988a) observed that floral bud formation was not inhibited by transferring TCL explants to auxin-free medium four days after culture initiation. They concluded that by the fourth day of culture, target cells have already committed to flower initiation and auxin is no longer important.

Bud formation on tobacco TCLs occurs mainly at the base of the explant (Croes et al. 1985; Smulders et al. 1988a, b), however, by increasing the NAA concentration above 2.2 μ M, Smulders et al. (1988a) observed that flower bud formation became uniformly distributed along the entire explant. This observation raised the question of auxin transport within the explant. To identify auxin movement within the explant, Smulders et al. (1988b) added the longitudinal auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and 1-naphthylphthalamic acid (NPA) to the culture medium. Both auxin transport inhibitors significantly decreased bud formation at the basal end of the explants. This resulted in uniform bud distribution along the entire explant surface without affecting floral bud number. Basipetal movement of NAA within the explant has been confirmed by adding radioactive NAA to the culture medium. Smulders et al. 1988b observed that radio-labelled NAA accumulated in the basal portion of the explants 6 hours after culture initiation. The addition of NPA to the culture medium inhibited

basipetal transport of labelled NAA. The movement of NAA to the basal portion of the explants corresponds with increased flower bud formation in that area. Uniform distribution of floral buds can be obtained by inhibiting the basipetal transport of auxin (NAA) within the explant.

Anti-auxins

Anti-auxins have been used to stimulate *in vitro* flower production by counteracting the inhibitory effects of high endogenous auxin concentrations. Tanimoto and Harada (1984) discovered that bud formation on *Torenia* explants could be increased by adding 0.015 μM 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) or 0.15 μM TIBA to the culture medium. Increased bud formation also occurred when explants were cultured on media without exogenously supplied cytokinin. Therefore, anti-auxins may be used to increase bud formation *in vitro* if high endogenous levels of auxin are thought to be a problem.

Cytokinin

In plant cells, cytokinin acts to stimulate cell division (McGaw 1987), and is required for *in vitro* floral bud formation on tobacco explants (Wardell and Skoog 1969a; Cousson and Tran Thanh Van 1981; van den Ende et al. 1984b; Barendse et al. 1988; Heylen and Vendrig 1988). Optimal cytokinin concentrations for *in vitro* flower production on tobacco explants range from 0.1-1 μM (Table 1). Higher concentrations (10 μM) inhibit flower formation (Hillson and LaMonte 1977) or cause gross abnormalities (van den Ende et al. 1984b; van der Krieken et al. 1988).

A direct correlation between cytokinin and the number of floral buds produced on tobacco (*N. tabacum* 'Samsun') TCLs has been demonstrated by van den Ende et al. (1984a). They reported that the number of floral buds produced on tobacco TCLs was dependent upon the cytokinin concentration in the medium, and not on the presence of auxin. They stressed that the latter must be present for floral bud initiation, but did not affect bud number.

The cytokinins generally used to promote *in vitro* floral bud formation on tobacco explants have been kinetin (Wardell and Skoog 1969a), and N⁶-benzyladenine (BA) (Cousson and Tran Thanh Van 1981; van den Ende et al. 1984b; Barendse et al. 1987; van der Krieken et al. 1988; Heylan and Vendrig 1988). Zeatin has been shown to promote meristematic activity in *Torenia* explants (Tanimoto and Harada 1984), but delayed direct floral bud formation on tobacco TCLs (Meeks-Wagner et al. 1989). In the latter, flower bud formation occurred after 4-5 leaves per *de novo* shoot had been produced. Heylan and Vendrig (1988) reported that *de novo* floral bud formation was greater when synthetic cytokinins (BA, kinetin) were used. Therefore, investigators would be wise to test several different cytokinins when developing TCL techniques on new plant species.

BA uptake during the first 24 hours of culture has been shown to be less rapid than NAA (Barendse et al. 1987), and 79.4% of the exogenously supplied BA was converted to 7- β -D-glucopyranosyl-BA (7G-BA) after absorption by the tissue. Only 7-12% (0.4 μ M) of the internal BA concentration was found in the free form; however, they were unable to determine if the BA metabolite(s) played a direct role in flower bud formation.

The above account demonstrates that cytokinin is indeed necessary for floral bud formation on TCL explants. Cytokinins may act by stimulating cell division in target cells

which form meristematic centers, and may eventually differentiate into flower buds. Although cytokinin uptake is slower than auxin during the first 24 hours after culture initiation, its presence in the medium is necessary to sustain cell division and further development of floral buds *in vitro*.

Anti-cytokinins

Anti-cytokinins have been used to test the importance of cytokinin in bud formation. Tanimoto and Harada (1984) observed that 2 μ M 4-cyclobutylamino-2-methylpyrrolo[2,3-*d*]pyrimidine (CB-P) reduced the formation of meristematic zones on *Torenia* explants by 50%, and that a complete reduction of meristem formation occurred when cytokinin was withheld. This confirmed that cytokinin is required for *in vitro* organogenesis.

Gibberellins

It has been hypothesized that gibberellins (GA) inhibit flower initiation and promote floral bud development (Lang 1965). Wardell and Skoog (1969a) reported that the addition of gibberellic acid (GA₃) to floral bud induction medium inhibited flower initiation on tobacco (*N. tabacum* 'Wisconsin 38') stem explants, but stimulated flower development when added to the same medium after flower buds were visible. Croes et al. (1985) observed that GA₃ decreased the number of floral buds per explant on tobacco (*N. tabacum* 'Samsun') TCLs (Table 1). When GA₃ was applied to emasculated tobacco flowers *in vivo*, the TCL explants derived from the tissue displayed decreased competence for *de novo* floral bud formation. Even though gibberellin has been illustrated to inhibit

in vitro floral bud formation, it may be beneficial to transfer explants to media containing gibberellin when flower buds are visible microscopically.

Polyamines

The involvement of polyamines in flowering has yet to be clearly determined. Recently, polyamines have been implicated in tomato fruit development (Teitel et al. 1985), somatic embryogenesis in eggplant (Fobert and Webb 1988), androgenesis in tobacco and *Datura* (Villanueva et al. 1985), and *de novo* flower formation in tobacco (*Nicotiana tabacum*) (Apelbaum et al. 1988; Kaur-Sawhney et al. 1988; Tiburcio et al. 1988; Torrigiani et al. 1987) (Table 1).

Torrighiani et al. (1987) observed that putrescine was the most abundant polyamine during floral and vegetative bud differentiation on tobacco (*Nicotiana tabacum* 'Samsun') TCL explants. An increase in free putrescine corresponded to the occurrence of floral meristem initials on pedicel explants, and vegetative bud formation on stem internode explants. The concentration of free spermidine also increased during culture, but was less than putrescine. They also reported that putrescine and spermidine were found mainly in conjugated form, and that these forms may be responsible for explant growth and differentiation. In contrast, Apelbaum et al. (1988), Tiburcio et al. (1988), and Kaur-Sawhney et al. (1988) have reported that spermidine was responsible for floral bud initiation, and that putrescine promoted vegetative bud formation on tobacco (*Nicotiana tabacum* 'Wisconsin 38') TCL explants. In the latter report, the addition of 0.5 mM spermidine to vegetative bud induction medium promoted floral bud formation. This suggests that spermidine may be involved in floral bud formation, and that the lack of spermidine or the presence of putrescine in the tissue increased vegetative bud formation.

The addition of polyamine biosynthesis inhibitors has aided in determining the importance of polyamines in flower and shoot organogenesis. Kaur-Swahney et al. (1988) reported that the addition of cyclohexylamine, a spermidine synthesis inhibitor, inhibited floral bud formation, but promoted the formation of vegetative buds. Tiburcio et al. (1988) observed that DL- α -difluoromethylarginine (DFMA), a specific inhibitor of arginine decarboxylase (ADC), inhibited bud formation on tobacco TCLs; however, bud formation progressed normally when DL- α -difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase (ODC), was added to the culture medium. They concluded that ADC and the polyamines derived through ADC may be involved in bud formation, but not bud development, whereas ODC and the polyamines derived through ODC do not promote bud initiation, but may aid in bud development.

Experiments with radioactive spermidine have been conducted to determine the role that polyamines play in tobacco TCL morphogenesis. In radio-labelled spermidine experiments, Apelbaum et al. (1988) demonstrated that [^3H]spermidine was rapidly absorbed by the explants and that binding of the [^3H]spermidine to a trichloroacetic acid (TCA) insoluble precipitate was greatest at day 4 (bud initiation) and decreased thereafter until day 10. Using HPLC, they identified 82% of the TCA precipitate as spermidine, 14% as putrescine and 3% as spermine. They also discovered that spermidine was covalently bound to an 18kd protein, and that hydrolysis of the protein identified its composition as mainly spermidine (81%). They concluded that [^3H]spermidine was not significantly altered before binding to the protein, and that post-translational modification of this protein by attachment of spermidine may be connected to floral bud production on tobacco TCL explants. Croes et al. (1986b) discovered the production of a similar size protein during morphogenesis of tobacco (*N. tabacum*) TCLs, but they did not identify the protein.

This evidence suggests that polyamines play a role in *de novo* morphogenesis in tobacco TCLs; however, further research is needed to confirm the role of polyamines during reproductive development in a variety of plant species.

Other Growth Promoting Substances

Various substances have been reported to promote flowering *in vitro*. Wardell and Skoog (1969b) observed that the RNA base analogues 2-thiouracil and 8-azahyanine increased flower formation of tobacco (*N. tabacum* 'Wisconsin 38') stem segments from apical regions. The addition of these RNA base analogues permitted flowering of segments collected from basal internodes of flowering plants. They concluded that once the floral condition was achieved, all stem tissue is capable of producing flowers given the proper stimulus.

Carbon Source

Sugars are necessary sources of carbon in many plant tissue culture media. Tran Thanh Van and Trinh (1978) suggested that the optimal tobacco flower-inducing medium contains 30 g ℓ^{-1} glucose. Cousson and Tran Thanh Van (1981) reported that equimolar concentrations (83 mM) of glucose and sucrose increased *de novo* flower production of tobacco compared to sucrose or glucose alone (167 mM). Therefore, it may be beneficial to use a glucose-sucrose mixture instead of glucose or sucrose alone.

Physical State of the Medium

Thin cell layers need support (Cousson and Tran Thanh Van 1981). This can be accomplished by using agar-solidified medium, or by providing either filter paper bridges or glass beads when using liquid medium. Tran Thanh Van and Trinh (1978) observed 100% flower formation on tobacco (*N. tabacum* 'Xanthi') TCLs when 1% agar was used. A decline in *de novo* flower bud production occurred as the agar concentration was reduced. In order to culture TCL's on liquid medium, glass beads (Cousson and Tran Thanh Van 1981), or filter paper bridges (Tran Thanh Van and Trinh 1978) have been used, but have been less successful than agar-solidified medium.

Light

Under continuous light (50 w m^{-2}), 100% flower bud formation has been observed on tobacco (*N. tabacum* 'Samsun') TCLs (Cousson and Tran Thanh Van 1983). The flowers formed also had well-developed anthers and pistils. Only 20% of explants cultured in the dark produced floral buds, and these buds failed to produce reproductive organs. Along with these observations, Cousson and Tran Thanh Van (1983) stated that light was especially critical on days 6-11.

Genotypic Influences

The response of different *Nicotiana* genotypes to thin cell layer flower morphogenesis is summarized in Table 2. Not all *Nicotiana* genotypes are capable of *de novo* flower morphogenesis *in vitro*; however, 80% (25/31) of the genotypes surveyed have produced

flowers (Table 2). Most *Nicotiana* genotypes capable of *de novo* flower morphogenesis are day-neutral (DN) plants (i.e., flowering is not dependent on day length), whereas *Nicotiana* species incapable of *de novo* flower morphogenesis are either long-day (LD), i.e., plants require short night to promote flowering, or short-day (SD), i.e., plants require long nights to promote flowering (Trinh and Tran Thanh Van 1981; Kamate et al. 1981; Tran Thanh Van and Cousson 1982; Bridgen 1984; Bridgen and Veilleux 1988; Mulin and Tran Thanh Van 1989). *In vitro* flowering of one LD species, *Nicotiana plumbaginifolia*, has been observed only after the formation of leafy shoots (Trinh and Tran Thanh Van 1981). However, factors other than photoperiod must determine competence for *de novo* flower formation *in vitro*. For example, when Bridgen and Veilleux (1985) incubated TCLs of *N. tabacum* 'Maryland Mammoth' (SD) and *N. sylvestris* (LD) in photoperiods that would normally stimulate flowering of *in vivo* plants, the explants were incapable of flower formation. Even the grafting of competent *N. tabacum* 'Samsun' TCLs to TCLs of 'Maryland Mammoth' and *N. sylvestris* could not overcome "floral inhibitors" present in the latter genotypes (Bridgen and Veilleux 1985).

Competence for *de novo* flower morphogenesis has been transferred to F₁ hybrids between responsive and non-responsive genotypes (Kamate et al. 1981; Tran Thanh Van and Cousson 1982; Bridgen 1984), and 44-66% of the F₂ progeny formed *de novo* flowers (Bridgen 1984) (Table 2). In one instance, 30% of the TCL explants from F₁ hybrids between *N. sylvestris* (LD) x *N. tomentosiformis* (SD) (non-responsive genotypes) formed *de novo* flower buds (Kamate et al. 1981). They concluded that competence for *in vitro* flower morphogenesis in this hybrid was induced by the combination of the two genomes.

Reduction of *N. tabacum* 'Samsun' ($2n = 4x = 48$) and *N. plumbaginifolia* ($2n = 2x = 20$) genomes to the haploid level increased flower size and decreased the amount of time necessary for *de novo* flower formation without influencing the percentage of TCL explants forming flowers (Trinh and Tran Thanh Van 1981; Bridgen and Veilleux 1988). TCLs of haploid 'Samsun' plants produced more flower buds than TCLs of diploid plants (Bridgen and Veilleux 1988).

TCLs from partial somatic hybrids between *N. plumbaginifolia* (responsive) and *Petunia hybrida* (non-responsive) produced flowers after 2-3 months *in vitro* (Mulin and Tran Thanh Van 1989). Flowers from one hybrid (NpPh) were similar to *N. plumbaginifolia*, whereas flowers on another hybrid (C31p11) were pink (similar to *P. hybrida* sfla). In this instance, competence for *in vitro* flower formation was transferred to intergeneric hybrid plants via somatic hybridization.

Competence for *de novo* flower morphogenesis can be transferred to sexual and somatic hybrid plants between responsive and non-responsive genotypes. Thus this trait may be dominantly inherited. Trinh and Tran Thanh Van (1983) stated that the morphology and type of flowers produced *in vitro* is dependent on the nuclear and cytoplasmic genome combination. TCLs from F_1 hybrids between *N. tabacum* 'Samsun' ($\varnothing$) and *N. plumbaginifolia* (δ) form flowers directly on the explant, whereas TCLs from reciprocal hybrids only formed flowers on elongated shoots (characteristic of *N. plumbaginifolia*). Bridgen (1984) reported that the genotype of the maternal parent did not influence the number of floral buds or the frequency of TCL explants producing flowers in F_1 hybrids between responsive and non-responsive plants. However, Mulin and Tran Thanh Van (1989b) observed a significant difference in the frequency of *in vitro* flowers between reciprocal hybrids of *Petunia* sp.

De Novo Shoot Production

A greater number of species, *Begonia rex* (Chlyah and Tran Thanh Van 1975), *Beta vulgaris* (Detrez et al. 1988), *Brassica napus* (Klimaszewska and Keller 1985), *Bryophyllum daigremontianum* (Bigot 1976), *Petunia* sp. (Mulin and Tran Thanh Van 1989b.), *Psophocarpus tetragonolobus* (Trinh et al. 1981), *Nicotiana tabacum* (Tran Thanh Van 1973) and *Torenia fournieri* (Chlyah 1973), have demonstrated competence for *de novo* shoot proliferation on thin cell layers.

The factors that promote shoot morphogenesis are similar to those demonstrated in the classical experiments conducted by Skoog and Miller (1957) with tobacco cell cultures. Generally, high cytokinin-auxin ratios (10-100:1) lead to *de novo* shoot morphogenesis. Tran Than Van et al. (1974) observed that vegetative bud formation was greatest on tobacco TCLs at 10 μ M BA and 1 μ M IAA. Higher cytokinin concentrations (100 μ M) were toxic to the explants. Similar results were obtained by Klimaszewska and Keller (1985) and Detrez et al. (1988) for *Brassica napus* and *Beta vulgaris*, respectively; however, the PGR concentrations were greater in the latter reports. Meeks-Wagner et al. (1989), observed that tobacco TCLs formed vegetative buds instead of floral buds when media contained zeatin instead of kinetin. Klimaszewska and Keller (1985) reported that BA was more effective than BA riboside, kinetin, N⁶-[Δ^2 -isopentyl]adenine (2iP); and zeatin for shoot formation on *Brassica napus* TCL explants derived from inflorescences collected before anthesis. NAA was also more effective for shoot regeneration than 2,4-D, IBA, IAA, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and picloram. They also observed that shoot formation was greatest when KNO₃ (30-90mM) was utilized as the sole nitrogen source, and when 0.12 M sucrose or 0.15 M glucose was used.

De novo shoots on TCLs have been shown to originate from subepidermal parenchyma cells. Klimaszewska and Keller (1985), and Detrez et al. (1988) demonstrated that TCL shoots originate from subepidermal parenchyma cells of TCL explants collected from *Brassica napus* and *Beta vulgaris*, respectively. Cell divisions were detected as early as three days after culture initiation on *Brassica* explants (Klimaszewska and Keller 1985), and ten days on *Beta vulgaris* (Detrez et al. 1988) TCLs. In both cases, *de novo* shoot organogenesis occurred without prior callus formation.

Thin cell layers can be used as an explant source for genetic transformation. Charest et al. (1988) recently reported that up to 50% of the shoots regenerated from *Brassica napus* ssp. *oleifera* cv. Westar TCLs exhibited kanamycin resistance and nopaline synthase activity after cocultivation with *Agrobacterium tumefaciens*. The highest transformation rates occurred when TCL explants were preconditioned on a feeder layer of *B. napus* cells derived from suspension cultures for four days before a 30s inoculation period in an *Agrobacterium tumefaciens* cell suspension (10^9 cells mL^{-1}). TCL explants were cocultivated with *A. tumefaciens* for two days before the addition of 500 $\mu\text{g mL}^{-1}$ either carbenicillin, cefotaxime, Claforan or Clavulin 250. Longer inoculation and cocultivation periods resulted in explant necrosis and uncontrollable bacterial growth. Transformed shoots were selected for kanamycin resistance by transferring unrooted shoots to B₅ medium containing 15 $\mu\text{g mL}^{-1}$ kanamycin. The surviving shoots were transferred to higher levels (80 $\mu\text{g mL}^{-1}$) of kanamycin until toxic concentrations were reached. Kanamycin resistance and nopaline synthase activity were transferred to the selfed progeny of the transgenic plants in ratios of 9:6, 8:5, 7:4, 12:4 or 7:8 depending on the *Agrobacterium* strain used.

Summary

Thin cell layer technology can be adapted to accommodate a wide range of physiological, histological and genetic studies. By manipulating PGR concentrations, TCL explants of competent plant species can be programmed to display many different morphogenic patterns. Equimolar concentrations of auxin and cytokinin may result in flower formation, a phenomenon which can be used to study flower morphogenesis to gain a greater knowledge on how plants switch from the vegetative to the reproductive state. Higher concentrations of cytokinin lead to shoot production which may be advantageous for micropropagation or genetic transformation. By extending the number of species competent for *de novo* organogenesis, greater insight into these processes can be gained which may lead to a greater understanding of the switch in morphogenic status in plants.

Literature Cited

- Aghion-Prat, D. 1965. Floral meristems-organizing gradient in tobacco stems. *Nature* 207:1211.
- Altamura, M.M., P. Bassi, A. Cavallini, G. Cionini, R. Cremonini, B. Monacelli, G. Pasqua, O. Sassoli, K. Tran Thanh Van and P.G. Cionini. 1987. Nuclear DNA changes during plant development and the morphogenetic response *in vitro* of *Nicotiana tabacum* tissues. *Plant Sci.* 53:73-79.
- Apelbaum, A., Z.N. Canellakis, P.B. Applewhite, R. Kaur-Sawhney and A.W. Galston. 1988. Binding of spermidine to a unique protein in thin-layer tobacco tissue culture. *Plant Physiol.* 88: 996-998.
- Barendse, G.W.M., A.F. Croes, M. Bosveld, W.M. van der Krieken and G.J. Wullems. 1987. Uptake and metabolism of NAA and BAP in explants of tobacco in relation to *in vitro* flower bud formation. *J. Plant Growth Regul.* 6:193-200.
- Bilderback, D.E. 1972. The effects of hormones upon the development of excised floral buds of *Aquilegia*. *Amer. J. Bot.* 59:525-529.
- Bigot, C. 1976. Bourgeonnement *in vitro* à partir d'épiderme séparé de feuille de *Bryophyllum daigremontianum* (Crassulacées). *Can. J. Bot.* 54:852-867.
- Blake, J. 1969. The effect of environmental and nutritional factors on the development of flower apices cultured *in vitro*. *J. Exptl. Bot.* 20:113-123.
- Bridgen, M.P. 1984. Studies of *in vitro* flowering and *de novo* flowers of *Nicotiana tabacum*. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
- Bridgen, M.P. and R.E. Veilleux. 1985. Studies of *de novo* flower initiation from thin cell layers of tobacco. *J. Amer. Soc. Hort. Sci.* 110:233-236.
- Bridgen, M.P. and R.E. Veilleux. 1988. A comparison of *in vitro* flowers to *in vivo* flowers of haploid and diploid *Nicotiana tabacum* L. *Plant Cell Tissue Organ Culture* 13:3-13.
- Charest, P.J., L.A. Holbrook, J. Gabard, V.N. Iyer and B.L. Miki. 1988. *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. *Theor. Appl. Genet.* 75:438-445.
- Chlyah, H. 1973. Néof ormation dirigée à partir de fragments d'organes de *Torenia fournieri* (Lind.) cultivés *in vitro*. *Biol. Plant.* 15:80-87.
- Chlyah, H. 1974a. Inter-tissue correlations in organ fragments. *Plant Physiol.* 54:341-348.

- Chlyah, H. 1974b. Formation and propagation of cell division-centers in the epidermal layers of internodal segments of *Torenia fournieri* grown *in vitro*. Can. J. Bot. 52:867-872.
- Chlyah, A. and M. Tran Thanh Van. 1975. Differential reactivity in epidermal cells of *Begonia rex* excised and grown *in vitro*. Plant Physiol. 35:16-20.
- Cousson, A. and K. Tran Thanh Van. 1981. *In vitro* control of *de novo* flower differentiation from tobacco thin cell layers cultured on a liquid medium. Physiol. Plant. 51:77-84.
- Cousson, A. and K. Tran Thanh Van. 1983. Light- and sugar-mediated control of direct *de novo* flower differentiation from tobacco thin cell layers. Plant Physiol. 72:33-36.
- Croes, A.F., T. Creemers-Molenaar, G. van den Ende, A. Kemp. and G.W.M. Barendse. 1985. Tissue age as an endogenous factor controlling *in vitro* bud formation on explants from the inflorescence of *Nicotiana tabacum* L. J. Exptl. Bot. 36:1771-1779.
- Croes, A.F., R. Derksen, A. Kemp, J. van Wezel and G.W.M. Barendse. 1986a. Influence of the developing fruit on aging of the floral stalk tissue with respect to flower bud regeneration *in vitro*. J. Plant Physiol. 125:61-68.
- Croes, A.F., P.L.G.M. Heslen, A.A.J. van de Loo and G.W.M. Barendse. 1986b. Protein synthesis during *in vitro* flower bud formation in tobacco. Acta Bot. Neer. 35:161-167.
- Detrez, C., T. Tetu, R.S. Sangwan and B.S. Sangwan-Norreel. 1988. Direct organogenesis from petiole and thin cell layer explants in sugar beet cultured *in vitro*. J. Exptl. Bot. 39:917-926.
- Fobert, P.R. and D.T. Webb. 1988. Effects of polyamine, polyamine precursors, and polyamine biosynthetic inhibitors on somatic embryogenesis from eggplant (*Solanum melongena*) cotyledons. Can. J. Bot. 66:1734-1742.
- Heylen, C. and J.C. Vendrig. 1988. The influence of different cytokinins and auxins on flower neoformation in thin cell layers of *Nicotiana tabacum* L. Plant Cell Physiol. 29:665-671.
- Hillson, T.D. and D.E. LaMonte. 1977. *In vitro* flowering and development of floral buds on tobacco stem explants. Plant Physiol. 60:881-884.
- Kamate, K., A. Cousson, T.H. Trinh and K. Tran Thanh Van. 1981. Influence des facteurs génétique et physiologique chez le *Nicotiana* sur la néoformation *in vitro* de fleurs à partir d'assises cellulaires épidermiques et sous-épidermiques. Can. J. Bot. 59:775-781.
- Kaur-Sawhney, R., A.F. Tiburcio and A.W. Galston. 1988. Spermidine and flower-bud differentiation in thin-layer explants of tobacco. Planta 173:282-284.

- Klimaszewska, K. and W.A. Keller. 1985. High frequency plant regeneration from thin cell layer explants of *Brassic napus*. Plant Cell Tissue Organ Culture 4:183-197.
- Lang, A. 1965. Physiology of flower initiation. In: W. Ruthland (ed.). Encyclopedia of plant physiology. Springer-Verlag, New York. pp. 1380-1536.
- McGaw, B.A. 1987. Cytokinin biosynthesis and metabolism. In: Davies, P.J. Plant hormones and their role in plant growth and development. Martinus Nijhoff Publishers, Dordrecht. pp. 76-93.
- Meeks-Wagner, D. R., E.S. Dennis, K. Tran Thanh Van and W.J. Peacock. 1989. Tobacco genes expressed during *in vitro* floral initiation and their expression during normal plant development. Plant Cell 1:25-35.
- Mulin, M. and K. Tran Thanh Van. 1989a. *In vitro* flower formation from thin epidermal cell layers of a partial somatic hybrid between *Petunia hybrida* (Hort.) and *Nicotiana plumbaginifolia* (Viv.). Plant Cell Tissue Organ Culture 16:195-206.
- Mulin, M. and K. Tran Thanh Van. 1989b. Obtention of *in vitro* flowers from thin epidermal cell layers of *Petunia hybrida* (Hort.). Plant Sci. 62:113-121.
- Nitsch, C. and J.P. Nitsch. 1967. The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. II. The production of reproductive buds. Planta 72:371-384.
- Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. Symp. Soc. Exp. Biol. 11:118-131.
- Smulders, M.J.M., G.F.E. Janssen, A.F. Croes, G.W.M. Barendse and G.J. Wullems. 1988a. Auxin regulation of flower bud formation in tobacco explants. J. Exptl. Bot. 39:451-459.
- Smulders, M.J.M., A.F. Croes and G.J. Wullems. 1988b. Polar transport of 1-naphthaleneacetic acid determines the distribution of flower buds on explants of tobacco. Plant Physiol. 88:752-756.
- Tanimoto, S. and H. Harada. 1984. Roles of auxin and cytokinin in organogenesis in *Torenia* stem segments cultured *in vitro*. J. Plant Physiol. 115: 11-18.
- Teitel, D.C., E. Cohen, S. Arad, E. Birnbaum and Y. Mizrahi. 1985. The possible involvement of polyamines in the development of tomato fruits *in vitro*. Plant Growth Regul. 3: 309-317.
- Thorpe, T.A. and S. Biondi. 1981. Regulation of plant organogenesis. In: Maramorosch, K. (ed.). Advances in cell culture, Vol. 1. Academic Press, New York. pp. 213-239.
- Thorpe, T.A., M. Tran Thanh Van and T. Gaspar. 1978. Isoperoxidases in epidermal layers of tobacco and changes during organ formation *in vitro*. Physiol. Plant. 44:388-394.

- Tiburcio, A.F., R. Kaur-Sawhney and A.W. Galston. 1988. Polyamine biosynthesis during vegetative and floral bud differentiation in thin cell layer tobacco tissue cultures. *Plant Cell Physiol.* 29:1241-1249.
- Torrigiani, P., M.M. Altamura, G. Pasqua, B. Monacelli, D. Serafini-Fracassini and N. Bagni. 1987. Free and conjugated polyamines during *de novo* floral and vegetative bud formation in thin cell layers of tobacco. *Physiol. Plant.* 70:453-460.
- Tran Thanh Van, K. 1973a. Direct flower neoformation from superficial tissue of small explants of *Nicotiana tabacum* L. *Planta* 115:87-92.
- Tran Thanh Van, M. 1973b. *In vitro* control of *de novo* flower, bud, root, and callus differentiation from excised epidermal tissues. *Nature* 246:44-45.
- Tran Thanh Van, K.M. 1981. Control of morphogenesis in *in vitro* cultures. *Ann. Rev. Plant Physiol.* 32:291-311.
- Tran Thanh Van, K. and A. Cousson. 1982. Microenvironment-genome interactions in *de novo* morphogenetic differentiation on thin cell layers. In: Earle, E.D. and Y. Demarly (eds.). *Variability in plants regenerated from tissue culture*. Praeger Scientific, New York. pp. 121-139.
- Tran Thanh Van, K. and H. Trinh. 1978. Plant propagation: Non-identical and identical copies. In: R. Henke and M. Kroh. (eds.). *Propagation of higher plants through tissue culture*. Natl. Tech. Inf. Serv., U.S. Dept. Comm., Springfield, VA. pp. 134-158.
- Tran Thanh Van, M., N.T. Dien and A. Chlyah. 1974. Regulation of organogenesis in small explants of superficial tissue of *Nicotiana tabacum* L. *Planta* 119:149-159.
- Trinh, T.H. and K. Tran Thanh Van. 1981. *In vitro* flower formation from diploid and haploid thin layer of epidermal and subepidermal cells of *Nicotiana tabacum* L. and of *Nicotiana plumbaginifolia* Viv. *Z. Pflanzenphysiol.* 101:1-8.
- Trinh, T.H. and K. Tran Thanh Van. 1983. Influence de l'interaction g nome-cytoplasme sur la formation de fleurs *in vivo* et *in vitro* chez les hybrides entre *Nicotiana plumbaginifolia* et *Nicotiana tabacum*. *Can. J. Bot.* 61:3514-3522.
- Trinh, T.H., H. Lie-Schricke and K. Tran Thanh Van. 1981. Direct *in vitro* bud formation from fragments and thin cell layers of different organs of the winged bean (*Psophocarpus tetragonolobus* L. DC). *Z. Pflanzenphysiol.* 102:127-139.
- van den Ende, G., A.F. Croes, A. Kemp and G.W.M. Barendse. 1984a. Development of flower buds in thin-layer cultures of floral stalk tissue from tobacco: role of hormones in different stages. *Physiol. Plant.* 61:114-118.
- van den Ende, G., A.F. Croes, A. Kemp, G.W.M. Barendse and M. Kroh. 1984b. Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum*. *Physiol. Plant.* 62:83-88.

- van der Kriecken, W.M., A.F. Croes, G.W.M. Barendse and G.J. Wullems. 1988. Uptake and metabolism of benzyladenine in the early stage of flower bud development *in vitro* in tobacco. *Physiol. Plant.* 74: 113-118.
- Villanueva, V.R., V. Mathivet and R.S. Sangwan. 1985. RNA, proteins and polyamines during gametophytic and androgenic development of pollen in *Nicotiana tabacum* and *Datura innoxia*. *J. Plant Growth Regul.* 3: 293-307.
- Wardell, W.R. and F. Skoog. 1969a. Flower formation in excised tobacco stem segments; I. Methodology and effects of plant hormones. *Plant Physiol.* 44:1402-1406.
- Wardell, W.R. and F. Skoog. 1969b. Flower formation in excised tobacco stem segments; II. Reversible removal of IAA inhibition by RNA base analogues. *Plant Physiol.* 44:1407-1412.
- Wardell, W.R. and F. Skoog. 1973. Flower formation in excised tobacco stem segments; III. Deoxyribonucleic acid content in stem tissue of vegetative and flowering tobacco plants. *Plant Physiol.* 52:215-220.

Table 1. Response of tobacco thin cell layer explants to media containing different auxins, cytokinins, gibberellin and polyamines.

Cultivar	PGR	Response	Reference
<i>Auxin</i>			
Samsun	0.1 μ M IBA	100% flower formation	Cousson and Tran Thanh Van, 1981
	0.1 μ M NAA	100% flower formation	van den Ende et al., 1984a,b
	1 μ M NAA	optimal flower development	van den Ende et al., 1984b
	NAA	rapid NAA uptake during first 24h	Barendse et al., 1987
	0.45 μ M NAA 2.2 μ M NAA 4.5 μ M NAA	basal flower formation uniform bud distribution callus formation	Smulders et al., 1988
	TIBA & NPA	inhibited NAA transport	Smulders et al., 1988
Wisconsin 38	NAA	97% floral bud formation	Tiburcio et al., 1988
Xanthi	0.1 μ M IBA	100% flower formation	Tran Thanh Van and Trinh, 1978
Unknown	NAA, CTA, 2,4-D, IAA & IBA	synthetic auxins increased flower morphogenesis	Heylen and Vendrig, 1988
<i>Cytokinin</i>			
Samsun	0.1 μ M Kinetin	100% flower formation	Cousson and Tran Thanh Van, 1981
	1 μ M BA	100% flower formation	Cousson and Tran Thanh Van, 1981; van den Ende et al., 1984b
	10 μ M BA	floral bud abnormalities	van den Ende et al., 1984b
	0.22-1 μ M BA	optimal BA concentration	Barendse et al., 1987
	0.45-1 μ M BA	optimal BA concentration	van der Kriecken et al., 1988
Xanthi	0.1 μ M kinetin	100% flower formation	Tran Thanh Van and Trinh, 1978
Unknown	BA	best cytokinin; synthetic CK's best	Heylen and Vendrig, 1988
<i>Gibberellin</i>			
Samsun	GA ₃	inhibited flower initiation, but promoted bud development	Croes et al., 1985

Table 1 continued

Cultivar	PGR	Response	Reference
	<i>Polyamines</i>		
Samsun	putrescine	highest concentration during bud formation	Torrigiani et al., 1987
Wisconsin 38	spermidine	promoted floral buds	Kaur-Swahney et al., 1988
	putrescine spermidine	promoted vegetative buds promoted floral buds	Tiburcio et al., 1988
	[³ H]spermidine	promoted floral buds	Apelbaum et al., 1988

Table 2. *Nicotiana* genotypes capable of flower formation on thin cell layer explants.

Genotype	Chromosome Number	Photoperiod	Floral bud Frequency	Reference
Responsive				
<i>N. tabacum</i> cvs.				
Lacerata	48	DN†	82	Kamate et al., 1981
McNair 944	48	DN	100	Bridgen, 1984
NC82	48	DN	100	Bridgen, 1984
Samsun	48	DN	100	Tran Thanh Van and Cousson, 1982
Samsun (haploid)	24	DN	100	Trinh and Tran Thanh Van, 1981
Wisconsin 38	48	DN	100	Tran Thanh Van and Cousson, 1982
Xanthi	48	DN	100	Tran Thanh Van and Cousson, 1982
Other <i>Nicotiana</i> sp.				
<i>N. rustica</i>	48	DN	25-35	Kamate et al., 1981
<i>N. debneyi</i>	48	DN	100	Kamate et al., 1981
<i>N. plumbaginifolia</i>	20	DN	100	Kamate et al., 1981
<i>N. plumbaginifolia</i> haploid	10	DN	80-100	Trinh and Tran Thanh Van, 1981
<i>N. tabacum</i> hybrid plants				
McNair 944 x NC22NF	48	DN	100	Bridgen, 1984
NC22NF x McNair 944	48	DN	100	Bridgen, 1984
NC82 x NC22NF	48	DN	100	Bridgen, 1984
NC22NF x NC82	48	DN	100	Bridgen, 1984
F ₂ (McNair 944 x NC22NF)	48	Variable	66.8	Bridgen, 1984
F ₂ (NC82 x NC22NF)	48	Variable	44.2	Bridgen, 1984
Interspecific hybrid plants				
<i>N. tabacum</i> Samsun x <i>N. sylvestris</i>	36	DN	60	Kamate et al., 1981
<i>N. tabacum</i> Samsun x <i>N. tomentosiformis</i>	36	DN	75	Kamate et al., 1981
<i>N. sylvestris</i> x <i>N. tomentosiformis</i>	24	LD	30	Kamate et al., 1981

Table 2 continued

Genotype	Chromosome Number	Photoperiod	Floral bud Frequency	Reference
<i>N. plumbaginifolia</i> x <i>N. sylvestris</i>	22	LD	10	Kamate et al., 1981
(<i>N. debneyi</i> x <i>N. tabacum</i>) x <i>N. tabacum</i>	48	DN	100	Kamate et al., 1981
(<i>N. suaveolens</i> x <i>N. tabacum</i>) x <i>N. tabacum</i>	48	DN	89	Kamate et al., 1981
Partial somatic hybrids between <i>N. plumbaginifolia</i> and <i>Petunia hybrida</i> sfla				
NpPh	20	NA	100	Mulin and Tran Thanh Van, 1989
C31p11	20	NA	42-50	Mulin and Tran Thanh Van, 1989
Non-responsive				
<i>N. tabacum</i> cvs.				
Maryland Mammoth	48	SD	0	Bridgen and Veilleux, 1985
NC22NF	48	SD	0	Bridgen, 1984
Other <i>Nicotiana</i> sp.				
<i>N. sylvestris</i>	24	LD	0	Tran Thanh Van and Cousson, 1982
<i>N. tomentosiformis</i>	24	SD	0	Tran Thanh Van and Cousson, 1982
<i>N. alata</i>	18	DN	0	Kamate et al., 1981
<i>N. plumbaginifolia</i> x <i>N. tomentosiformis</i>	22	LD	0	Kamate et al., 1981

†DN, SD, LD = day neutral, short day or long day plants, respectively. NA = not applicable.

Chapter Three: Shoot, Root and Flower

Morphogenesis on Tomato Thin Cell Layers

Abstract

This paper reports the regeneration of *de novo* shoots, roots and flowers on thin cell layer explants excised from inflorescences of tomato (*Lycopersicon esculentum* Mill.). In preliminary experiments, indole-3-acetic (IAA), indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) were added in a 3×3×3 factorial combination with kinetin, each at 0.001, 0.1 and 10 μ M concentrations. Direct shoot formation occurred when media contained 10 μ M kinetin and 0.001 μ M IAA or NAA, but in subsequent experiments, shoot organogenesis was increased by substituting 10 μ M zeatin or N⁶-benzyladenine (BA) for kinetin. Root formation was observed when media contained 0.1-10 μ M IAA, IBA or NAA. Flower formation on elongated shoots with several leaves occurred when media contained 10 μ M IAA and 0.1 μ M kinetin. All tomato cultivars were capable of forming shoots directly on thin cell layer explants. The mean

number of shoots per explant ranged from 1.3 for 'Red Alert' to 5.3 for 'Large Red Cherry'. Active cell divisions were observed in subepidermal and cambial tissue during the first week of culture, and meristematic centers of dividing cells were evident by day 14. Well-developed shoot apices and leaf structures were observed on 50% of the explants 28 days after culture initiation.

Introduction

The commercial importance of tomato, *Lycopersicon esculentum* Mill. (1986 crop valued at more than \$1.4 billion; USDA 1986), and its position within the Solanaceae, a plant family with species for which many tissue and cell culture procedures were originally developed, has made it the focus of numerous tissue culture applications (Hille et al. 1989). Although shoot regeneration has been obtained on tomato cotyledon calli (Sibi et al. 1984), leaf disc calli (Padmanabhan et al. 1974; Evans and Sharp 1983) and calli derived from leaf mesophyll protoplasts (Shahin 1984; Neidez et al. 1985; Tan et al. 1987), morphogenesis of tomato in tissue culture systems has been less frequent than for other Solanaceous species. The development of thin cell layer technology may provide a more efficient organ regeneration system; for such a system could potentially be used to incorporate foreign genes with *Agrobacterium* vectors as for *Brassica napus* (Charest et al. 1988). The high nutritional value of tomato fruit (Grierson and Kadar 1986) has made it an excellent candidate for the National Aeronautics and Space Administration's (NASA) controlled-environment life-support system (CELSS) (Salisbury and Bugbee 1988). *De novo* production of flowers and fruit on tomato thin cell layers can be envi-

sioned as a means to supply fresh fruit under the constraint of limited growing conditions.

Controlling morphogenesis at the whole plant level can be complicated by the presence of high levels of endogenous plant growth regulators (PGR), and the presence of meristems, buds, flowers, or other metabolic sources and sinks (Chlyah, 1974). The development of thin cell layer techniques has allowed the study of direct morphogenesis *in vitro* (Tran Thanh Van, 1973b). Because of their small size (2×10 mm) and the absence of preformed buds, thin cell layer explants contain lower levels of endogenous plant growth regulators and other regulating substances than whole plants. Small explant size also allows exogenously supplied plant growth regulators to reach the target tissue easily, allowing the control of morphogenic patterns that may not be controllable at the whole plant level. Results gained through the use of the TCL culture system have been successfully related to whole plants (Tran Thanh Van and Trinh, 1978; Meeks-Wagner et al., 1989; Mulin and Tran Thanh Van, 1989a).

The production of flowers, shoots and roots on thin cell layer explants has been possible by manipulating the auxin-cytokinin ratio. Auxin concentrations ranging from 0.1-2.2 μ M combined with cytokinin (0.1-1 μ M) promote flower bud formation on thin cell layer explants of *Cichorium intybus* (Bouniols, 1974), *Nicotiana tabacum* (Tran Thanh Van, 1973a; Tran Thanh Van and Trinh, 1978; Tran Thanh Van and Cousson, 1982; van den Ende et al., 1984b; Croes et al., 1985) and *Torenia fournieri* (Chlyah, 1973). These PGR concentrations also stimulate flower formation on elongated shoots derived from thin cell layer explants of *Nicotiana plumbaginifolia* (Kamate et al., 1981) and *Petunia hybrida* (Mulin and Tran Thanh Van, 1989b). Higher auxin concentrations reduce flower formation, and stimulate root and/or callus formation (Tran Thanh Van, 1973b; Smulders

et al., 1988), whereas higher cytokinin levels ($10\mu\text{M}$) result in flower inhibition or morphological abnormalities (van den Ende et al., 1984b), or stimulate vegetative bud formation in *Begonia rex* (Chlyah and Tran Thanh Van, 1975:), *Beta vulgaris* (Detrez et al., 1988), *Brassica napus* (Klimaszewska and Keller, 1985), *Bryophyllum daigremontianum* (Bigot, 1976), *Nicotiana* spp. (Tran Thanh Van, 1973b), *Petunia hybrida* (Mulin and Tran Thanh Van, 1989b), *Psophocarpus tetragonolobus* (Trinh et al., 1981) and *Torenia fournieri* (Chlyah, 1973).

During thin cell layer morphogenesis, *de novo* organs develop from single, differentiated parenchyma cells that must dedifferentiate to form meristematic centers, and then redifferentiate into the desired tissues and organs (Thorpe and Biondi, 1981). *De novo* morphogenesis can occur directly on the explant without prior callus formation, thus reducing the chance of chromosomal aberrations that may occur during a callus phase.

Thin cell layer technology has been implemented in a wide range of physiological, histological and genetic studies. Direct flower formation occurs when equimolar concentrations of cytokinin and auxin are used. Studies of *de novo* flower formation can be used to investigate flower morphogenesis and may give added insight into the morphological, physiological, biochemical and genetic changes that occur during the switch from the vegetative to the reproductive state. In contrast, shoot organogenesis on TCLs may lead to a more efficient means of either micropropagation or genetic transformation. The factors which govern these processes may become clearer by expanding the number of species competent for *de novo* morphogenesis. The *Lycopersicon* genus has a rich genetic history including a well-mapped genome with many morphological and enzymatic markers. By adapting thin cell layer technology to tomato, greater insight may be gained into the genetic aspects of flower and shoot regeneration

in vitro. Hence, the objective of this study was to develop techniques for direct shoot, root and flower morphogenesis on tomato thin cell layers by: (1) testing the effects of different combinations and concentrations of plant growth regulators, (2) testing the ability of different tomato cultivars to undergo direct shoot organogenesis, and (3) tracing the origin and development of tomato shoots on thin cell layer explants.

Material and Methods

Effects of auxin on thin cell layer morphogenesis

To determine the optimal plant growth regulator for morphogenesis on tomato thin cell layers, several combinations and concentrations of auxin and cytokinin were tested. Tomato (*Lycopersicon esculentum* Mill. 'Red Alert') plants were grown in 7.57 ℓ pots that contained 1 weblite: 2 moss peat: 1 sand. The plants were fertilized weekly with a 20:20:20 (400 ppm N) nutrient solution. Inflorescences with 2-3 open flowers were collected from plants grown in the greenhouse under natural photoperiod at $20 \pm 3^\circ\text{C}$. After the flowers and buds were removed, inflorescences were surface-sterilized in 10% bleach² plus 0.1% 'Tween 20' for 10 min followed by three rinses in sterile, distilled water. Thin cell layer explants (2×10mm) were excised from pedicel and peduncle tissue, and placed into 25×150mm culture tubes (Bellco³) containing 20 ml of the following medium: MS salts (Murashige and Skoog, 1962) plus 0.1 mg ℓ^{-1} thiamine•HCl, 100

² Manufactured by Wonder Chemical Corp., Fairless Hills, Pa.

³ Manufactured by Bellco Glass, Inc., Vineland, N.J.

mg ℓ^{-1} myo-inositol and 3% sucrose (w/v). A separate 3×3 factorial experiment was conducted to examine the morphogenic effects on tomato thin cell layers of each of three auxins [indole-3-acetic acid (IAA⁴), indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA)], at three concentrations (0.001, 0.1 and 10 μ M) in combination with kinetin (6-furfurylaminopurine) at the same concentrations. Medium pH was adjusted to 5.8 with 1N HCl or KOH prior to the addition of 1% Difco Bacto⁵ agar. Kaputs⁶ were used to close the culture vessels prior to autoclaving at 121°C and 1 kg cm⁻² for 20 min. Cultures were incubated under a 16 h photoperiod at 25°C and a photosynthetic photon flux density (PPFD) of 50 μ E m⁻²s⁻¹ provided by 2 Philips (40 watt) cool white fluorescent lamps⁷. After 12 weeks, the following data were collected: number of explants forming flowers; number of flowers per explant; number of explants forming shoots; number of shoots per explant; number of explants forming roots; number of roots per explant. A completely random design was used with 10 replications. The experiment was conducted twice.

Surface-sterilization

To alleviate bacterial contamination encountered during the preliminary experiments, several surface-sterilization treatments were tested: a) 15% bleach/8 min, b) 15% bleach/10 min, c) 25% bleach/5 min, d) 25% bleach/10 min, e) 50% bleach/5 min, f)

⁴ Manufactured by Sigma Chemical Co., St. Louis, Mo.

⁵ Manufactured by Difco Laboratories, Detroit, Mich.

⁶ Manufactured by Bellco Glass, Inc., Vineland, N.J.

⁷ Manufactured by the North American Philips Lighting Corp. Somerset, N.J.

0.1% mercuric chloride⁸/5 min, g) 0.1% mercuric chloride/10 min, h) 0.2% mercuric chloride/5 min, and i) 0.2% mercuric chloride/10 min. After surface-sterilization, inflorescences were rinsed twice in sterile, distilled water, and thin cell layer explants were prepared as described above. Culture medium consisted of MS salts plus 0.1 mg ℓ^{-1} thiamine•HCl, 100 mg ℓ^{-1} myo-inositol, 10 μ M IAA, 0.1 μ M kinetin, and 3% sucrose (w/v). The pH was adjusted to 5.8 with 1N HCl or KOH prior to the addition of 1% Difco Bacto agar. After autoclaving at 121°C and 1 kg cm^{-2} , 20 ml of culture medium was aseptically dispensed into 90×15mm quad-4 petri plates⁹ (5ml quad⁻¹) and allowed to cool overnight at room temperature under the laminar flow hood. Two pedicel and peduncle thin cell layer explants were cultured in each quad-4 plate, and 4 plates were cultured for each treatment resulting in 8 pedicel and peduncle explants per treatment. Culture conditions were as described above. The frequency of contaminated explants and explant quality ratings (0=dead; 1=mostly necrotic with some green tissue; 2=good, moderate necrosis; 3=excellent, no necrosis) were recorded 30 days after culture initiation. At this time, the explants were subcultured; the number of explants producing flowers and the number of floral buds per explant were recorded 60 days later. A completely random design was used.

Effects of zeatin, benzyladenine, kinetin and 2iP on shoot morphogenesis

During the preliminary experiments, several studies were published indicating that N⁶-benzyladenine (BA) increased floral morphogenesis on tobacco thin cell layers (Barendse et al., 1987; Heylen and Vendrig, 1988), and that zeatin increased shoot organogenesis

⁸ Manufactured by Sigma Chemical Co., St. Louis, Mo.

⁹ Manufactured by Baxter Scientific Products, Columbia, Md.

on tomato leaf disc and protoplast calli (Tan et al., 1987). Therefore, a comparison of the effect of four cytokinins on shoot morphogenesis from tomato thin cell layers was made. Thin cell layer explants were prepared from 'Red Alert' inflorescences as above. Culture medium consisted of MS salts plus 0.1 mg ℓ^{-1} thiamine•HCl, 100 mg ℓ^{-1} myo-inositol, 250 mg ℓ^{-1} cefotaxime¹⁰ and 3% sucrose (w/v). Plant growth regulators consisted of 1 nM IAA, and 10 μ M of one of four cytokinins: kinetin, BA, zeatin (6-[4-hydroxy-3-methylbut-2-enylamino]purine) or 2iP (N⁶-[Δ^2 -isopentenyl]adenine). The pH was adjusted to 5.8 with 1N HCl or KOH prior to the addition of 1% Difco Bacto agar. Zeatin and cefotaxime were filter-sterilized and added to the medium after it was autoclaved at 121°C and 1 kg cm⁻² for 20 min. Culture vessels and incubation conditions were as previously described. The numbers of explants forming shoots and the number of shoots per explant were tabulated after 12 weeks. A completely random design was used with 10 replications. The experiment was conducted twice.

Shoot morphogenic competence among tomato cultivars

Competence for *de novo* shoot morphogenesis was tested on thin cell layers of 11 tomato cultivars. The cultivars (Table 3) were selected for their previous success in either callus or protoplast culture, or because of economic importance. Thin cell layer explants were taken from pedicel tissue derived from mature (5-9 wks after sowing) plants grown in the greenhouse at 20 $\pm$ 3°C and natural photoperiods (summer 1989). Culture medium consisted of MS salts plus 0.1 mg ℓ^{-1} thiamine•HCl, 100 mg ℓ^{-1} myo-inositol, 3% sucrose, 1 nM IAA, 10 μ M zeatin and 250 mg ℓ^{-1} cefotaxime. The pH was adjusted to 5.8 prior to the addition of 10 mg ℓ^{-1} Difco Bacto agar. Zeatin and cefotaxime were filter-

¹⁰ Manufactured by Sigma Chemical Co., St. Louis, Mo.

sterilized and added after the medium was autoclaved at 121°C and 1 kg cm⁻² for 20 min. Explants were initially cultured in 24-well plates¹¹ which contained 1.5ml of medium per well. After 3 weeks, the explants were subcultured to 25×100mm borosilicate (Pyrex) tubes¹² which contained 10ml of the same medium. Incubation periods were as previously described. The number of explants forming shoots and the number of shoots per explant were tabulated after 12 weeks. A completely random design was used with 12 replications. The experiment was conducted twice.

Histological development of tomato thin cell layers

Histological development of thin cell layer explants was examined to determine the origin of *de novo* shoots. Thin cell layer explants of the tomato cultivar 'Ohio 7814' were collected as described above. The medium and culture conditions were as described for shoot competence studies. For a period of four weeks, ten thin cell layer explants were removed each week from culture, fixed in formalin-acetic-alcohol (10%-5%-50%), and dehydrated using a Fisher Histomatic Tissue Processor (model 166)¹³. Samples were imbedded in paraffin, sectioned at 9 µm on a rotary microtome¹⁴, mounted on microscope slides, cleared and stained with hemalum (Sass, 1958).

¹¹ Manufactured by Flow Laboratories, Inc., McLean, Va.

¹² Manufactured by Corning Glass Works, Corning, N.Y.

¹³ Fisher Scientific, Lexington, Mass.

¹⁴ Spencer Lens Co., Buffalo, N.Y.

Results and Discussion

Effects of auxin on thin cell layer morphogenesis

All morphogenic patterns which have been reported for tobacco thin cell layers (Tran Thanh Van, 1973b) were expressed to varying degrees by tomato thin cell layer explants (Table 4). Direct shoot formation occurred when media contained 10 μ M kinetin and either IAA or NAA, and to a limited extent, IBA. The frequency of explants forming shoots was greatest (75%) at 10 μ M kinetin and 0.001 μ M IAA (Table 5). A decline in the frequency of explants forming shoots from 75% to 25% occurred as the IAA concentration increased from 0.001 to 10 μ M, but the increase did not influence the number of shoots per explant. Shoot organogenesis was also observed at 10 μ M kinetin and 0.001 μ M NAA (Table 5); however, the frequency of explants forming shoots was lower (57%) than with IAA (75%). The number of shoots per explant did not differ between the IAA and NAA treatments. Callus formation without shoot formation occurred at higher (0.1 and 10 μ M) NAA concentrations. Callus formation was the predominant morphogenic pattern when IBA was used.

Direct root formation was observed on tomato thin cell layer explants when media contained a higher auxin to cytokinin ratio than required for shoot formation (Table 4). The frequency of explants producing roots was greatest (100%) when media contained 0.001 μ M kinetin and 10 μ M IAA or IBA (Table 5). The number of roots per explant was also greatest on the same two media (6.0 ± 1.8 and 3.9 ± 0.6 roots per explant for IAA or IBA, respectively), or with equimolar (0.01 μ M) concentrations of kinetin and NAA (4.5 ± 0.1 roots per explant).

Flower formation (25% of explants; 4.5 ± 0.5 flowers per explant) was observed on tomato thin cell layers when media contained $0.1 \mu\text{M}$ kinetin and $10 \mu\text{M}$ IAA (data not shown). This is contrary to reports in the literature stating that auxin levels greater than $1 \mu\text{M}$ inhibited flower production on tobacco stem segments (Wardell and Skoog, 1969). Flower formation did not occur directly on the explant as occurs on thin cell layers of cultivated tobacco (Tran Thanh Van, 1973a; Croes et al., 1985), but inflorescences developed on elongated shoots with several leaves (Fig. 1a). This is similar to reports of *in vitro* flower formation on thin cell layer explants of *Nicotiana plumbaginifolia* (Kamate et al., 1981) and *Petunia hybrida* (Mulin and Tran Thanh Van, 1989b). Flower formation was not observed when IBA or NAA was the auxin source. Flowers on tomato plantlets derived from thin cell layers eventually formed parthenocarpic fruit (Fig. 1b).

Surface-sterilization

All surface-sterilization treatments were effective at removing surface contamination on thin cell layer explants with the exception of 25 or 50% bleach for 5 min (Table 6). Contamination rates were approximately 50% in these treatments. The sterilization treatments influenced flower production and explant quality based on visual observations of necrotic tissue 30 days after culture initiation. Flower production was greatest (31%) when thin cell layer explants were treated with 15% bleach for 10 min. Explants in this treatment also exhibited less browning. Surface contamination was eliminated when explants were treated with 0.1% mercuric chloride (HgCl_2) for 5 min; however, flower morphogenesis and explant quality were reduced. Increased HgCl_2 concentrations and incubation times inhibited morphogenesis. Therefore, tomato inflorescences were surface-sterilized in 15% bleach for 10 min in further investigations.

Effects of zeatin, BA, kinetin and 2iP on shoot morphogenesis

In a comparison of the effect of four cytokinins at the same concentration (10 μ M) in combination with 1 nM IAA, the frequency of tomato thin cell layer explants which formed shoots was greatest (50%) when media contained either zeatin or BA (Table 7). There was no significant difference in the number of shoots per explant between zeatin and BA (2.8 and 2.5, respectively), but the number of shoots per explant was significantly greater for zeatin (2.8) than for kinetin or 2iP (1.7 and 1.3, respectively). Explants incubated on medium containing BA produced more callus than explants cultured on medium containing zeatin (data not shown). Therefore, zeatin was chosen for further shoot regeneration studies. The percentage of explants forming shoots was less (20%; Table 7) than observed in the same treatment (1 nM IAA, 10 μ M kinetin) in the preliminary experiments (75%; Table 5). This could have resulted from the addition of cefotaxime to the medium, or from the age of the explant donor plants. Charest et al. (1988) reported that cefotaxime reduced shoot organogenesis on thin cell layer explants of *Brassica napus*. We have observed that plants which have been flowering for more than two months displayed reduced morphogenesis.

Shoot morphogenic competence among tomato cultivars

All tomato cultivars responded to thin cell layer techniques to varying degrees. The number of shoots per explant ranged from 1.3 for 'Red Alert' to 5.3 for 'Large Red Cherry' (Table 8). The mean number of shoots per thin cell layer explant was greater for 'Large Red Cherry', 'Ohio 7814' (3.9) and 'BL 6807' (3.7) than for 'Campbell 1327' (1.8) and 'Red Alert.' The other six cultivars exhibited an intermediate response (Table 8).

The frequency of responding explants also varied among the cultivars and ranged from 29% ('Campbell 37') to 63% ('Ohio 7814'). When comparing two cultivars with similar mean shoot numbers (e.g., 'Saladette' and 'VF 36'), the frequency of responding explants differ to such an extent that total shoot production may be greater for one (e.g., 'VF36'). However, most cultivars that had high shoot numbers also had a high frequency of responding explants.

Histological development of tomato thin cell layers

Tomato thin cell layer explants taken from pedicel tissue of flowering tomato plants are composed of epidermis, cortex (to be referred to as subepidermis so as to be consistent with previous TCL reports; Tran Thanh Van 1973b), xylem and phloem tissues (Fig. 2). Because of the small diameter of the pedicel tissue composing the tomato cyme, these explants are composed of more tissue types than thin cell layer explants previously reported for other species (Klimaszewska and Keller, 1985; Detrez et al., 1988). We observed that subepidermal cells of freshly excised thin cell layer explants were small (5-8 μ m) and lacked actively dividing cells as determined by the absence of densely stained areas (Fig. 3). After one week in culture (using a medium leading to shoot formation), some explants had undergone several cell divisions as determined by heavily stained regions of densely cytoplasmic cells with little or no vacuole (Fig.4). The remaining subepidermal cells had increased approximately 2 fold in size (10-15 μ m), and contained large vacuoles. Cell division areas occurred mainly in the subepidermal tissue, but some cell divisions were also detected in cambial tissue.

After 2 weeks in culture, cell division centers were distributed throughout the subepidermal layer (Fig. 5). Meristematic structures with vascular tissue (Fig. 6) appeared to protrude through displaced epidermal cells on some explants (Fig. 7). Different stages of cell division were evident across the explant. Small amounts of callus developed from cambial tissue present on the cut surface of the explant; however, callus production was not obvious macroscopically.

Explants at 21 days after culture initiation frequently exhibited visible vegetative structures with well-developed xylem and phloem tissue (Fig. 8). Smaller meristems were scattered over the explant surface of some explants. Many of the meristematic structures located on the epidermis were of subepidermal origin. Callus formation was limited to the cut surfaces of the explants, and was not structurally organized like cell division centers located in the epidermal and subepidermal region.

Four weeks after culture initiation, 50% of the explants examined had formed shoots and other vegetative structures (Fig. 9). Some explants formed a greater number of shoot apices at the base of the explant (Fig. 10). Shoots that continued to develop were harvested after the eighth week of culture (Fig. 11). Developing leaves without shoot meristems were also observed. Explants that did not produce shoots by day 28 exhibited few cell division areas in the subepidermal tissue, but contained actively dividing cambial cells that produced callus. It appeared that these explants had begun cell division in the subepidermal region, but active cell division must have ceased sometime during the culture period.

Summary

These experiments have demonstrated that morphogenic patterns similar to those reported for tobacco were programmable on tomato thin cell layers excised from pedicel tissue; however, the frequency of responding explants and the number of shoots and floral buds per explant were reduced. Direct shoot formation was optimal when the cytokinin to auxin ratio was approximately 100 to 1. Zeatin was the preferred cytokinin and IAA the preferred auxin. The frequency of explants forming shoots and the number of shoots per explant were similar with either zeatin or BA at the same concentration, but callus formation was increased with BA. All tomato cultivars tested were capable of direct shoot formation on thin cell layer explants to varying degrees.

Direct root formation was observed on thin cell layer explants when media contained 10-100× more auxin than cytokinin. Flower formation occurred on 'Red Alert' thin cell layers when media contained 10 μ M IAA and 0.1 μ M kinetin. Flower formation did not occur directly on the explant, but occurred on elongated shoots similar to *in vitro* flower formation on *Nicotiana plumbaginifolia* (Kamate et al., 1981) and *Petunia hybrida* (Mulin and Tran Thanh Van, 1989b) thin cell layer explants. The percentage (25-31%) of tomato explants forming flowers was less than has been observed on tobacco (90-100%) thin cell layers (Tran Thanh Van, 1973a), but was similar to flower production reported for *Petunia hybrida* (15%; Mulin and Tran Thanh Van, 1989b).

Tomato thin cell layer explants consist of epidermal, subepidermal, and vascular tissues. Cell divisions were observed during the first week of culture with well-developed meristematic regions by day 14. Vegetative meristems were observed by the third week

of culture, and well-formed vegetative shoots and leaves were observed both micro- and macroscopically by day 28. Shoots were of harvestable size (1-2 cm) 8-12 weeks after culture initiation depending on the cultivar. Some callus formation generally preceded shoot regeneration on tomato thin cell layers, and the amount of callus produced varied considerably among cultivars. For example, the 'Ohio' cultivars were characterized by very little callus whereas 'VF36' exhibited copious callus growth. Callus on 'Ohio 7814' explants originated from the cut surfaces of the explants and not from the region of shoot origin (subepidermal cells). Therefore, shoots originate from subepidermal cells and not from callus tissue.

Subsequent studies have demonstrated that shoot regeneration from tomato thin cell layer explants is more efficient than shoot organogenesis from either micropropagated shoot tips or cotyledon callus (Compton and Veilleux, 1989) making the thin cell layer system more versatile for regeneration of tomato organs *in vitro*. Currently, tomato transformation procedures utilize shoot organogenesis from leaf-disc or protoplast calli that have been inoculated with *Agrobacterium*. The adaptation of thin cell layer procedures for *Agrobacterium*-mediated transformation of tomato may increase the efficiency of obtaining transformed plants by increasing the number of shoots produced. *Agrobacterium*-mediated transformation of *Brassica napus* TCLs has proven successful (Charest et al. 1988). In addition, the tomato TCL system may be used to study plant/pathogen interactions. Radin (personal communication) has successfully used our techniques for *de novo* root production to identify tomato cultivars resistant to nematode infestation.

Literature Cited

- Barendse GWM, Croes AF, Bosveld M, van der Krieken WM, Wullems GJ (1987) Uptake and metabolism of NAA and BAP in explants of tobacco in relation to *in vitro* flower bud formation. *J Plant Growth Regul* 6:193-200
- Bigot C (1976) Bourgeonnement *in vitro* à partir d'épiderme séparé de feuille de *Bryophyllum daigremontianum* (Crassulacées). *Can J Bot* 54:852-867
- Bouniols A (1974) Néof ormation de bourgeons floraux *in vitro* à partir de fragments de racine d'endive *Cichorium intybus* L.: Influence du degré d'hydratation des tissus et ses conséquences sur la composition en acides amines. *Plant Sci Lett* 2:363-371
- Charest PJ, Holbrook LA, Gabard J, Iyer VN, Miki BL (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L. *Theor Appl Genet* 75:438-445
- Chlyah H (1973) Néof ormation dirigée à partir de fragments d'organes de *Torenia fournieri* (Lind) cultivés *in vitro*. *Biol Plant* 15:80-87
- Chlyah H (1974) Inter-tissue correlations in organ fragments. *Plant Physiol* 54:341-348
- Chlyah A, Tran Thanh Van M (1975) Differential reactivity in epidermal cells of *Begonia rex* excised and grown *in vitro*. *Plant Physiol* 35:16-20
- Compton ME, Veilleux RE (1989) *De novo* shoot organogenesis on tomato thin cell layers. 86th Annual Meeting of ASHS, Tulsa, OK. (Abstract)
- Cousson A, Tran Thanh Van K (1981) *In vitro* control of *de novo* flower differentiation from tobacco thin cell layers cultured on a liquid medium. *Physiol Plant* 51:77-84
- Croes AF, Creemers-Molenaar T, van den Ende G, Kemp A, Barendse GWM (1985) Tissue age as an endogenous factor controlling *in vitro* bud formation on explants from the inflorescence of *Nicotiana tabacum* L. *J Exptl Bot* 36:1771-1779
- Detrez C, Tetu T, Sangwan RS, Sangwan-Norreel BS (1988) Direct organogenesis from petiole and thin cell layer explants in sugar beet cultured *in vitro*. *J Exptl Bot* 39:917-926
- Evans D, Sharp W (1983) Single gene mutations in tomato plants regenerated from tissue culture. *Science* 221:949-951
- Grierson D, Kader AA (1986) Fruit ripening and quality. In: Atherton JG, Rudich J (eds) *The tomato crop: a scientific basis for improvement* (pp 241-280) Chapman and Hall Ltd, London

- Heylen C, Vendrig JC (1988) The influence of different cytokinins and auxins on flower neoformation in thin cell layers of *Nicotiana tabacum* L. *Plant Cell Physiol* 29:665-671
- Hille J, Koornneef M, Ramanna MS, Zabel P (1989) Tomato: a crop species amenable to improvement by cellular and molecular methods. *Euphytica* 42:1-23
- Kamate K, Cousson A, Trinh TH, Tran Thanh Van K (1981) Influence des facteurs génétique et physiologique chez le *Nicotiana* sur la néoformation *in vitro* de fleurs a partir d'assises cellulaires épidermiques et sous-épidermiques. *Can J Bot* 59:775-781
- Klimaszewska K, Keller WA (1985) High frequency plant regeneration from thin cell layer explants of *Brassic napus*. *Plant Cell Tissue Organ Culture* 4:183-197
- Meeks-Wagner DR, Dennis ES, Tran Thanh Van K, Peacock WJ (1989) Tobacco genes expressed during *in vitro* floral initiation and their expression during normal plant development. *Plant Cell* 1:25-35
- Mulin M, Tran Thanh Van K (1989a) *In vitro* flower formation from thin epidermal cell layers of a partial somatic hybrid between *Petunia hybrida* (Hort) and *Nicotiana plumbaginifolia* (Viv). *Plant Cell Tissue Organ Culture* 16:195-206
- Mulin M, Tran Thanh Van K (1989b) Obtention of *in vitro* flowers from thin epidermal cell layers of *Petunia hybrida* (Hort). *Plant Sci* 62:113-121
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473-497
- Niedz R, Rutter S, Handley L, Sink K (1985) Plant regeneration from leaf protoplasts of six tomato cultivars. *Plant Sci* 39:199-204
- Padmanabhan V, Paddock E, Sharp W (1974) Plantlet formation from *Lycopersicon esculentum* leaf callus. *Can J Bot* 52:1429-1432
- Salisbury FB, Bugbee B (1988) Plant productivity in controlled environments. *HortScience* 23:293-299
- Sass JE (1958) Botanical microtechnique. The Iowa State University Press, Ames, Iowa
- Shahin EA (1984) Isolation and culture of protoplasts: tomato. In: Vasil IK (ed) *Cell culture and somatic cell genetics of plants* (pp 370-380) Academic Press, Inc., Orlando
- Sibi M, Biglary M, Demarly Y (1984) Increase in the rate of recombinants in tomato (*Lycopersicon esculentum* L.) after *in vitro* regeneration. *Theor Appl Genet* 68:317-321
- Smulders MJM, Janssen GFE, Croes AF, Barendse GWM, Wullems GJ (1988) Auxin regulation of flower bud formation in tobacco explants. *J Exptl Bot* 39:451-459

- Tan M, Rietveld E, van Marrewijk G, Kool A (1987) Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and plant regeneration. *Plant Cell Reports* 6:172-175
- Thorpe TA, Biondi S (1981) Regulation of plant organogenesis. In: Maramorosch, K. (ed) *Advances in cell culture*, Vol 1. (pp 231-239) Academic Press, New York
- Tran Thanh Van K (1973a) Direct flower neoformation from superficial tissue of small explants of *Nicotiana tabacum* L. *Planta* 115:87-92
- Tran Thanh Van M (1973b) *In vitro* control of *de novo* flower, bud, root, and callus differentiation from excised epidermal tissues. *Nature* 246:44-45
- Tran Thanh Van K, Cousson A (1982) Microenvironment-genome interactions in *de novo* morphogenetic differentiation on thin cell layers. In: Earle ED, Demarly Y (eds.) *Variability in plants regenerated from tissue culture* (pp 121-139) Praeger Scientific, New York
- Tran Thanh Van K, Trinh H (1978) Plant propagation: Non-identical and identical copies. In: Henke R, Kroh M (eds.) *Propagation of higher plants through tissue culture* (pp134-158) Natl Tech Inf Serv, US Dept Comm, Springfield, VA
- Trinh TH, Lie-Schricke H, Tran Thanh Van K (1981) Direct *in vitro* bud formation from fragments and thin cell layers of different organs of the winged bean (*Psophocarpus tetragonolobus* L. DC). *Z Pflanzenphysiol* 102:127-139
- USDA Agricultural Statistics (1986) United States Government Printing Office, Washington
- van den Ende G, Croes AF, Kemp A, Barendse GWM (1984a) Development of flower buds in thin-layer cultures of floral stalk tissue from tobacco: role of hormones in different stages. *Physiol Plant* 61:114-118
- van den Ende G, Croes AF, Kemp A, Barendse GWM, Kroh M (1984b) Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum*. *Physiol Plant* 62:83-88
- Wardell WR, Skoog F (1969) Flower formation in excised tobacco stem segments; I. Methodology and effects of plant hormones. *Plant Physiol* 44:1402-1406

Table 3. Names and sources of tomato cultivars used in thin cell layer shoot morphogenic competence experiments.

Cultivar	Source
Large Red Cherry	Self-pollinated plants
Red Alert	Self-pollinated plants
Ohio 7814	S.Z. Berry, Ohio State Research and Development Center, Wooster, Oh
Ohio 7663	S.Z. Berry
Ohio 8243	S.Z. Berry
BL 6807	S.Z. Berry
Saladette	C.M. Rick, Dept. of Vegetable Crops, Univ. of California, Davis, Cal.
VF36	C.M. Rick
Ferry Morse 6203	DNA Plant Technology Corp., Cinnaminson, N.J.
Campbell 37	DNA Plant Technology Corp.
Campbell 1327	Twilley Seed Co., Inc., Trevoise, Pa.

Table 4. Morphogenic response of tomato thin cell layers from pedicel and peduncle tissue to media containing different levels of kinetin, IAA, IBA and NAA.

Kinetin (μ M)	Auxin		Morphogenic Response			
	(μ M)	(Type)	Callus	Shoots	Roots	Flowers
0.001	0.001	IAA	- †	-	-	-
		IBA	-	-	-	-
		NAA	-	-	-	-
	0.1	IAA	-	-	+	-
		IBA	-	-	+	-
		NAA	-	-	+	-
	10	IAA	-	-	+	-
		IBA	-	-	+	-
		NAA	-	-	+	-
0.1	0.001	IAA	+	-	-	-
		IBA	+	-	-	-
		NAA	+	-	+	-
	0.1	IAA	+	-	-	-
		IBA	+	-	-	-
		NAA	+	-	+	-
	10	IAA	+	-	+	+ ††
		IBA	+	-	-	-
		NAA	+	-	-	-
10	0.001	IAA	-	+	-	-
		IBA	+	+	-	-
		NAA	-	+	-	-
	0.1	IAA	-	+	-	-
		IBA	+	-	-	-
		NAA	+	-	+	-
	10	IAA	-	+	-	-
		IBA	+	-	-	-
		NAA	+	-	-	-

†Either a positive (+) or no (-) morphogenic response of the explants by the imposed treatment.

††Flowering occurred on plantlets with elongated shoots.

Table 5. Shoot and root formation on tomato thin cell layer explants from pedicel tissue as influenced by different concentrations of kinetin, IAA, IBA and NAA.

Kinetin (μ M)	Auxin		Shoots		Roots	
	(μ M)	(Type)	Frequency (%)	Number explant ⁻¹ †	Frequency (%)	Number explant ⁻¹ ††
0.001	0.001	IAA	0 (0/14)	—	0 (0/14)	—
		IBA	0 (0/20)	—	0 (0/20)	—
		NAA	0 (0/16)	—	0 (0/16)	—
	0.1	IAA	0 (0/16)	—	14 (2/16)	2.5
		IBA	0 (0/20)	—	20 (4/20)	1.5 ± 0.5†††
		NAA	0 (0/16)	—	38 (6/16)	1.7 ± 0.3
	10	IAA	0 (0/8)	—	100 (8/8)	6.0 ± 1.8
		IBA	0 (0/20)	—	100 (20/20)	3.9 ± 0.6
		NAA	0 (0/16)	—	63 (10/16)	2.2 ± 0.5
0.1	0.001	IAA	0 (0/16)	—	0 (0/16)	—
		IBA	0 (0/10)	—	0 (0/10)	—
		NAA	0 (0/10)	—	20 (2/10)	2.0
	0.1	IAA	0 (0/16)	—	0 (0/16)	—
		IBA	0 (0/20)	—	0 (0/20)	—
		NAA	0 (0/10)	—	80 (8/10)	4.5 ± 0.1
	10	IAA	0 (0/16)	—	25 (4/16)	1.5 ± 0.5
		IBA	0 (0/11)	—	0 (0/11)	—
		NAA	0 (0/16)	—	0 (0/16)	—
10	0.001	IAA	75 (12/16)	2.7 ± 0.8†††	0 (0/16)	—
		IBA	5 (1/19)	1.0	0 (0/19)	—
		NAA	57 (8/14)	1.8 ± 0.6	0 (0/14)	—
	0.1	IAA	62 (10/16)	1.8 ± 0.6	0 (0/16)	—
		IBA	0 (0/20)	—	0 (0/20)	—
		NAA	0 (0/20)	—	20 (4/20)	1.5 ± 0.5
	10	IAA	25 (4/16)	2.0 ± 0.7	0 (0/16)	—
		IBA	0 (0/20)	—	0 (0/20)	—
		NAA	0 (0/10)	—	0 (0/10)	—

†Mean number of shoots per explant.

††Mean number of roots per explant.

†††Standard error of the mean.

Table 6. Flower production and explant quality ratings for tomato thin cell layer explants from pedicel and peduncle tissue as influenced by eight different surface-sterilization treatments.

Sterilization treatment	N†	Explants with floral buds	Floral buds explant ⁻¹ ††	Explant quality†††
15% Bleach/8 min.	13 (81%)	15%	0.5	1.4±0.3
15% Bleach/10 min.	13 (81%)	31%	2.0	2.8±0.2
25% Bleach/5 min.	7 (44%)	29%	1.5	1.6±0.5
25% Bleach/10 min.	13 (81%)	0%	0.0	1.9±0.4
50% Bleach/5 min.	9 (56%)	0%	0.0	1.9±0.5
0.1% HgCl ₂ /5 min.	16 (100%)	6%	1.1	2.1±0.4
0.1% HgCl ₂ /10 min.	16 (100%)	0%	0.0	1.0±0.4
0.2% HgCl ₂ /5 min.	16 (100%)	0%	0.0	0.3±0.2
0.2% HgCl ₂ /10 min.	16 (100%)	0%	0.0	0.0±0.0

†Number of uncontaminated explants 30 days after culture initiation (16 total).

††Mean number of floral buds per uncontaminated explant.

†††Explant quality (assessed at day 30)

0 = Dead

1 = Mostly necrotic, with some green tissue

2 = Good; Moderately necrotic

3 = Excellent; no necrosis evident

Table 7. Shoot organogenesis on thin cell layer explants from tomato pedicel tissue on media containing 1nM IAA and 10 μ M of either zeatin, BA, kinetin or 2iP.

Cytokinin	Shoot regeneration†	
	Frequency (%)	Number explant ⁻¹ ††
Zeatin	50	2.8 a†††
BA	50	2.5 ab
Kinetin	20	1.7 b
2iP	15	1.3 b

†Based on 20 explants per treatment

††Mean number of flower buds per responding explant.

†††Mean separation by LSD, 5% level.

Table 8. Mean shoot number and the frequency of responding explants among thin cell layers explants from pedicel tissue of 11 tomato cultivars.

Cultivar	Shoot		
	Number† explant ⁻¹ ††	Frequency (%)	Response
Large Red Cherry	5.3 a†††	42	(10/24)
Ohio 7814	3.9 ab	63	(15/24)
BL 6807	3.7 ab	54	(13/24)
Saladette	3.1 bc	42	(10/24)
VF36	3.0 bc	58	(14/24)
Ohio 8243	2.6 bc	54	(13/24)
Ferry Morse 6203	2.4 bc	38	(9/24)
Campbell 37	2.4 bc	29	(7/24)
Ohio 7663	2.1 bc	50	(12/24)
Campbell 1327	1.8 c	33	(8/24)
Red Alert	1.3 c	38	(9/24)

†Based on the mean shoot number of responding explants.

††Mean number of shoots per responding explant.

†††Mean separation by Duncan's Multiple Range Test, 5% level.

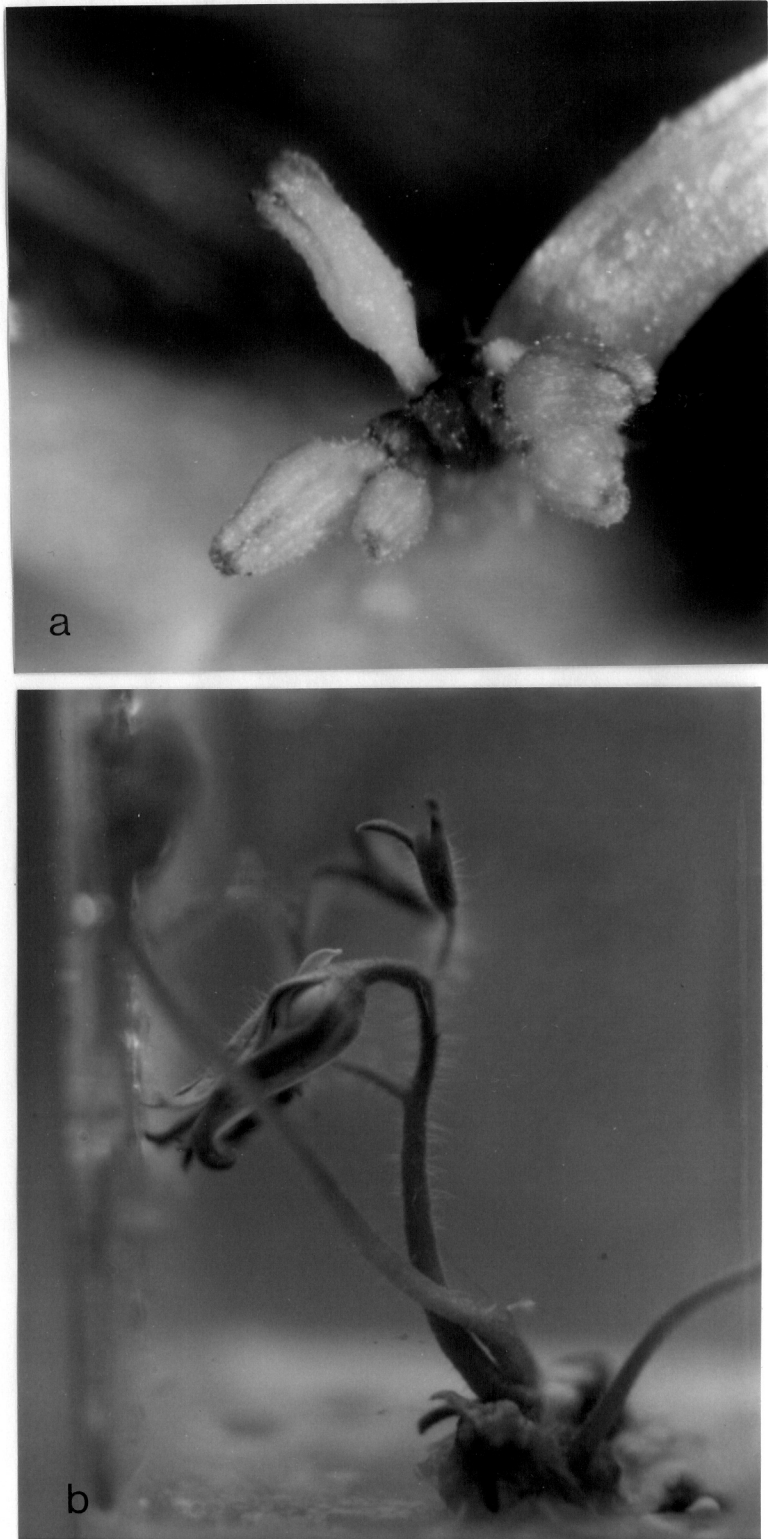


Figure 1. Floral morphogenesis on tomato ('Red Alert') thin cell layer explants from pedicel tissue: a) small flower buds were present on 12 wk old explants (32 $\times$); b) fruit formed after an additional 4 wks (4 $\times$).

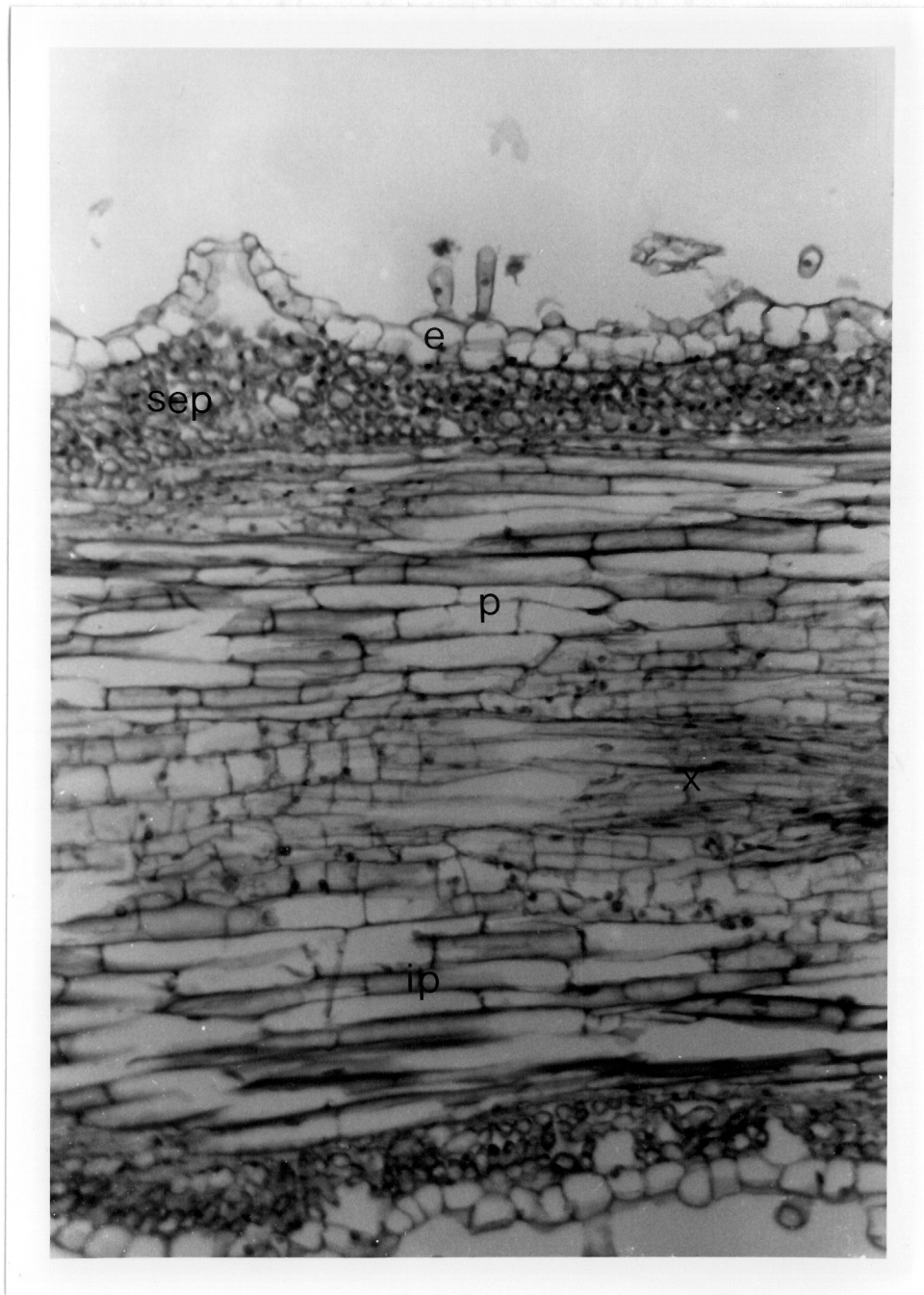


Figure 2. Longitudinal section of a freshly isolated tomato 'Ohio 7814' thin cell layer explant derived from pedicel tissue: such explants consisted of epidermis (e), subepidermal parenchyma cells (sep), phloem (p), xylem (x) and inner phloem (ip) (500 $\times$)

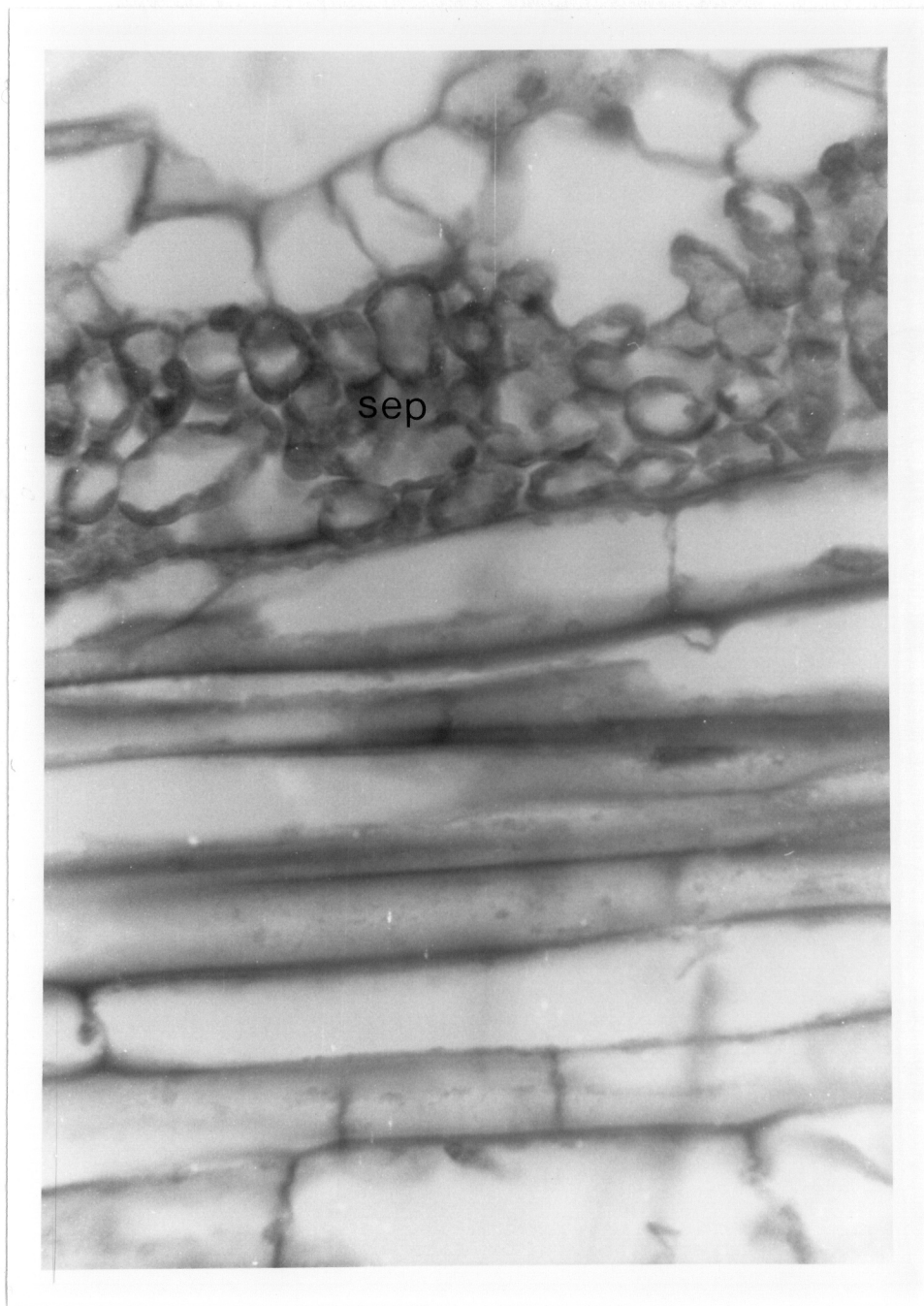


Figure 3. Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 7 days after culture initiation: explants were excised from pedicel tissue and consisted of small subepidermal parenchyma cells (sep) which lacked areas of active cell division (2000 $\times$).

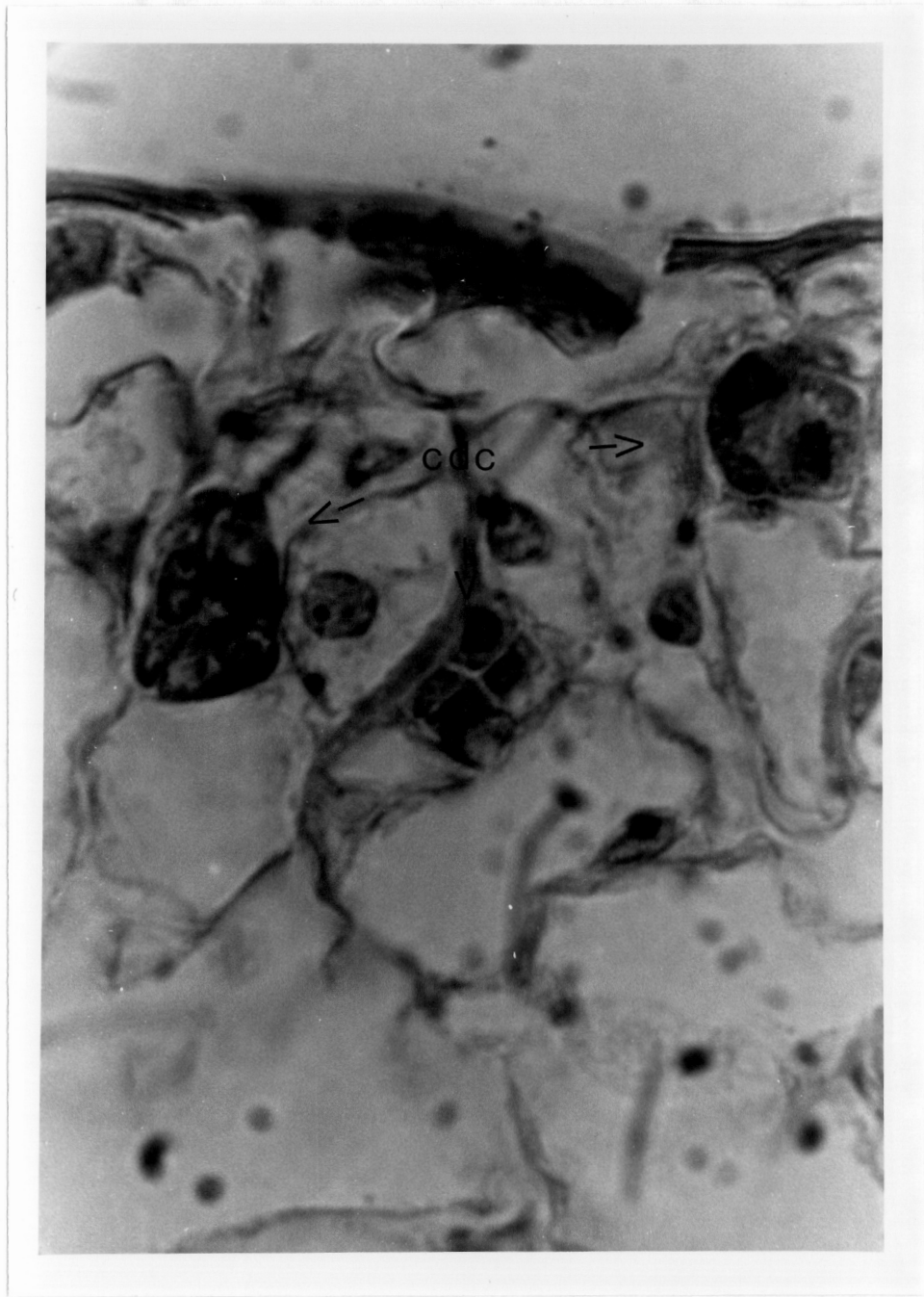


Figure 4. Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 7 days after culture initiation: notice the presence of active cell division centers (cdc) scattered throughout the explant (3150x).

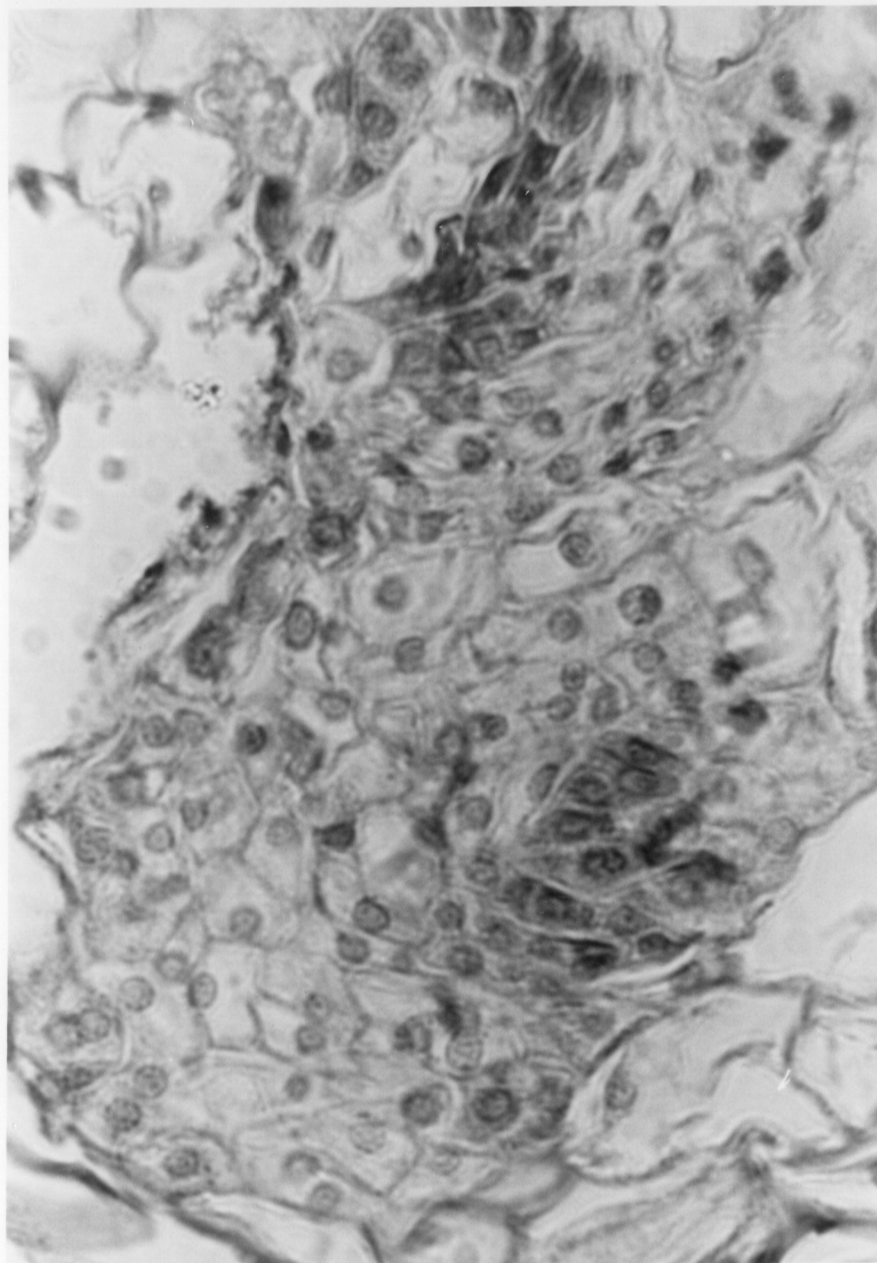


Figure 5. Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation: large areas with numerous cell divisions were scattered throughout the subepidermal layer of the explants (2000 $\times$).

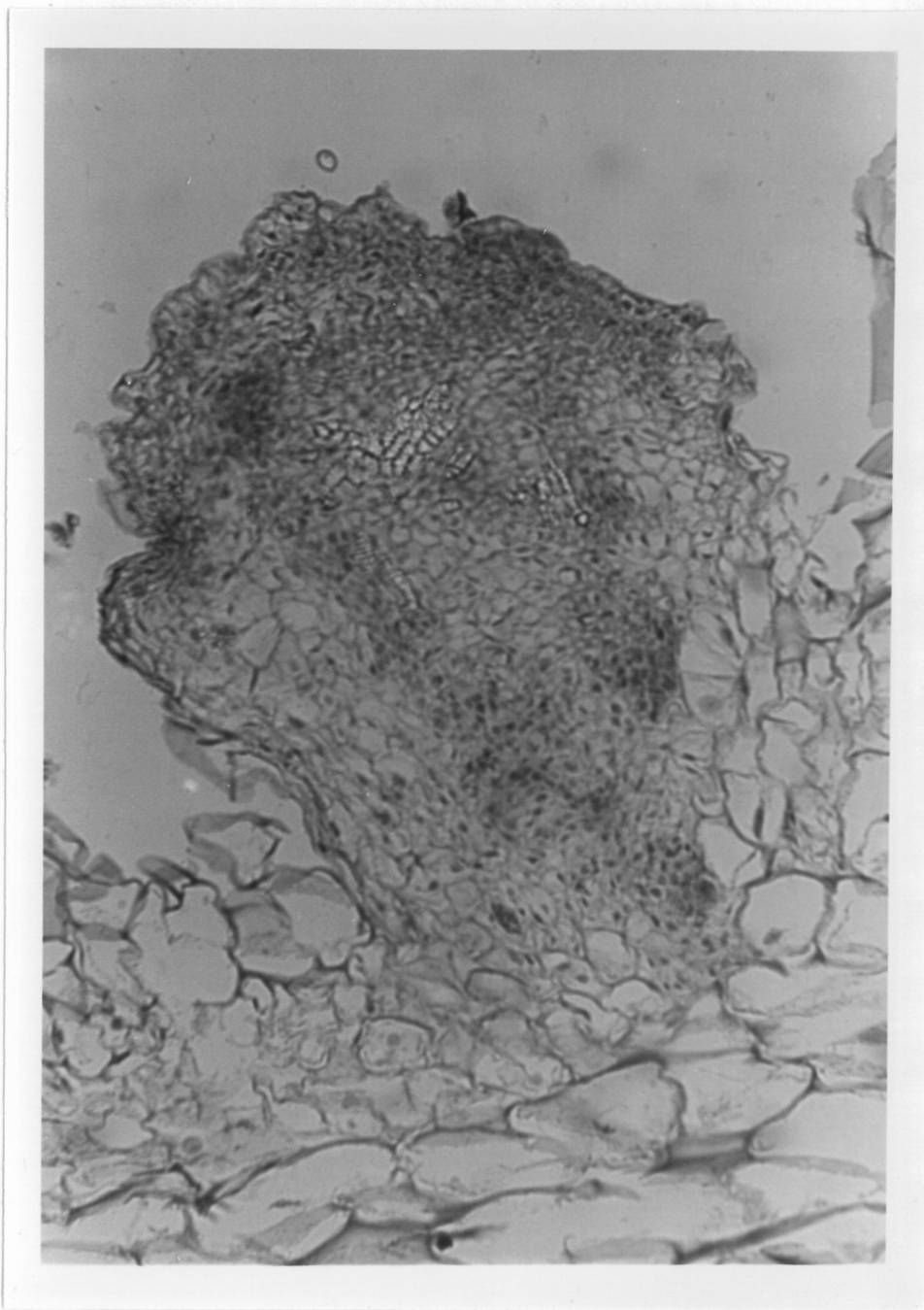


Figure 6. Longitudinal section through a meristematic center of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation: meristematic centers appeared to have originated from subepidermal tissue (500 $\times$).

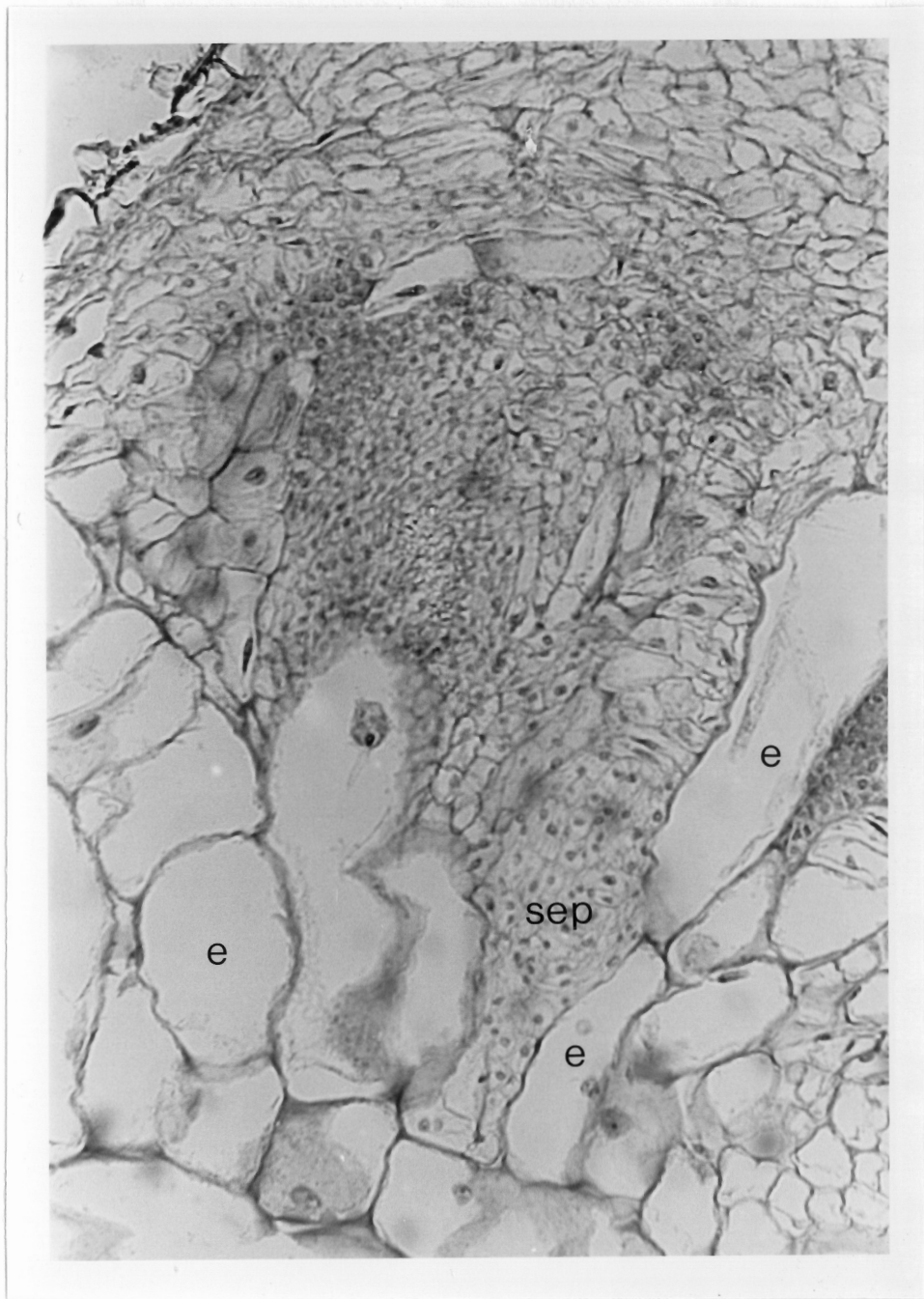


Figure 7. Longitudinal section through a meristematic center of a tomato 'Ohio 7814' thin cell layer explant 14 days after culture initiation: actively dividing subepidermal (sep) cells have protruded through the original epidermal (e) cells (2000 $\times$).

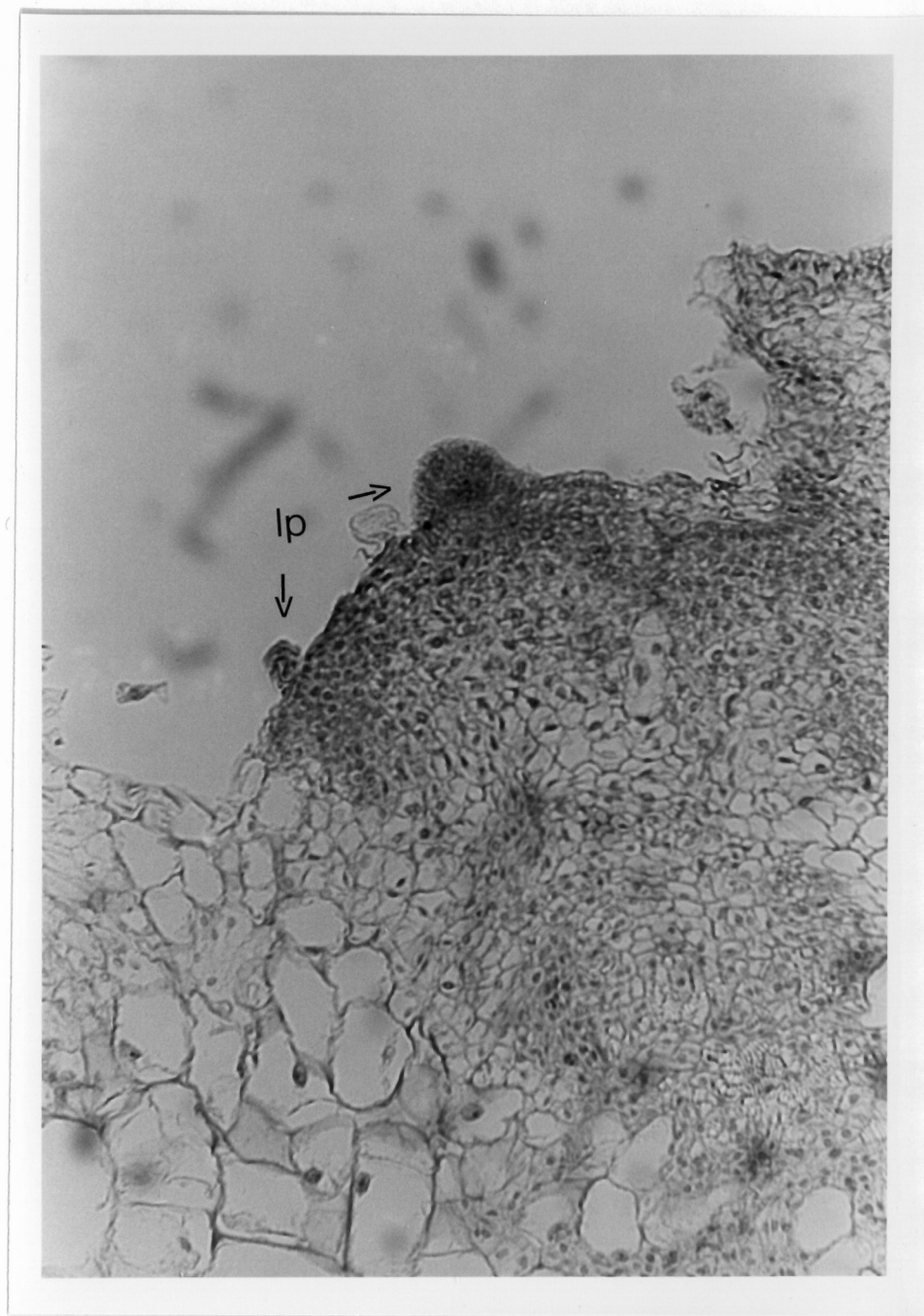


Figure 8. Transverse section of a meristematic region of a tomato 'Ohio 7814' thin cell layer explant 21 days after culture initiation: this figure shows an oblique longitudinal view of a meristematic region with small leaf primordia (lp) that originated from subepidermal cells (500 $\times$).

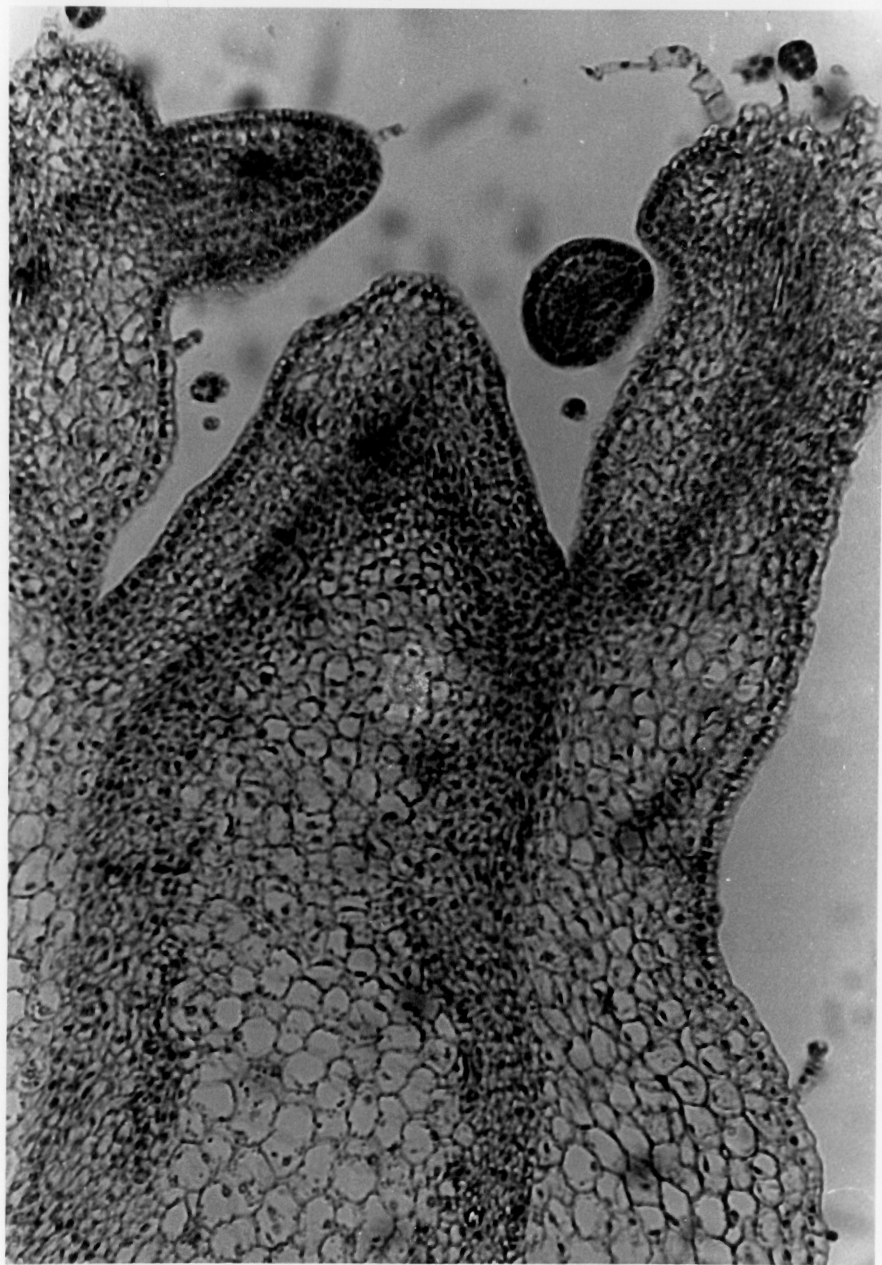


Figure 9. Longitudinal section through a shoot tip present on a tomato 'Ohio 7814' thin cell layer explant 28 days after culture initiation: shoot apices with well-developed leaves were observed on 50% of the explants examined (500 $\times$).



Figure 10. Longitudinal section of a tomato 'Ohio 7814' thin cell layer explant 28 days after culture initiation: multiple shoot apices with differentiated leaves developed on the basal region of many explants (125×)



Figure 11. Shoot morphogenesis on a tomato 'Ohio 7814' thin cell layer explant 8 wks after culture initiation (20 $\times$).

Chapter Four: Variation for Genetic Recombination Among Tomato Plants Regenerated from Three Tissue Culture Systems

Abstract

Shoot morphogenesis was compared among 2 tomato inbred lines, 2 mutant lines and their 8 reciprocal F₁ hybrids in three tissue culture systems. The number of shoots per explant was greatest on tomato thin cell layer explants (3.9), intermediate on cotyledon calli (1.6) and poor on micropropagated shoot tips (1.3). Genetic recombination rates of hybrid plants regenerated from different tissue culture treatments were compared by backcrossing regenerated plants with mutant parents and comparing the observed crossover frequencies with those expected based on control plants raised from seed in the greenhouse. Increased recombination rates and map distance were observed among plants from micropropagated shoot tips (4.5%-5.9%), cotyledon calli (3.7%-8.5%) and

thin cell layers (2.8%-6.5%) between the *sunny* (*sy*) and *baby leaf syndrome* (*bls*) markers which flank the centromere on chromosome 3. Similar increases in recombination rates and map distance among plants from micropropagated shoot tips (4.5%-6.1%), cotyledon calli (3.2%-3.9%) and thin cell layers (3.9%-5.7%) were observed between *white virescence* (*wv*) and *anthocyanin reduced* (*are*) on chromosome 2. Conversely, a decreased map distance was observed between *bls* and the *solanifolia* (*sf*) locus which is more distal to the centromere on the same arm of chromosome 3 as *bls*. Increased map distance between the former loci may be stimulated by elevated levels of nutrients present in tissue culture media combined with the incubation of cultures under environmental conditions that promote rapid growth.

Introduction

A prolonged callus phase for plants may lead to change in ploidy (aneuploidy or polyploidy), single gene mutation, change in DNA sequence copy number, DNA amplification, or chromosome breakage which may subsequently result in inversion, translocation, deletion, deficiency, or mitotic recombination (Larkin and Scowcroft 1981; Evans and Sharp 1983; Evans et al. 1984; Larkin 1987; Lee and Phillips 1988). However, not all culture induced changes are deleterious. Increased disease resistance has been reported in plants regenerated from protoplast-derived calli of potato (Shepard et al. 1980) and tomato (Shahin and Spivey 1986), celery cell cultures (Heath-Paliuso et al. 1988), and callus cultures of maize (Gengenbach et al. 1977) and sugar cane (Lorz and Scowcroft 1983). In addition, increased freezing tolerance has been reported in wheat (Lazar et al. 1988) and herbicide tolerance in carrot (Nafziger et al. 1984). It is well

known that genetic and epigenetic changes occur during tissue culture, but whether one tissue culture process is more conducive to such change than another has not been extensively addressed.

Caulogenesis (shoot organogenesis) from leaf tissue or protoplast calli allows the opportunity to study genetic changes that may occur during culture. Plants, heterozygous for selected marker loci, can be regenerated from sectors of calli, or individual cells, and self-pollinated or backcrossed to plants that are homozygous for the markers to determine if a genetic change has occurred. Any deviation from the expected distribution indicates a genetic change, and the regenerants are classified as somaclonal variants if the change is persistent for several sexual generations (Schaeffer et al. 1985).

Mitotic recombination may occur as somatic crossing over or sister chromatid exchange (Larkin and Scowcroft 1981). The former has been identified in tobacco, tomato and soybean, and occurrences may be increased by environmental factors, mutagenic agents (Evans and Paddock 1976, 1980) or repeated subculture (Lorz and Scowcroft 1983). Somatic crossing over has been demonstrated to occur at high rates (20.6/dividing cell) in barley (Schubert et al. 1980). A somatic crossover during callus culture of heterozygous plants would result in sectors of calli that are homozygous for the studied loci. For example, all sugary (*su/su*) kernels have been observed on ears of a self-pollinated maize plant regenerated from callus tissue derived from plants heterozygous for the sugary allele (McCoy 1980).

Elevated recombination rates during meiosis have been identified in plants regenerated from callus culture. Sibi et al. (1984) reported an increase in recombination rates between genetic loci in tomato (*Lycopersicon esculentum* L. 'Monalbo') plants regenerated from callus cultures. An 11-13% increase in map distance was observed between two

pairs of loci in the somaclones. They concluded that enhanced map distance could be attributed to DNA amplification or rearrangement, or may be related to a tissue culture influence on meiosis. Increases in DNA content have been attributed to DNA amplification (Berlyn 1983) and may involve repeated DNA sequences (Lapitan et al. 1988).

DNA amplification in plants from tissue culture may have serious implications to their use in a breeding program. Reed and Burns (1989) observed that F_1 hybrid plants generated by crossing tobacco plants of an anther-derived doubled haploid (DH) line with its anther-donor displayed spindle abnormalities (lagging chromosomes, multipolar spindles) during mitosis and meiosis. No gross abnormalities were observed in either parental line; however, the anther-derived line had 41% more nuclear DNA than its anther-donor plant. Analysis of mitotic cells of F_1 plants revealed that 26% of the plants had fewer chromosomes (13-30) than the parents ($2n=4x=48$). All F_1 plants produced seed, but pollen fertility was reduced by 21%. No reduction in female fertility was observed. The difference in nuclear DNA between the parents was believed to be responsible for the occurrence of spindle abnormalities in the F_1 hybrids. Reduced fertility and chromosome loss in plants regenerated from tissue culture may limit their utilization as breeding stock.

Changes in plant phenotype and DNA content have not been limited to plants propagated *in vitro*. Heritable changes in plant height and weight (Durrant 1962a, b), hairiness of capsule septa (Durrant and Nicholas 1970; McLellan and Durrant 1973), nuclear DNA content (Evans et al. 1966), peroxidase isozyme banding patterns (Cullis 1981) and ribosomal DNA (rDNA) amounts (Cullis and Charlton 1981) have been induced in flax (*Linum usitatissimum* L.) genotrophs. Durrant (1962a) defined flax genotrophs as plants that underwent heritable phenotypic changes induced by different fertilizer combinations

when the plants were grown under environment conditions that promoted rapid growth. Large genotrophs (based on plant weight) were induced by supplying flax cultivars 'Stormont Cirrus' and 'Lyrar Prince' elevated levels of combined nitrogen, phosphorus and potassium, whereas small genotrophs were induced by increased nitrogen and potassium or phosphorus alone at temperature regimes of 19°C day/11°C night and 14-16 h photoperiods (Durrant 1962a, b, 1971). Reciprocal grafts between stocks and scions of large and small genotrophs confirmed that nutrient application was the contributing factor in the induction of large and small genotrophs (Durrant 1962a). Genotropic characteristics (plant weight) were dominant in diallel crosses among large and small genotrophs, and other flax and linseed varieties (Durrant and Tyson 1964). Large genotrophs had 10-16% more DNA than small genotrophs which had 5-10% less DNA than control plants (Evans et al. 1966). Increased DNA content of large genotrophs was attributed to an elevated number of intermediately repeated DNA sequences (Cullis 1973), whereas the decrease in DNA content of small genotrophs was attributed to a loss of some repeated and conserved sequences (Cullis 1977). Differences in specific DNA sequences in flax leaves from control plants, callus tissue, leaves of plants regenerated from callus, and *Agrobacterium* transformed calli have also been characterized (Cullis and Cleary 1986). Changes in flax tissue cultures were similar to those found when the genotrophs were induced.

Somaclonal variation can be an advantageous source of novel variation, but when genetically uniform plants are the desired end product of a tissue culture process, a high frequency of variants is a disadvantage. It has generally been accepted that micropropagated shoot tips or single node explants are genetically uniform with less variation than would occur among plants regenerated from callus (Beauchesne 1982; Bhojwani and Razdan 1983). However, Cassells et al. (1983) observed similar variation among potato

(*Solanum tuberosum* 'Golden Wonder') plants regenerated from single node explants, stem-derived callus and cell cultures. Therefore, the objective of this study was to compare the frequency and types of variation observed among tissue culture derived tomato plants obtained from: 1) micropropagated shoot tips, 2) cotyledon calli, and 3) thin cell layer (TCL) explants taken from pedicels of flowering plants grown in the greenhouse. The effect of the tissue culture processes on both mitotic and meiotic recombination was assessed by the segregation of known genetic markers in the test plants.

Materials and Methods

Plant Material

Self-pollinated tomato lines (*Lycopersicon esculentum* Mill. cvs. Red Alert and Large Red Cherry), and 2 mutant lines (LA 1430 and LA 1526¹⁵), each of which has at least 2 gene mutations located on a single chromosome, were used. LA 1430 is homozygous recessive for *sunny* (*sy*), *baby leaf syndrome* (*bls*) and *solanifolia* (*sf*) which appear phenotypically as whitish cotyledons and leaves that emerge yellow before turning green (*sy*), anthocyanin lacking and compact growth habit (*bls*), and entire primary leaves (*sf*) (Mutschler et al. 1987). The centromere is located between the *sy* and *bls* genes, and the *sf* locus is located more distally to the centromere than *bls* on the same arm of chromosome 3. LA 1526 is homozygous recessive for *white virescence* (*wv*), *anthocyanin reduced* (*are*) and *dwarf* (*d*) plant height. These markers appear phenotypically as whitish-yellow

¹⁵ Obtained from Dr. C.M. Rick, Dept. of Vegetable Crops, University of California, Davis, Cal.

cotyledons and leaves that emerge white and become speckled before greening (*wv*), almost no anthocyanin until fruit set (*are*) and dwarf (*d*) height. All three genes for LA 1526 are located on chromosome 2 which has a telocentric centromere that is located 41 map units from the *wv* locus. Each set of mutations occur within 40 map units along the respective chromosome. Crosses were made between each of the *L. esculentum* cultivars and mutant lines to generate F₁ hybrids. All hybrids (including reciprocals) and the parents (total of 12 genetic lines) were placed in micropropagation, callus and TCL culture systems.

Micropropagation

Seeds of each genotype were surfaced-sterilized in 50% bleach¹⁶ (2.5% NaOCl) plus 0.1% 'Tween 20' for 30 min, rinsed 3× in sterile distilled water and placed into autoclaved 150×20 mm glass petri dishes¹⁷ that contained sterile filter paper (Whatman No. 50¹⁸), moistened with sterile distilled water. After germination (10-14 days), each epicotyl with a portion of the hypocotyl was aseptically removed and transferred to 25×150mm borosilicate glass tubes that contained 20 ml of MS basal medium (Murashige and Skoog 1962), 2% sucrose, and 5 μM N⁶-benzyladenine (BA) (Schnapp and Preece, 1986). The medium pH was adjusted to 5.8 with 1N KCl or KOH prior to the addition of 7 mg ℓ⁻¹ agar (Difco Bacto¹⁹). Kaputs²⁰ were used to cover the culture vessels prior to autoclaving at 121°C and 1 kg m⁻² s⁻¹ for 20 min. The cultures were

¹⁶ Manufactured by Wonder Chemical Corp, Fairless Hills, Pa.

¹⁷ Manufactured by Corning Glass Works, Corning, N.Y.

¹⁸ Obtained from Fisher Scientific, Pittsburgh, Pa.

¹⁹ Manufactured by Difco Laboratories, Detroit, Mich.

²⁰ Manufactured by Bellco Glass, Inc., Vineland, N.J.

incubated under a 16 h photoperiod at 25°C and a photosynthetic photon flux density (PPFD) of $50 \mu\text{E m}^{-2}\text{s}^{-1}$ provided by 2 cool white fluorescent lamps²¹ (40 watts) for four weeks. At the end of this period, shoot tip and single node explants (5 per culture vessel) were transferred to Magenta²² (GA7) boxes that contained 50 ml of the same medium. Microshoots were subcultured to fresh medium of the same composition every 4 weeks for an additional 12 weeks at which time the number of axillary shoots per explant was recorded for each genotype. A completely random design was used.

Callus culture

Sterilized cotyledon explants, 50 for LA 1430 hybrids or 80 for LA 1526 hybrids, were prepared according to Sibi et al. (1984), and cultured in 100×15mm petri plates²³ that contained 20ml of MS salts, Bourgin and Nitsch vitamins (1967), 3% sucrose, $22.8 \mu\text{M}$ indole-3-acetic acid (IAA) and $18.6 \mu\text{M}$ kinetin (Padmanbham et al. 1974). The medium pH was adjusted to 5.8 with 1N KCl or KOH prior to the addition of $7 \text{ g } \ell^{-1}$ Difco Bacto agar, and was aseptically dispensed into petri plates after it was autoclaved. In preliminary studies, shoot regeneration was increased when Red Alert and Large Red Cherry cotyledon explants were incubated on medium described by Padmanbhan et al. (1974) (Table 19). Therefore, the medium described by Padmanbham et al. (1974) was used. Cultures were incubated as above, and after 3 weeks, the calli were subcultured to 100×80mm deep dish petri plates²⁴ that contained 65ml of the same medium, but with $5 \text{ mg } \ell^{-1}$ GA₃ and 1% agar. There were approximately 5 (LA 1430 hybrids) or 8 (LA

²¹ Manufactured by North American Philips Lighting Corp., Somerset, N.J.

²² Manufactured by Magenta Corp., Chicago, Ill.

²³ Manufactured by Fisher Scientific Co., Pittsburgh, Pa.

²⁴ Manufactured by Corning Glass Works, Corning, N.Y.

1526 hybrids) plates (each of which contained 10 calli) per inbred line and reciprocal F_1 hybrid. The number of calli producing shoots and the number of shoots per calli were recorded after 12 weeks. A completely random design was used.

Thin cell layers

Thin cell layer explants (80) were taken from pedicel tissue collected from inflorescences of each genetic line during flowering in the greenhouse. Whole inflorescences minus flowers were sterilized in 15% bleach plus 0.1% 'Tween 20' for 10 min., and rinsed twice in sterile distilled water. Medium consisted of MS salts plus 0.1 mg ℓ^{-1} thiamine•HCl, 100 mg ℓ^{-1} myo-inositol, 3% sucrose, 10 μ M zeatin, 1 nM IAA, 250 mg ℓ^{-1} cefotaxime. The pH was adjusted to 5.8 prior to the addition 1% Difco Bacto agar. Zeatin and cefotaxime were filter-sterilized and added to the medium after it was autoclaved. Explants (6) from each genetic line were cultured in 100×15mm square petri dishes²⁵ that contained 25 ml of medium. The number of explants producing shoots and the number of shoots per explant were recorded for each genetic line 12 weeks after culture initiation. A completely random design was used.

Statistical analysis of shoot numbers from each tissue culture treatment was performed using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS). Significant differences among the treatments and genetic lines were detected by using the plate(explant) mean square as the error term. Mean comparisons were conducted using the standard error of the mean.

²⁵ Manufactured by Miles Laboratories, Inc., Naperville, Ill.

Seed set in F₁ hybrids backcrossed to mutant parents

Shoots regenerated from each tissue culture system were rooted on MS basal medium after reaching 2 cm in height. After 2-4 weeks, rooted plants were transferred to plastic flats that contained 1 moss peat:1 vermiculite:1 perlite (ProMix BX²⁶) in the greenhouse, and acclimatized under intermittent mist (8s 16min⁻¹) for 1 week. A sample of 5 normal appearing (exhibiting wild type characteristics) plants per F₁ reciprocal hybrid family and culture system was selected and potted into classic 600²⁷ containers that contained 2 moss peat:1 weblite:1 sand, grown to maturity in the greenhouse under natural photoperiod at 20 ± 3°C. All plants were fertilized weekly with a 20:20:20 (400 ppm N) nutrient solution. F₁ hybrid plants from seed were used as controls. A minimum of 15 backcross pollinations were performed on each regenerated plant, and control F₁ hybrid. The regenerated plants and control F₁ hybrids were used as female parents because of low fruit and seed set of the mutants (Data not shown). F₁ hybrid plants were emasculated as described by Rick (1980), and fresh pollen was used in all pollinations. Backcross pollinations were conducted from July, 1988 to October, 1989. Seeds were extracted from each fruit and soaked in a 0.1% pectinase²⁸ and 1% HCl solution for 24 h to remove the mucilaginous layer surrounding the seed coat. The seeds were rinsed and treated in warm (52°C) distilled water for 30 min prior to planting in the greenhouse. The number of seed per fruit was counted for each treatment and analyzed using SAS GLM procedures. Mean comparisons were made using Duncans Multiple Range Test at the 5% level.

²⁶ Premier Brands, Inc., New Rochelle, N.Y.

²⁷ Obtained from Wetsel Seed Co., Inc., Harrisonburg, Va.

²⁸ Obtained from Sigma Chemical Co., St. Louis, Mo.

Mitotic Recombination

The effects of each tissue culture process on mitotic recombination was assessed by screening the regenerated plants for the expression of the respective mutant trait(s) after a minimum of four weeks in the greenhouse.

Map Distance Between Selected Marker Loci

The backcross progeny (44,583) were germinated in flats that contained 1 moss peat:1 vermiculite:1 perlite (Sunshine LC1²⁹), and scored for the presence of the mutant trait(s). Comparisons of map distance between the mutant loci were made among progeny of the regenerated plants and control plants grown from seed in the greenhouse. Map distance for each treatment was calculated by adding the number of single crossovers divided by the total, and the number of double crossovers divided by the total and then multiplying the frequency by 100 (Strickberger 1968). Data were analyzed by Chi-Square and interesting differences were compared using Z test for normal distribution. The percent change (absolute) in map distance was calculated by multiplying 95% confidence intervals by 100 (Koopmans 1987).

²⁹ Fisons Western (US) Inc., Downers Grove, Ill.

Results and Discussion

Tissue Culture Treatments

Micropropagation

Axillary shoot production was greater for Red Alert (1.8) and Large Red Cherry (1.6) than for LA 1430 (1.3) and LA 1526 (1.0; Table 9). The number of axillary shoots per explant on micropropagated shoot tips from F_1 hybrids between the cultivars and mutants was similar to the mutant parents.

Callus culture

Shoot production from cotyledon callus was greatest for Large Red Cherry (2.0), intermediate for LA 1430 (1.7) and poor for both Red Alert (1.1) and LA 1526 (1.1; Table 9). The number of shoots per explant for F_1 hybrids was similar to Large Red Cherry (LRC) when LRC was used as the female parent in crosses with LA 1430 (LRC $\times$ LA 1430); however, shoot organogenesis was equal to LA 1430 for the reciprocal F_1 hybrid (LA 1430 $\times$ LRC). The number of shoots per calli for F_1 hybrids between LRC and LA 1526 was equal to LA 1526. Shoot organogenesis for the F_1 hybrid Red Alert $\times$ LA 1430 was equal to the better parent (LA 1430), but the reciprocal hybrid (LA 1430 $\times$ Red Alert) produced more shoots per explant than either parent. Shoot regeneration for F_1 hybrids between Red Alert and LA 1526 was not significantly different from either par-

ent. Shoot organogenesis was generally greater for F₁ hybrids between the tomato cultivars and LA 1430 than for hybrids between the cultivars and LA 1526.

Thin cell layers

The number of shoots per thin cell layer explant was greatest for LRC (6.1), intermediate for LA 1430 (2.4) and poor for both Red Alert (1.4) and LA 1526 (1.1; Table 9). The mean number of shoots per explant was intermediate in hybrids between LRC and LA 1430 when the former was used as the female parent (LRC × LA 1430), and equal to LA 1430 for the reciprocal hybrid. Shoot regeneration was intermediate for F₁ hybrids between LRC and LA 1526. Shoot organogenesis was equal to LA 1430 for F₁ hybrids between Red Alert and LA 1430; however, the frequency of responding explants was less for the hybrids. In hybrids between Red Alert and LA 1526, more shoots were produced when the former was used as the female parent. The number of shoots per explant in the reciprocal hybrid was equal to LA 1526.

All hybrid families were capable of shoot regeneration in all of the tissue culture systems. The number of shoots per explant was greatest for thin cell layer explants (3.9 ± 0.37), intermediate for cotyledon calli (1.6 ± 0.07) and poor for micropropagated shoot tips (1.3 ± 0.04 ; Table 9). This trend was observed for all the parents and F₁ hybrids except for Red Alert and F₁ hybrids between Red Alert and LA 1430 (Figures 12, 13). More shoots were produced on micropropagated shoot tips of Red Alert (1.8) than on thin cell layer explants (1.4) or cotyledon calli (1.1). There was no difference in the number of shoots per explant among callus and thin cell layer cultures of the former; however, the frequency of responding explants was less for thin cell layers (Table 9). Although the frequency of responding explants was less for thin cell layer explants (37%) than

cotyledon calli (48%; Table 9), the difference in the number of shoots per explant compensated for the lower regeneration rate.

Seed set for F₁ hybrids backcrossed to mutant parents

There was no difference in the number of seed per fruit among reciprocal hybrids, and between cultivars with the same mutant parent. Therefore, data were pooled for all backcrosses within each mutant family, and the mean number of seed per fruit was compared for the different propagation methods. The number of seed per fruit was greatest when F₁ hybrid plants from micropropagated shoot tips (40.6) or plants grown from seed (37.5) were backcrossed to LA 1430 (Table 10). Seed set was reduced in backcrosses between hybrid plants regenerated from cotyledon calli (31.6) or thin cell layers (22.2) and LA 1430. Propagation method had no effect on seed set in backcrosses with LA 1526.

All micropropagated plants and plants from seed were fertile and produced seed after pollination; however, three plants each from cotyledon calli and thin cell layers were infertile on backcrossing, but produced limited seed after self-pollination. These plants were suspected to be tetraploid because they had larger flowers and leaves. The mean number of chloroplasts per guard cell pair in fully expanded leaves of these plants ranged from 14.3 to 19.7 which corresponds to that found for tetraploid tomato plants (Jacobs and Yoder 1989).

One explanation for reduced seed set in backcrosses between LA 1430 and plants regenerated from callus or thin cell layers could be the season in which the backcrosses were made. Backcrosses between F₁ hybrid plants from seed or from micropropagated

shoot tips and LA 1430 were performed in the summer and fall of 1988, respectively, whereas those for cotyledon calli and thin cell layers were performed in the spring of 1989. The spring of 1989 was very cloudy so that lower seed set may have been a result of low light conditions (Picken 1984). However, the same seasonal variation occurred with backcrosses involving LA 1526 for which the number of seed per fruit was generally low, but no differences in seed set among propagation methods were observed.

Mitotic recombination

A majority of the 1185 F₁ hybrid plantlets regenerated from tissue culture displayed the expected dominant phenotypic characteristics of the cultivar parents. Exceptions included the aforementioned sterile plants that were assumed to be tetraploid, and two plantlets from thin cell layer explants of LRC×LA 1430 with variegated leaves. However, the variegation was not persistent once the plants were transferred to the greenhouse.

Four possible mitotic recombinants were observed in F₁ hybrids between Red Alert and LA 1526 plants regenerated from cotyledon calli (2) and thin cell layers (2). Hybrid plants between LA 1526 (indeterminate) and Red Alert (bushy) were indeterminate like the LA 1526 parent. However, the regenerated plants were bushy like the Red Alert parent. These plants may have originated from cells that had undergone a somatic crossover between the centromere and the *bu* gene on chromosome 8. No evidence of mitotic crossing over between the marker genes on chromosome 2 (*wv*, *are*, *d*) or 3 (*sy*, *bls*, *sf*) was detected.

Map Distance Between Selected Marker Loci.

Heterogeneity χ^2 was used to detect differences for recombination rates among plants within the same treatment, and between reciprocal hybrids and cultivars. There were no differences among F_1 hybrid plants with the same mutant parent and treatment (Tables 11, 12, 13, 14, 23, 24, 25, 26) except for Red Alert $\times$ LA 1430 and LA 1430 $\times$ Large Red Cherry hybrid plants regenerated from cotyledon calli. Therefore, individual χ^2 analyses were conducted for the latter treatments, and will be discussed later. No differences were observed within the same treatment between the reciprocal hybrids (Tables 20, 21, 27, 28) and between the cultivars (Table 18, 29). Therefore, all data within the same mutant family and treatment were combined.

Recombination rates of F_1 hybrid control plants grown from seed and backcrossed to LA 1430 were similar to estimated frequencies calculated from previously published map distances (Mutschler et al. 1987). These plants had a map distance of 28 between *sy* and *bls* and 37 between *bls* and *sf* (Figure 14). An increase of single recombinations (+ *bls sf* and *sy* + +) between loci flanking the centromere was observed in plants regenerated from all of the tissue culture treatments (Table 15). Increased recombination rates between the *sy* and *bls* loci can be translated into an increased map distance between these markers. Plants derived from micropropagated shoot tips had a map distance of 33 between *sy* and *bls*. This represents an increase of 5 map units between *sy* and *bls*, or a relative change of 18.8%. The resulting 95% confidence interval for the absolute change in map distance between *sy* and *bls* for micropropagated plants was 4.4%-5.9%. Map distances between *sy* and *bls* for plants derived from cotyledon calli (32) and thin cell layers (31) were similarly increased by 3.7%-8.5% (17.3% relative increase) and 2.8%-6.5% (13.5% relative increase), respectively (Figure 14). The increase between *sy*

and *bls* was greater for plants from micropropagated shoot tips than for plants derived from thin cell layers.

Recombination rates for Red Alert×LA 1430 (Table 16) and LA 1430×LRC (Table 17) F₁ hybrid plants derived from cotyledon calli were not as uniform as the other treatments. Plants from these treatments had significant heterogeneity χ^2 values and were analyzed individually. Of the five Red Alert×LA 1430 plants derived from cotyledon calli, a significant increase in map distance between *sy* and *bls* was observed for three (Figure 15). A map distance of 33 was calculated between *sy* and *bls* for the latter plants which represents an increase of 5 map units. This represents an absolute change of 2.5%-7.3%, or a relative increase of 17.5%-18.5%. Only one of three LA 1430×LRC plants derived from cotyledon calli had a significantly increased map distance between *sy* and *bls* (Figure 16). This plant had a map distance of 37 between these loci which represents an increase of 11 map units between *sy* and *bls*, or a relative increase of 41.6%. The resulting 95% confidence interval for the absolute change between these loci was 8.7%-13.3%.

A similar increase in recombination rates was observed between the *white virescence* (*wv*) and *reduced anthocyanin* (*are*) markers (chromosome 2) among plants regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers (Table 18). Recombinants between *are* and *d* were not tabulated because it was impossible to distinguish between *wv* - + and *wv* - *d* seedlings. Plants grown from seed had a map distance of 16 between *wv* and *are*; however, plants from micropropagated shoot tips, cotyledon calli and thin cell layers had map distances of 21, 20 and 21, respectively (Figure 17). This represents an increase of 4.5-6.1% (33% relative increase) for plants

from micropropagated shoot tips, 3.2%-5.0% (25% relative increase) for plants from cotyledon calli and 3.9%-5.7% (29% relative increase) for plants from thin cell layers.

Increased map distance between selected marker loci has previously been reported for tomato (Sibi et al. 1984) and potato (Singsit and Veilleux 1990). Sibi et al. (1984) observed that tomato plants derived from cotyledon calli had a 13% relative increase in map distance between *bs* and *ms-32* (chromosome 1) and a 11% relative increase between *aa* and *d* (chromosome 2) compared to the controls. Singsit and Veilleux (1990) observed a range in relative increase of 22.4% to 64.1% in the distance between the centromere and the *y* (*yellow tuber flesh*) gene in potato plants derived from callus culture. However, this is the first report of an increase in map distance between selected marker loci among plants regenerated from micropropagated shoot tips and thin cell layers of tomato. This suggests that increased recombination rates and map distance may be typical of tomato plants regenerated from any tissue culture system.

Conversely, a decrease in the number of single crossovers (+ + *sf* and *sy bls* +) between the more distal loci on chromosome 3 of the LA 1430 families was observed in plants derived from micropropagated shoot tips and cotyledon calli, but not in plants derived from thin cell layers. Plants grown from seed had a map distance of 37 between the *bls* and *sf* loci (Figure 14). Plants derived from micropropagated shoot tips and cotyledon calli had map distances of 33 and 34, respectively, which represent a 2.8%-4.2% (9.5% relative decrease) and a 1.2%-3.4% (6.3% relative decrease) change from the control, respectively. There was no significant difference in map distance among plants from micropropagated shoot tips and cotyledon calli. Map distance between *bls* and *sf* among thin cell layer (36) plantlets and control (37) plants from seed was similar (Figure 14). The distance between *bls* and *sf* was 1.9%-3.7% (8.5% relative

increase) and 0.4%-2.8% (4.7% relative increase) greater among plants from thin cell layers (36) than plants from micropropagated shoot tips (33) and cotyledon calli (34), respectively.

Heritable changes in plant height and weight, nuclear DNA content, rDNA content, and peroxidase isozyme banding patterns have been observed in flax plants grown in the greenhouse (Durrant 1962a, b; Evans et al. 1966; Cullis 1981; Cullis and Charlton 1981). Plants that received elevated levels of combined nitrogen, phosphorus and potassium had greater plant weights and DNA content than the controls. The increased DNA content was evident across the entire genome of the affected plants (Evans et al. 1966), and was attributed to an increase in the number of intermediately repeated sequences (Cullis 1973). We suggest that the high levels of nitrogen, phosphorus and potassium in tissue culture media, combined with the incubation of cultures in an environment that promotes rapid growth, influences the meiotic recombination rates of *in vitro* derived tomato plants. Generally, increased recombination rates and map distance can be translated into an increase in the physical distance, or the amount of DNA between genetic markers. Therefore, these plants may have a greater DNA content per nucleus than plants grown from seed. An increase in DNA content could possibly be attributed to an increase in the number of repeated sequences located in heterochromatic regions of the chromosome.

Decreased recombination rates and map distance between the *bls* and *sf* loci may be the result of a structural change, such as a deletion or inversion, or a secondary effect of the increased recombination rates between the *sy* and *bls* loci. Stevens and Rick (1986) suggested that increased recombination rates in plants from callus culture may be a result of decreased recombination in other areas caused by "chromosome structural

hybridity." Cornu et al. (1989) demonstrated that recombination modifiers exist in *Petunia hybrida* which influence meiotic recombination frequencies between marker loci located on chromosomes 1, 2, 3, 5, 6 and 7. They observed that meiotic modifiers increased recombination between marker loci on chromosomes 1, 2, 5, 6 and 7, and decreased recombination frequencies between markers located on chromosome 3. Our data demonstrate that plants with the greatest distance between *sy* and *bls* have the shortest distance between *bls* and *sf*. For example, plants derived from micropropagated shoot tips and cotyledon calli have the greatest map distance between *sy* and *bls* (33 and 32, respectively), and the shortest distance between *bls* and *sf* (33 and 34, respectively). Therefore, the increase in map distance between *sy* and *bls*, and *wv* and *are* may be caused by a recombination modifier that is activated when plants are placed *in vitro*. The decrease in recombination frequencies observed between *bls* and *sf* may be controlled by the same, or a similar recombination modifying factor.

Summary

These experiments have demonstrated that shoot morphogenesis was greater on thin cell layer explants (3.9) than on cotyledon calli (1.6) or micropropagated shoot tips (1.3). This trend was observed for 11 of 14 genetic stocks.

We have also demonstrated that recombination rates between selected marker loci were more uniform among plants regenerated from thin cell layers than among plants regenerated from cotyledon calli. Inflated recombination rates and map distances were observed in all the tissue culture treatments. Increased map distance was observed among

plants from micropropagated shoot tips (4.5% to 5.9%; 18.8% relative change) cotyledon calli (3.7%-8.5%; 17.3% relative change) and thin cell layers (2.8%-6.5%; 13.5% relative change) between the *sy* and *bls* loci which flank the centromere on chromosome 3. There was also an increase in map distance between *wv* and *are* loci located 41 map units from the centromere on chromosome 2. Plants from micropropagated shoot tips, cotyledon calli and thin cell layers had an increased map distance of 4.5%-6.1% (33% relative change), 3.2%-5.0% (25% relative change) and 3.9%-5.7% (29% relative change), respectively, between *wv* and *are* compared to plants grown from seed. The distance between *are* and *d* was not determined because it was impossible to distinguish between *wv*-+ and *wv*-*d* seedlings. Sibi et al. (1984) observed a similar increase in map distance between *bs* and *ms*-32 (13%) located on chromosome 1, and *aa* and *d* (11%) located on chromosome 2 for plants derived from callus culture. The increase in recombination rates and map distance may be due to an increase in nuclear DNA content, or the activation of a recombination modifier which may be brought about by the high levels of macro-nutrients (N, P and K) supplied under environmental conditions that promote rapid growth. Evans et al. (1966) have reported that elevated levels of combined nitrogen, phosphorus and potassium increased the nuclear DNA content in the form of intermediately repeated sequences of flax plants grown under environmental conditions that promote rapid growth.

A decrease in recombination rates and map distance was observed between the *bls* and *sf* loci located on the same arm of chromosome 3. We suggest that this decrease is a result of the increased frequency of *sy* and *bls* recombinants, or may be controlled by the activation of recombination modifiers as observed for *Petunia hybrida*. This is supported by the observation that plants with the greatest recombination rates in the *sy* - *bls* region had the lowest recombination frequency in the *bls* - *sf* region. Further experiments

should be conducted to determine if the increased map distance corresponds to an increase in nuclear DNA content, and to determine if the increased map distance could be attributed to an increase in the number of repeated or specific sequences. It would also be of interest to determine if the change in map distance could be controlled by manipulating the macro-nutrient concentration of the tissue culture media, and if changes in map distance are passed to future generations.

Literature Cited

- Beauchesne, G. 1982. Appearance of plants not true to type during *in vitro* plant propagation. In: Earle, E.D., Demarly, Y. (eds). Variability in plants regenerated from tissue culture. Praeger Publishers, New York. pp. 268-272.
- Berlyn, M.B. 1983. Patterns of variability in DNA content and nuclear volume in regenerating cultures of *Nicotiana tabacum*. Can. J. Genet. Cytol., 25:354-360.
- Bhojwani, S.S. and Razdan, M.K. 1983. Plant tissue culture: theory and practice. Elsevier, Amsterdam, pp. 313-372.
- Bourgin, J.P. and Nitsch, J.P. 1967. Obtention de *Nicotiana* haploids à partir d'étamines cultivées in vitro. Ann. Physiol. Veg., 9:377-382.
- Cassells, A.C., E.M. Goetz and S. Austin. 1983. Phenotypic variation in plants produced from lateral buds, stem explants and single-cell-derived callus of potato. Potato Res., 26:367-372.
- Cornu, A., Farcy, E. and Mousset, C. 1989. A genetic basis for variations in meiotic recombination in *Petunia hybrida*. Genome, 32:46-53.
- Cullis, C.A. 1973. DNA differences in flax genotrophs. Nature, 243:515-516.
- Cullis, C.A. 1977. Molecular aspects of the environmental induction of heritable changes in flax. Heredity, 38:129-154.
- Cullis, C.A. 1981. Environmental induction of heritable changes in flax: defined environments inducing changes in rDNA and peroxidase isozyme band pattern. Heredity, 47:87-94.
- Cullis, C.A. and L. Charlton. 1981. The induction of ribosomal DNA changes in flax. Plant Sci. Lett., 20:213-217.
- Cullis, C.A. and W. Cleary. 1986. DNA variation in flax tissue culture. Can. J. Genet. Cytol., 28:247-251.
- Durrant, A. 1962a. The environmental induction of heritable change in *Linum*. Heredity, 17:27-61.
- Durrant, A. 1962b. Induction, reversion and epitrophism of flax genotrophs. Nature, 196:1302-1304.
- Durrant, A. 1971. Induction and growth of flax genotrophs. Heredity, 27:277-298.
- Durrant, A. and D.B. Nicholas. 1970. An unstable gene in flax. Heredity, 25:513-527.

- Durrant, A. and H. Tyson. 1964. A diallel cross of genotypes and genotrophs of *Linum*. *Heredity*, 19:207-227.
- Evans, D.A. and E.F. Paddock. 1976. Comparison of somatic crossing over frequency in *Nicotiana tabacum* and three other crop species. *Can. J. Genet. Cytol.*, 18:57-65.
- Evans, D.A. and E.F. Paddock. 1980. Characterization of X-ray induced increase of mitotic cross-overs in *Glycine max*. *Theor. Appl. Genet.*, 56:245-251.
- Evans, D.A. and W.R. Sharp. 1983. Single gene mutations in tomato plants regenerated from tissue culture. *Science*, 221:949-951.
- Evans, D.A., W.R. Sharp and H.P. Medina-Filho. 1984. Somaclonal and gametoclonal variation. *Amer. J. Bot.*, 759-774.
- Evans, G.M., A. Durrant and H. Rees. 1966. Associated nuclear changes in the induction of flax genotrophs. *Nature*, 212:697-699.
- Gengenbach, B.G., C.E. Green and C.M. Donovan. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. *Proc. Natl. Acad. Sci.*, 74:5113-5117.
- Heath-Pagliuso, S., J. Pullman and L. Rappaport. 1988. Somaclonal variation in celery: screening for resistance to *Fusarium oxysporum* f. sp. *apii*. *Theor. Appl. Genet.*, 75:446-451.
- Jacobs, J.P. and J.I. Yoder. 1989. Ploidy levels in transgenic tomato plants determined by chloroplast number. *Plant Cell Reports*, 7:662-664.
- Koopmans, L.H. 1987. Introduction of contemporary statistical methods. Duxbury Press, Boston, pp. 412-451.
- Lapitan, N.L.V., R.G. Sears and B.S. Gill. 1988. Amplification of repeated DNA sequences in wheatxrye hybrids regenerated from tissue culture. *Theor. Appl. Genet.*, 75:381-388.
- Larkin, P.J. 1987. Somaclonal variation: history, method, and meaning. *Iowa State J. Res.*, 61:393-434.
- Larkin, P.J. and W.R. Scowcroft. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.*, 60:197-214.
- Lazar, M.D., T.H.H. Chen, L.V. Gusta and K.K. Kartha. 1988. Somaclonal variation for freezing tolerance in a population derived from Norstar winter wheat. *Theor. Appl. Genet.*, 75:480-484.
- Lee, M. and R.L. Phillips. 1988. The chromosomal basis of somaclonal variation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 39:413-437.
- Lorz, H. and W.R. Scowcroft. 1983. Somaclonal variation and eyespot toxin tolerance in sugarcane. *Plant Cell Tissue Organ Culture*, 2:111-121.

- McCoy, T.J. 1980. Cytogenetic stability of tissue cultures and regenerated plants of oats (*Avena sativa* L.) and corn (*Zea mays* L.). PhD Thesis, Univ. Minn., St. Paul.
- McLellan, J.C. and A. Durrant. 1973. Instability of *Hh* heterozygotes in flax genotrophs. *Heredity*, 30:63-71.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Mutschler, M.A., S.D. Tanksley and C.M. Rick. 1987. 1987 linkage maps of the tomato (*Lycopersicon esculentum*). Tomato Genetics Cooperative Report No., 37:5-8.
- Nafziger, E.D., J.M. Widholm, H.C. Steinrucken and J.L. Killmer. 1984. Selection and characterization of a carrot cell line tolerant to glyphosate. *Plant Physiol.*, 76:571-574.
- Padmanbham, V., E.F. Paddock and W.R. Sharp. 1974. Plantlet formation from *Lycopersicon esculentum* leaf callus. *Can. J. Bot.*, 52:1429-1432.
- Picken, A.J.F. 1984. A review of pollination and fruit set in the tomato (*Lycopersicon esculentum* Mill.). *J. Hort. Sci.*, 59:1-13.
- Reed, S.M. and J.A. Burns. 1989. Cytological consequences of DNA amplification in an anther-derived doubled haploid line of *Nicotiana tabacum* Amer. J. Bot., 76:958-965.
- Rick, C.M. 1980. Tomato. In: Fehr, W.R. and Hadley, H.H. (eds.). Hybridization of crop plants. Amer. Soc. Agron.-Crop Sci. Soc. Amer., Madison, Wis., pp. 669-680.
- Schaeffer, W.I., Makino, R.K., Moehring, T.J., Sanford, K., Schneider, I. 1984. Usage of vertebrate, invertebrate and plant cell, tissue and organ culture terminology. *In Vitro*, 20:19-24.
- Schnapp, S.R. and J.E. Preece. 1986. *In vitro* growth reduction of tomato and carnation microplants. *Plant Cell Tissue Organ Culture*, 6:3-8.
- Schubert, I., G. Kunzel, H. Bretschneider and R. Rieger. 1980. Sister chromatid exchanges in barley. *Theor. Appl. Genet.*, 56:1-4.
- Shahin, E.A. and R. Spivey. 1986. A single dominant gene for Fusarium wilt resistance in protoplast-derived tomato plants. *Theor. Appl. Genet.*, 69:235-240.
- Shepard, J.F., D. Bidney and E. Shahin. 1980. Potato protoplasts in crop improvement. *Science*, 28:17-24.
- Sibi, M., M. Biglary and Y. Demarly. 1984. Increase in the rate of recombinants in tomato (*Lycopersicon esculentum* L.) after *in vitro* regeneration. *Theor. Appl. Genet.*, 68:317-321.
- Singsit, C. and Veilleux, R. 1990. Genetic analysis of androgenetic competence and plants regenerated from callus culture of diploid potato species. *Genome*, 33:(in press).

Strickberger, M.W. 1968. Genetics. The MacMillan Company, New York.

Stevens, A.M. and Rick, C.M. 1986. Genetics and breeding. In: Atherton, J.G. and Rucich, J. (eds.). The tomato crop: a scientific basis for improvement. Chapman and Hall, Ltd., New York, pp. 35-109.

Table 9. Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of 12 tomato genetic lines.

Genotype	Micropropagation		Cotyledon calli		Thin cell layers	
	N†	Shoots explant ⁻¹	Regeneration frequency (%)	Shoots explant ⁻¹ ††	Regeneration frequency (%)	Shoots explant ⁻¹ ††
Large Red Cherry (LRC)	25	1.6 ± 0.1†††	74 (59/80)	2.0 ± 0.1	46 (30/65)	6.1 ± 1.0
LA 1430	40	1.3 ± 0.1	73 (29/40)	1.7 ± 0.1	51 (34/66)	2.4 ± 0.3
LRC ♀ x LA 1430 ♂	33	1.2 ± 0.1	70 (35/50)	2.1 ± 0.2	35 (17/48)	4.6 ± 0.9
LA 1430 ♀ x LRC ♂	26	1.3 ± 0.1	85 (34/40)	1.6 ± 0.2	51 (36/71)	3.1 ± 0.5
Large Red Cherry (LRC)	25	1.6 ± 0.1	74 (59/80)	2.0 ± 0.1	46 (30/65)	6.1 ± 1.0
LA 1526	24	1.0 ± 0.1	19 (15/80)	1.1 ± 0.1	22 (16/72)	1.7 ± 0.2
LRC ♀ x LA 1526 ♂	46	1.0 ± 0.1	44 (31/70)	1.0 ± 0.1	36 (19/53)	3.5 ± 0.6
LA 1526 ♀ x LRC ♂	45	1.1 ± 0.1	48 (24/50)	1.1 ± 0.1	42 (25/60)	4.0 ± 0.6
Red Alert	43	1.8 ± 0.2	30 (24/80)	1.1 ± 0.1	38 (25/66)	1.4 ± 0.1
LA 1430	40	1.3 ± 0.1	73 (29/40)	1.7 ± 0.1	51 (34/66)	2.4 ± 0.3
Red Alert ♀ x LA 1430 ♂	40	1.1 ± 0.1	72 (36/50)	1.9 ± 0.2	15 (8/54)	1.8 ± 0.4
LA 1430 ♀ x Red Alert ♂	38	1.2 ± 0.1	70 (35/50)	2.2 ± 0.3	23 (12/52)	2.1 ± 0.3
Red Alert	43	1.8 ± 0.2	30 (24/80)	1.1 ± 0.1	38 (25/66)	1.4 ± 0.1
LA 1526	24	1.0 ± 0.1	19 (15/80)	1.1 ± 0.1	22 (16/72)	1.7 ± 0.2
Red Alert ♀ x LA 1526 ♂	45	1.1 ± 0.1	14 (11/80)	1.5 ± 0.3	43 (26/60)	2.8 ± 0.6
LA 1526 ♀ x Red Alert ♂	20	1.1 ± 0.1	36 (29/80)	1.3 ± 0.1	35 (25/71)	2.0 ± 0.3
Overall mean	425	1.3 ± 0.04	48 (362/750)	1.6 ± 0.07	37 (273/738)	3.9 ± 0.37

†N = Total number of shoot tips examined.

††Mean number of shoots per responding explant.

†††Standard error of the mean.

Table 10. Seed set for F₁ hybrids derived by four propagation methods and used as female parents in backcrosses with LA 1430 and LA 1526.

Propagation method	Backcross mutant parent (♂)			
	LA 1430		LA 1526	
	Seed fruit ⁻¹	Number of fruit	Seed fruit ⁻¹	Number of fruit
Micropropagation	40.6 a†	199	23.3 a	199
Seed	37.5 a	199	24.3 a	194
Cotyledon calli	31.6 b	170	22.1 a	170
Thin cell layers	22.2 c	140	21.0 a	173

†Mean separation by Duncan's Multiple Range at the 5% level.

Table 11. Recombination between marker loci of Red Alert×LA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Phenotype	Seed (n = 1610)		Micropropagation (n = 2287)		Cotyledon calli (n = 2562)		Thin cell layers (n = 201)	
	Obs.	Exp.†	Obs.	Exp.††	Obs.	Exp.††	Obs.	Exp.††
+ + +	378	354.2	479	515.2	593	577.2	39	45.3
+ <i>bls</i> <i>sf</i>	150	144.9	297	209.2	266	234.4	31	18.4
<i>sy</i> + +	164	144.9	250	209.2	294	234.4	24	18.4
+ + <i>sf</i>	208	217.4	286	314.6	286	352.5	28	27.7
<i>sy bls</i> +	217	217.4	257	314.6	308	352.5	28	27.7
+ <i>bls</i> +	69	83.7	113	104.4	142	116.9	8	9.2
<i>sy</i> + <i>sf</i>	69	83.7	99	104.4	120	116.9	8	9.2
<i>sy bls sf</i>	355	354.2	506	515.2	553	577.2	35	45.3
χ^2	9.9	0.10-0.25	61.64	0.001	44.47	0.001	13.88	0.05-0.10
Heterogeneity χ^2	35.53	0.025-0.05	16.9	0.95-0.975	47.6	0.01-0.025	—	—

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

+ , *sy*, *bls*, *sf* Represent either wild type (+), or the mutant loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solanifolia* (*sf*), respectively.

The totals represent the sums of 4, 5, 3, and 1 individually backcrossed plant(s) derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 12. Recombination between marker loci of LA 1430×Red Alert hybrid plants grown in from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Phenotype	Seed (n = 1612)		Micropropagation (n = 2765)		Cotyledon calli (n = 2739)		Thin cell layers (n = 1663)	
	Obs.	Exp.†	Obs.	Exp.††	Obs.	Exp.††	Obs.	Exp.††
+ + +	352	354.6	594	622.9	611	617.1	365	374.7
+ <i>bls</i> <i>sf</i>	146	145.1	337	253	323	250.6	184	152.1
<i>sy</i> + +	141	145.1	345	253	308	250.6	202	152.1
+ + <i>sf</i>	224	217.6	324	380.4	334	376.8	217	228.8
<i>sy bls</i> +	205	217.6	318	380.4	323	376.8	230	228.8
+ <i>bls</i> +	89	80.6	117	126.2	134	125	75	75.9
<i>sy</i> + <i>sf</i>	81	80.6	145	126.2	135	125	76	75.9
<i>sy bls sf</i>	374	354.6	585	622.9	571	617.1	314	374.7
χ^2	4.1	0.75-0.90	87.14	0.001	51.59	0.001	33.7	0.001
Heterogeneity χ^2	25.5	0.50-0.75	30.7	0.25-0.50	37.75	0.10-0.25	26.28	0.50-0.75

†Expected values were calculated from map distances supplied by the Tomato Genetics Cooperative.

††Expected values were calculated from frequencies observed of plants grown from seed.

+ , *sy*, *bls*, *sf* Represent either wild type (+), or the mutant loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solanifolia* (*sf*), respectively.

The totals represent the sums of 5 individually backcrossed plants derived from each propagation method.

Table 13. Recombination between marker loci of LRCxLA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Phenotype	Seed (n = 1658)		Micropropagation (n = 3170)		Cotyledon calli (n = 1759)		Thin cell layers (n = 1010)	
	Obs.	Exp.†	Obs.	Exp.††	Obs.	Exp.††	Obs.	Exp.††
+ + +	357	364.8	745	714.2	406	396.3	224	227.5
+ <i>bls sf</i>	148	149.2	334	290.0	180	161.0	97	92.4
<i>sy</i> + +	146	149.2	394	290.0	176	161.0	119	92.4
+ + <i>sf</i>	249	223.8	386	436.1	207	242.0	134	139.0
<i>sy bls</i> +	227	223.8	385	436.1	223	242.0	135	139.0
+ <i>bls</i> +	72	86.2	142	144.7	88	80.3	53	46.1
<i>sy</i> + <i>sf</i>	85	86.2	143	144.7	97	80.3	46	46.1
<i>sy bls sf</i>	374	364.8	641	714.2	382	396.3	202	227.5
χ^2	5.72	0.5-0.75	64.6	0.001	15.2	0.025-0.05	12.1	0.05-0.1
Heterogeneity χ^2	30.98	0.25-0.50	47.4	0.01-0.025	26.2	0.10-0.25	12.4	0.50-0.75

†Expected values were calculated from map distances supplied by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

+ , *sy*, *bls*, *sf* Represent either wild type (+), or the mutant loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solanifolia* (*sf*), respectively.

The totals represent the sums of 5, 5, 4, and 3 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 14. Recombination between marker loci of LA 1430×LRC hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Phenotype	Seed (n = 1807)		Micropropagation (n = 3011)		Cotyledon calli (n = 1354)		Thin cell layers (n = 1055)	
	Obs.	Exp.†	Obs.	Exp.††	Obs.	Exp.††	Obs.	Exp.††
+ + +	421	397.5	624	678.3	323	305.0	281	237.7
+ <i>bls</i> <i>sf</i>	158	162.6	335	275.5	157	123.9	95	96.5
<i>sy</i> + +	164	162.6	347	275.5	138	123.9	113	96.5
+ + <i>sf</i>	257	244.0	391	414.3	163	186.3	141	145.1
<i>sy bls</i> +	246	244.0	353	414.3	155	186.3	141	145.1
+ <i>bls</i> +	69	94.0	118	137.4	68	61.8	48	48.2
<i>sy</i> + <i>sf</i>	87	94.0	152	137.4	64	61.8	47	48.2
<i>sy bls sf</i>	405	397.5	691	678.3	286	305.0	189	237.7
χ^2	9.53	0.1-0.25	50.96	0.001	21.58	0.001-0.005	20.97	0.001-0.005
Heterogeneity χ^2	34.19	0.10-0.25	38.25	0.05-0.10	42.37	0.001	11.97	0.50-0.75

†Expected values were calculated from map distances supplied by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

+, *sy*, *bls*, *sf* Represent either wild type (+), or the mutant loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solanifolia* (*sf*), respectively.

The totals represent the sums of 5, 5, 3, and 3 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 15. Recombination between marker loci of cultivars LA 1430 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.†

Phenotype	Seed (n = 6687)		Micropropagation (n = 11233)		Cotyledon calli (n = 2841)		Thin cell layers (n = 3929)	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
+ + +	1508	1506.5	2442	2530.6	716	640.0	909	885.1
+ <i>bls sf</i>	602	611.8	1303	1027.7	325	259.9	407	359.4
<i>sy</i> + +	615	611.8	1336	1027.7	275	259.9	458	359.4
+ + <i>sf</i>	938	920.0	1387	1545.4	313	390.9	520	540.6
<i>sy bls</i> +	895	920.0	1313	1545.4	348	390.9	534	540.6
+ <i>bls</i> +	299	305.2	490	512.7	172	129.7	184	179.3
<i>sy</i> + <i>sf</i>	322	305.2	539	512.7	144	129.7	177	179.3
<i>sy bls sf</i>	1508	1506.5	2423	2530.6	548	640.0	740	885.1
Treatment Heterogeneity χ^2	339.43		0.001					

†Expected values were calculated from observed frequencies of plants grown from seed.

+ , *sy*, *bls*, *sf* Represent either wild type (+), or the mutant loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solanifolia* (*sf*), respectively.

The totals represent the sums of 19, 20, 9, and 12 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 16. Recombination between marker loci among 5 individual Red Alert×LA 1430 plants regenerated from cotyledon calli.

Plant number		++	+	+	++	+	+	+	++	+	+	++	+	+	++	+	+	Total	χ^2	α
1	obs	89	41	64	47	53	59.2	19.6	0.02	0.02	0.02	0.01	19	19	19	98	430			
	exp†	96.9	39.3	39.3	59.2	59.2	59.2	19.6	19.6	19.6	19.6	96.9								
	<u>(obs-exp)²</u>	0.64	0.07	15.5	2.5	0.6	0.6	0.02	0.02	0.02	0.02	0.01							19.36	0.005-0.01
	exp																			
2	obs	133	58	45	47	40	65.9	21.9	7.9	0.2	0.4	101	35	20	101	479				
	exp	107.9	43.8	43.8	65.9	65.9	65.9	21.9	21.9	21.9	107.9									
	<u>(obs-exp)²</u>	5.8	4.6	0.03	5.4	10.2	10.2	7.9	7.9	0.2	0.4								34.55	0.001
	exp																			
3	obs	81	45	44	45	56	55.3	18.3	18.3	1.6	1.2	101	17	13	101	402				
	exp	90.6	36.8	36.8	55.3	55.3	55.3	18.3	18.3	1.6	1.2	90.6								
	<u>(obs-exp)²</u>	1.0	1.8	1.4	1.9	0.01	0.01	0.01	0.01	0.01	1.2								9.06	0.10-0.25
	exp																			
4	obs	101	44	46	58	50	58.2	23	14	87	423									
	exp	95.3	38.7	38.7	58.2	58.2	58.2	19.3	19.3	19.3	95.3									
	<u>(obs-exp)²</u>	0.34	0.7	1.4	0.001	1.2	1.2	0.7	1.5	0.7									6.49	0.25-0.5
	exp																			
5	obs	189	78	97	89	109	113.9	48	54	166	828									
	exp	186.5	75.8	75.8	113.9	113.9	113.9	37.8	37.8	37.8	186.5									
	<u>(obs-exp)²</u>	0.03	0.07	4.9	5.4	0.2	0.2	2.8	2.8	6.9	2.3								22.62	0.001-0.005
	exp																			

†Expected values were calculated from observed frequencies of plants grown from seed.

+, - Represent either wild type (+), or the mutant (-) loci *sunny* (*sy*), *baby leaf syndrome* (*bls*), or *solaniifolia* (*sf*), respectively.

Numbers in bold represent significant individual Chi-square comparisons at 1 df.

Table 18. Recombination between marker loci of tomato cultivars×LA 1526 grown from seed and re-generated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+ +		\ + are	wv +		wv are			
	obs	exp		obs	exp	obs	exp		
Seed (n = 4106)	1764	1704.0†	331	349.0	332	349.0	1679	1704.0	4.24 NS
Micropropagation (n = 5146)	2077	2160.3††	529	412.7	576	412.7	1962	2160.3	118.83 ***
Cotyledon calli (n = 3556)	1451	1492.8††	360	285.2	359	285.2	1386	1492.8	47.54 ***
Thin cell layers (n = 3169)	1281	1330.3††	327	254.2	337	254.2	1224	1330.3	58.22 ***

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

*** significant at 0.1% level.

NS = nonsignificant.

+, wv, are Represent either wild type (+), or the mutant loci *white virescence* (wv) or *anthocyanin reduced* (are).

The totals represent the sums of 12, 18, 17, and 14 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

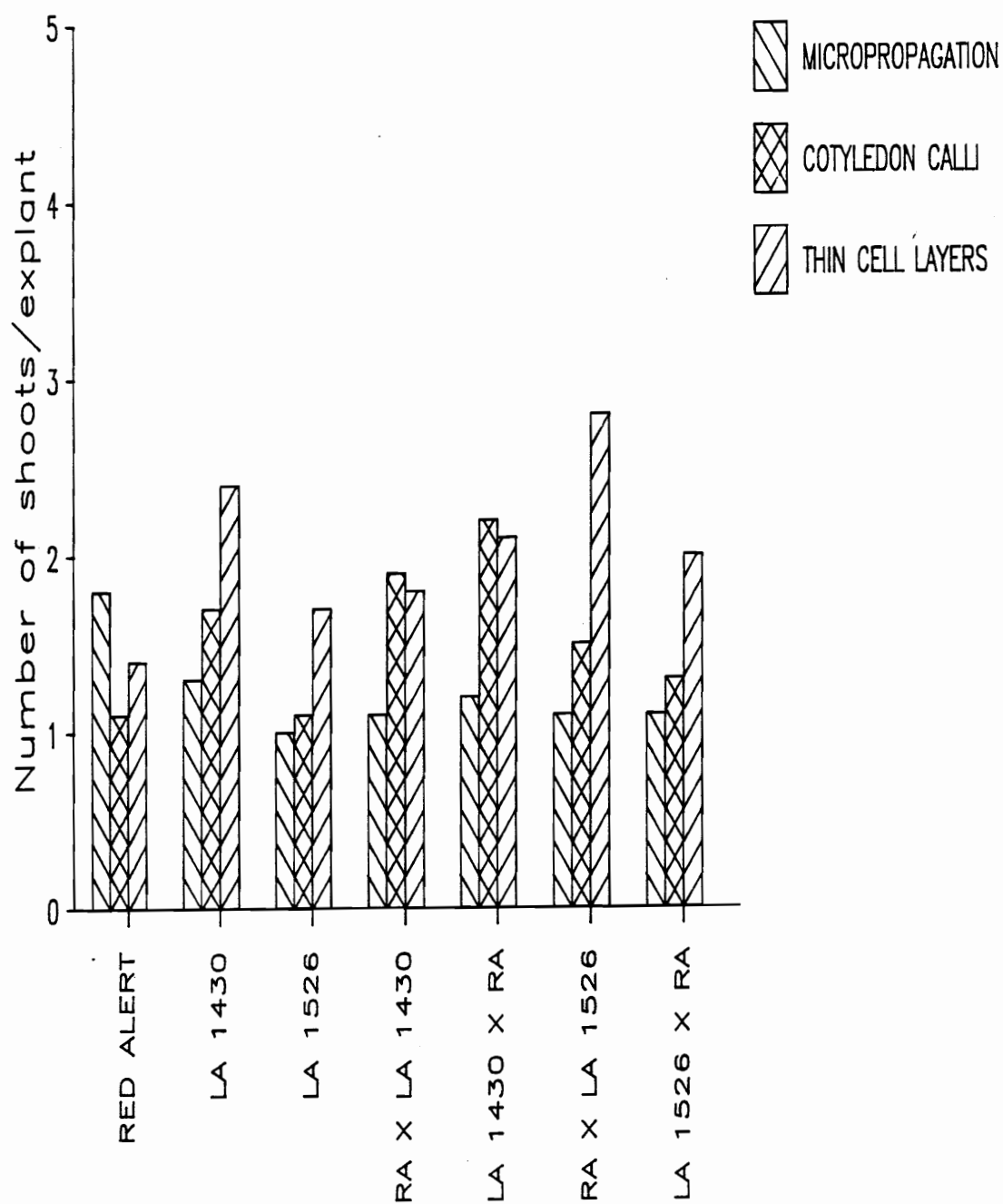


Figure 12. Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of Red Alert, LA 1430, LA 1526 and their reciprocal F_1 hybrids.

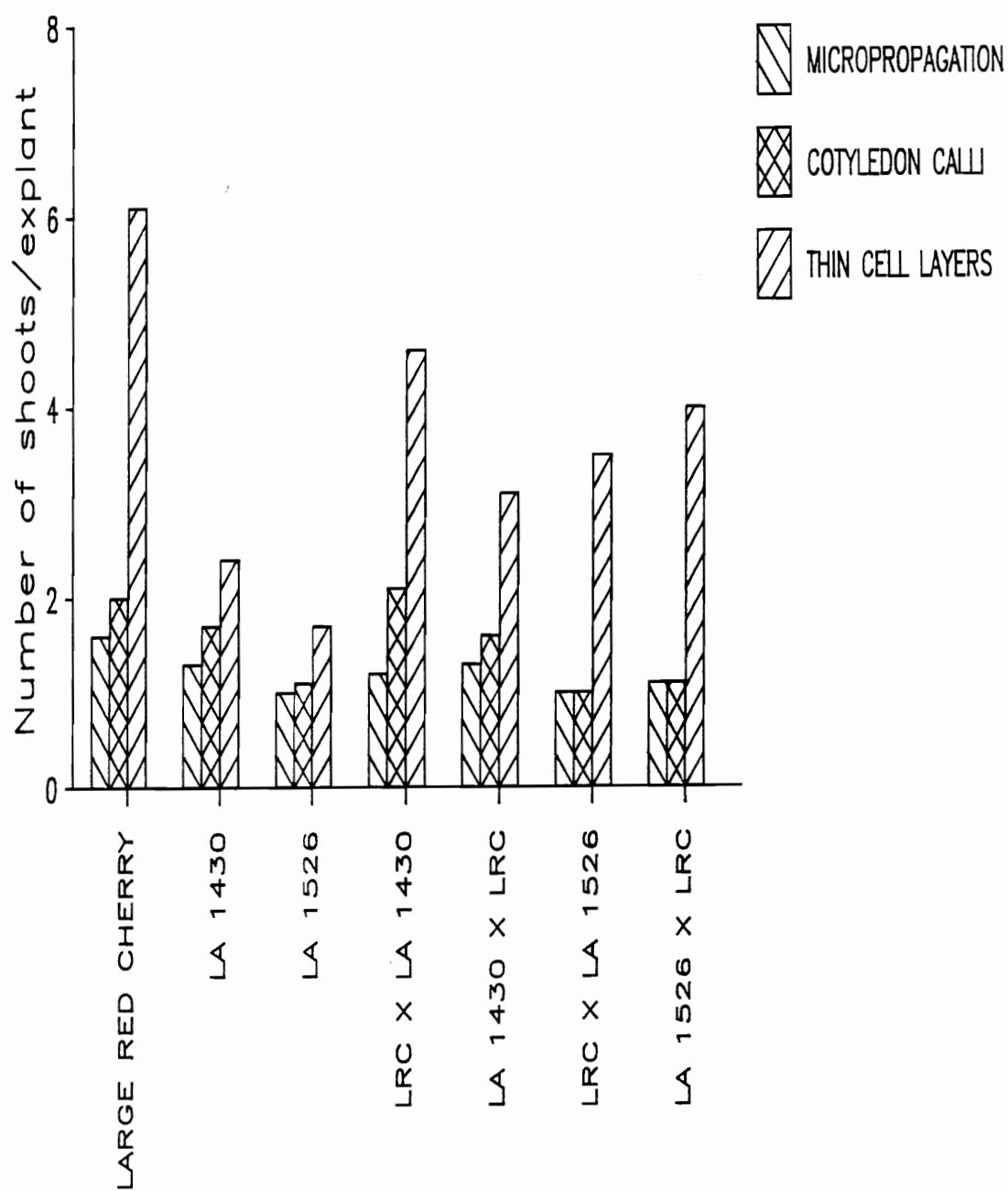


Figure 13. Shoot organogenesis on micropropagated shoot tips, cotyledon calli and thin cell layers of Large Red Cherry, LA 1430, LA 1526 and their reciprocal F_1 hybrids.

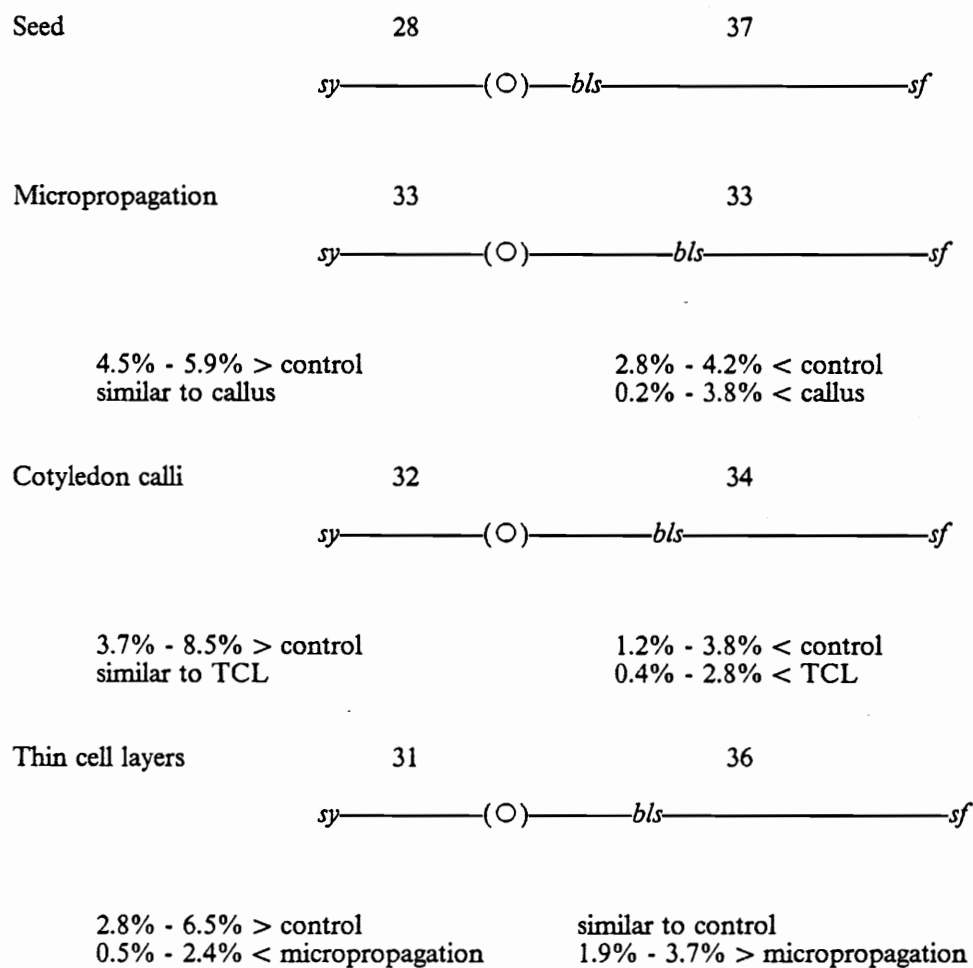


Figure 14. Map distance between marker loci of LA 1430 F₁ hybrid plants grown from seed and re-generated from micropropagation, cotyledon calli and thin cell layers: *sy* = sunny; *bls* = baby leaf syndrome; *sf* = solanifolia; ○ represents the centromere.

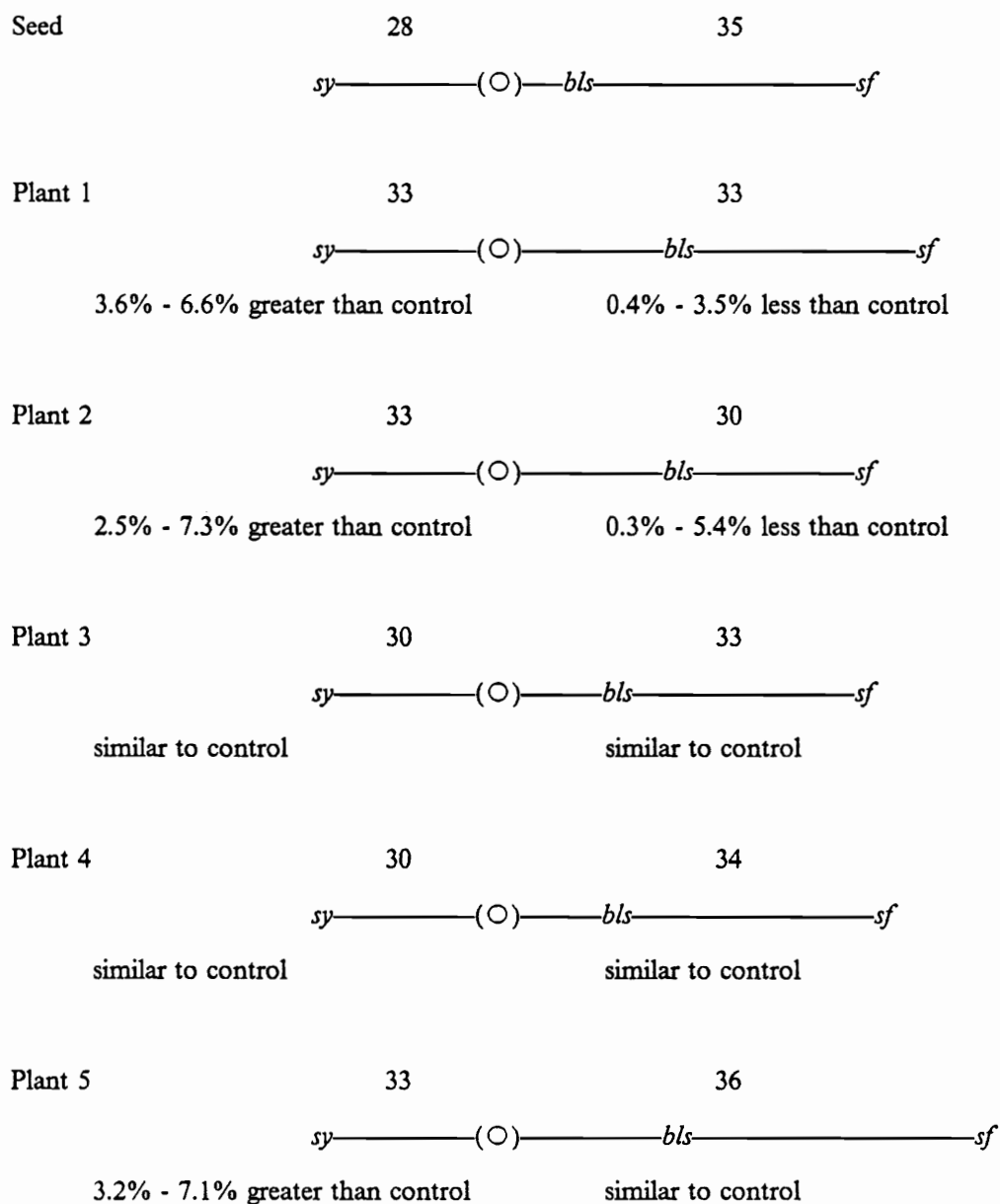


Figure 15. Map distance between marker loci of Red Alert×LA 1430 F₁ hybrid plants grown from seed and regenerated from cotyledon calli: sy = sunny; bls = baby leaf syndrome; sf = solanifolia; ○ represents the centromere.

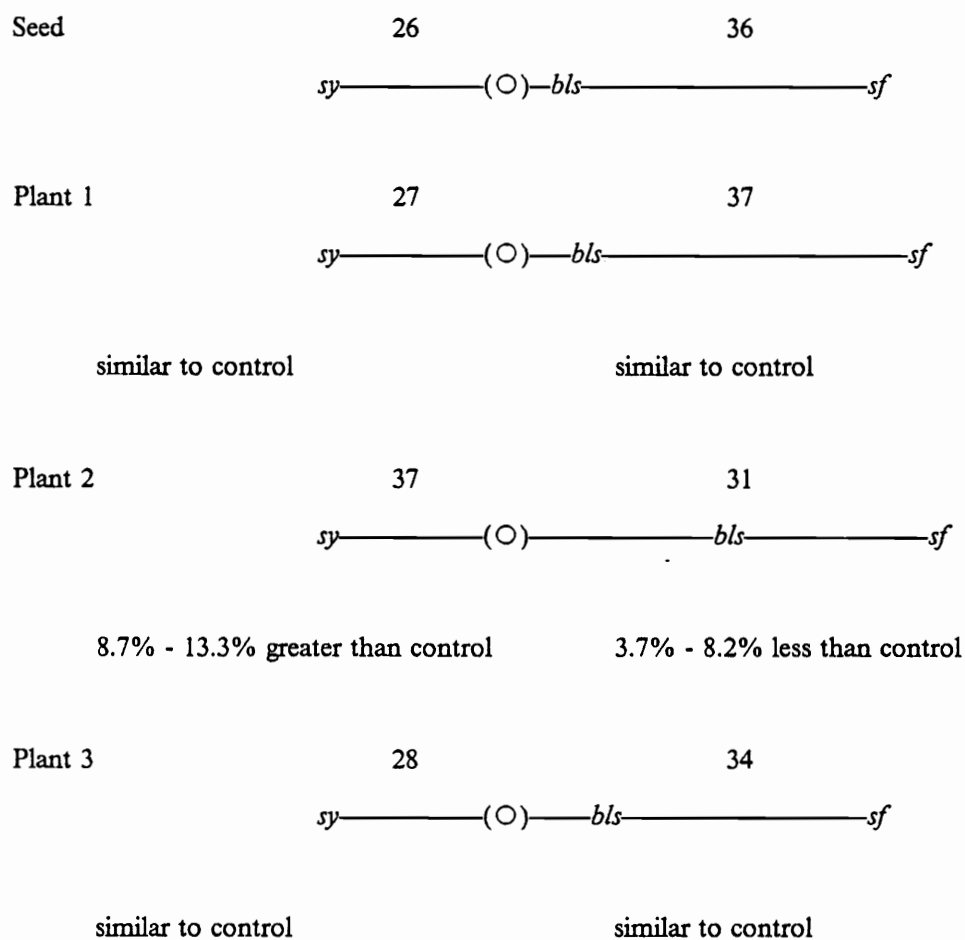


Figure 16. Map distance between selected marker loci of LA 1430 x LRC F₁ hybrid plants grown from seed and regenerated from cotyledon calli: *sy* = sunny; *bls* = baby leaf syndrome; *sf* = *solanifolia*; ○ represents the centromere.

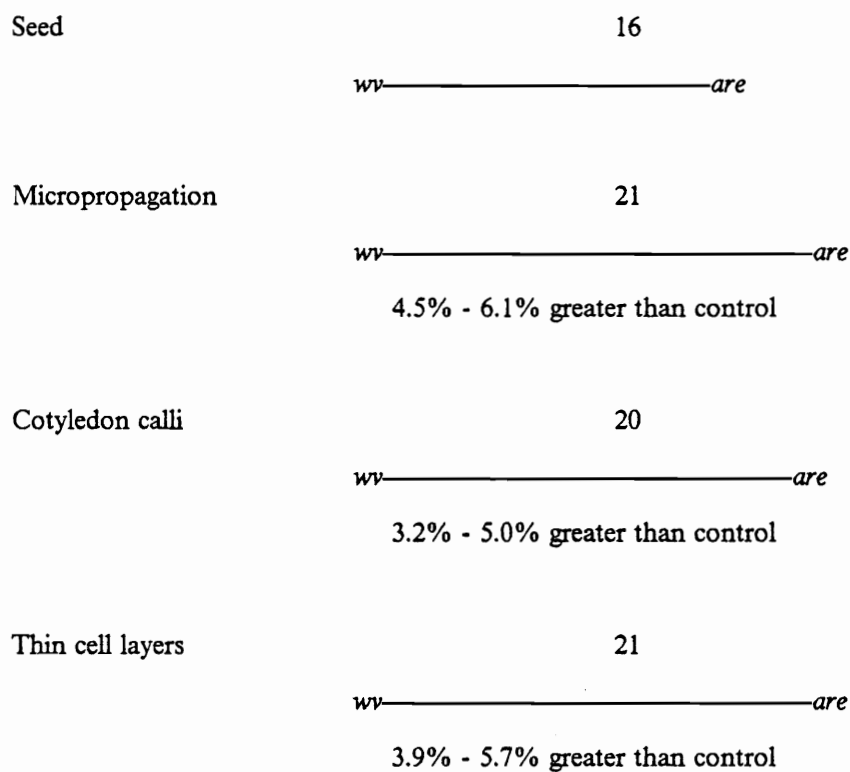


Figure 17. Map distance between marker loci of LA 1526 F₁ hybrid plants grown from seed and re-generated from micropropagated shoot tips, cotyledon calli and thin cell layers: *wv* = *white virescence*; *are* = *anthocyanin reduced*.

Appendix A. Shoot regeneration from cotyledon calli of Large Red Cherry and Red Alert on two different media

Table 19.. Shoot regeneration from cotyledon calli of Large Red Cherry and Red Alert on two different media.

Cultivar	Medium	Shoot Number explant ⁻¹ †	Explants with shoots
Large Red Cherry	P††	3.1 ± 0.6	81% (21/26)
	S†††	1.9 ± 0.2	85% (22/26)
Red Alert	P	2.1 ± 0.3	45% (10/24)
	S	1.0 ± 0.0	10% (5/49)

†Mean number of shoots per responding explant.

††Shoot regeneration medium described by Padmanbhan et al. (1974).

†††Shoot regeneration medium described by Sibi et al. (1984).

Appendix B. Chi-square analysis of recombination rates among tomato hybrids regenerated from tissue culture

Table 20. Recombination between marker loci of hybrids between Red Alert and LA 1430 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment		+++	++	+-	++	+-	++	+-	++	+-	---	χ^2	α
Seed†													
RA×LA 1430	obs	378	150	164	208	217	69	69	355				
	exp	372.5	152.4	152.4	228.6	228.6	88.0	88.0	372.5				
LA 1430×RA	obs	352	146	141	224	205	89	81	374				
	exp	354.6	145.1	145.1	217.6	217.6	83.8	83.8	354.6	9.95	0.25-0.50		
Micropropagation††													
RA×LA 1430	obs	479	297	250	286	257	113	99	506				
	exp	515.2	209.2	209.2	314.6	314.6	104.4	104.4	515.2				
LA 1430×RA	obs	594	337	345	324	318	117	145	585				
	exp	622.9	253.0	253.0	380.4	380.4	126.2	126.2	622.9	9.13	0.25-0.50		
Thin cell layers††													
RA×LA 1430	obs	39	31	24	28	28	8	8	35				
	exp	45.3	18.4	18.4	27.7	27.7	9.2	9.2	45.3				
LA 1430×RA	obs	365	184	202	217	230	75	76	314				
	exp	374.7	152.1	152.1	228.8	228.8	75.9	75.9	374.7	4.77	0.75-0.90		

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

+, - Represents either wild type (+), or the mutant (-) loci *sunny* (*sy*), *baby leaf syndrome* (*bls*) or *solanifolia* (*sf*), respectively.

The totals represent the sums of 9, 10, and 6 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 21. Recombination between marker loci of hybrids between Large Red Cherry and LA 1430 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	++ +	+ ++	- + +	+ +-	-- +	+ - +	- + -	---	χ^2	α
Seed†										
LRC×LA 1430 obs	357	148	146	249	227	72	85	374		
exp	364.8	149.2	149.2	223.8	223.8	86.2	86.2	364.8		
LA 1430×LRC obs	421	158	164	257	246	69	87	405		
exp	397.5	162.6	162.6	243.9	243.9	94.0	94.0	397.5	2.36	0.90-0.95
Micropropagation††										
LRC×LA 1430 obs	745	334	394	386	385	142	143	641		
exp	714.2	290.0	290.0	436.1	436.1	144.7	144.7	714.2		
LA 1430×LRC obs	624	335	347	391	353	118	152	691		
exp	678.3	275.5	275.5	414.3	414.3	137.3	137.3	678.3	15.33	0.05-0.10
Thin cell layers††										
LRC×LA 1430 obs	224	97	119	134	135	53	46	202		
exp	227.5	92.4	92.4	139.0	139.0	48.2	48.2	227.5		
LA 1430×LRC obs	281	95	113	141	141	48	47	189		
exp	237.7	96.5	96.5	145.1	145.1	48.2	48.2	237.7	6.96	0.50-0.75

+Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

†††Expected values were calculated from plants grown from seed.

+, - Represents either wild type (+), or the mutant (-) loci *sunny* (*sy*), *baby leaf syndrome* (*bls*) or *solaniifolia* (*sf*), respectively.

The totals represent the sums of 10, 10, and 6 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 22. Recombination between marker loci of F₁ hybrids between cultivars and LA 1430 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment		+++	++	+	+-	---	+++	++	+	+-	---	χ^2	α
Seed													
Red Alert	obs	730	296	305	432	422	158	150	729				
	exp†	727.1	297.5	297.5	446.2	446.2	171.1	171.1	727.1				
Large Red Cherry	obs	778	306	310	506	473	141	172	779				
	exp	762.3	311.8	311.8	467.7	467.7	180.2	180.2	762.3	5.47	0.5-0.75		
Micropropagation													
Red Alert	obs	1073	634	595	610	575	230	244	1091				
	exp††	1138.1	462.2	462.2	623.0	623.0	230.6	230.6	1138.1				
Large Red Cherry	obs	1369	669	741	777	738	260	232	1332				
	exp	1392.5	565.5	565.5	850.4	850.4	282.1	282.1	1392.5	12.09	0.05-0.1		
Cotyledon calli													
LA 1430×RA	obs	611	323	308	334	323	134	135	571				
	exp††	617.1	250.6	250.6	376.8	376.8	125.0	125.0	617.1				
LRC×LA 1430	obs	406	180	176	207	223	88	97	382				
	exp	396.3	161.0	161.0	242.0	242.0	80.3	80.3	396.3	6.88	0.25-0.5		
Thin cell layers													
Red Alert	obs	404	215	226	245	258	83	84	349				
	exp††	420.0	170.5	170.5	256.5	256.5	85.1	85.1	420.0				
Large Red Cherry	obs	505	192	232	275	276	101	93	391				
	exp	465.2	188.9	188.9	284.1	284.1	94.3	94.3	465.2	10.18	0.10-0.25		

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

+, - Represents either wild type (+), or the mutant (-) loci *sunny* (*sy*), *baby leaf syndrome* (*bls*) or *solanifolia* (*sf*), respectively.

The totals represent the sums of 19, 20, 9, and 12 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 23. Recombination between marker loci of Red Alert×LA 1526 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+	+	+	<i>are</i>	<i>wv</i>	+	<i>wv</i>	<i>are</i>	
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 646)	285	268.1†	59	54.9	52	54.9	250	268.1	2.75 NS
Micropropagation (n = 857)	337	359.8††	102	68.7	98	68.7	320	359.8	34.4 ***
Cotyledon calli (n = 586)	251	246.0††	62	47.0	55	47.0	218	246.0	9.44 *
Thin cell layers (n = 1082)	431	454.2††	120	86.8	125	86.8	406	454.2	35.86 ***

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

*, *** significant at 5% and 0.1% levels, respectively.

NS = nonsignificant.

The totals represent the sums of 3, 4, 3, and 5 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 24. Recombination between marker loci of LA 1526×Red Alert hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+ +		+ are		wv +		wv are		
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 897)	367	372.3†	80	76.2	79	76.2	371	372.3	3.36 NS
Micropropagation (n = 1411)	581	592.3††	144	113.2	146	113.2	540	592.3	22.77 ***
Cotyledon calli (n = 795)	322	333.7††	90	63.8	74	63.8	309	333.7	14.69 **
Thin cell layers (n = 829)	337	348.0††	84	66.5	86	66.5	322	348.0	12.63 **

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

, * significant at 1% and 0.1% levels, respectively.

NS = nonsignificant.

The totals represent the sums of 3, 4, 5, and 4 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 25. Recombination between marker loci of LRCxLA 1526 hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+ +		+ are		wv +		wv are		
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 1309)	563	543.2†	97	111.3	107	111.3	542	543.2	2.17 NS
Micropropagation (n = 1315)	529	552.0††	136	105.5	153	105.5	495	552.0	36.72 ***
Cotyledon calli (n = 1463)	591	614.2††	142	117.3	150	117.3	580	614.2	17.06 **
Thin cell layers (n = 262)	109	110.0††	26	21.0	27	21.0	100	110.0	3.81 NS

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

, * significant at 1% and 0.1% levels, respectively.

NS = nonsignificant.

The totals represent the sums of 3, 5, 5, and 2 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 26. Recombination between marker loci of LA 1526xLRC hybrid plants grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2	
	+		+		+		+			χ^2
	obs	exp	obs	are	obs	exp	obs	are		
Seed (n = 1254)	549	520.4†	95	106.6	94	106.6	516	520.4	4.36 NS	
Micropropagation (n = 1563)	630	656.1††	147	125.4	179	125.4	607	656.1	31.42 ***	
Cotyledon calli (n = 712)	287	298.9††	66	57.1	80	57.1	279	298.9	12.37 **	
Thin cell layers (n = 996)	404	418.1††	97	79.9	99	79.9	396	418.1	9.89 *	

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

*, **, *** significant at 5%, 1% and 0.1% levels, respectively.

NS = nonsignificant.

The totals represent the sums of 3, 5, 4, and 3 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 27. Recombination between marker loci of Red Alert and LA 1526 hybrids grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	<u>+</u> <u>+</u>		<u>+</u> <u>are</u>		<u>wv</u> <u>+</u>		<u>wv</u> <u>are</u>		
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 1543)									
RA×LA 1526	285	268.1†	59	54.9	52	54.9	250	268.1	1.84 NS
LA 1526×RA	367	372.3	80	76.2	79	76.2	371	372.3	
Heterogeneity									
Micropropagation (n = 2268)									
RA×LA 1526	337	359.8††	102	68.7	98	68.7	320	359.8	3.25 NS
LA 1526×RA	581	592.3	144	113.2	146	113.2	540	592.3	
Heterogeneity									
Cotyledon calli (n = 795)									
RA×LA 1526	251	246.0††	62	47.0	55	47.0	218	246.0	0.89 NS
LA 1526×RA	322	333.7	90	63.8	74	63.8	309	333.7	
Heterogeneity									
Thin cell layers (n = 1911)									
RA×LA 1526	431	454.2††	120	86.8	125	86.8	406	454.2	1.62 NS
LA 1526×RA	337	348.0	84	66.5	86	66.5	322	348.0	
Heterogeneity									

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

NS = nonsignificant.

The totals represent the sums of 6, 8, 8, and 9 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 28. Recombination between marker loci of Large Red Cherry and LA 1526 hybrids grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+ +		+ <i>are</i>		<i>wv</i> +		<i>wv are</i>		
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 2563)									
LRC×LA 1526	563	543.2†	97	111.3	107	111.3	542	543.2	0.47 NS
LA 1526×LRC	549	520.4	95	106.6	94	106.6	516	520.4	
Heterogeneity									
Micropropagation (n = 2878)									
LRC×LA 1526	529	552.0††	136	105.5	153	105.5	495	552.0	1.06 NS
LA 1526×LRC	630	656.1	147	125.4	179	125.4	607	656.1	
Heterogeneity									
Cotyledon calli (n = 2175)									
LRC×LA 1526	591	614.2††	142	117.3	150	117.3	580	614.2	0.72 NS
LA 1526×LRC	287	298.9	66	57.1	80	57.1	279	298.9	
Heterogeneity									
Thin cell layers (n = 1258)									
LRC×LA 1526	109	110.0††	26	21.0	27	21.0	100	110.0	1.22 NS
LA 1526×LRC	404	418.1	97	79.9	99	79.9	396	418.1	
Heterogeneity									

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

NS = nonsignificant.

The totals represent the sums of 6, 10, 9, and 5 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Table 29. Recombination between marker loci of hybrids between tomato cultivars and LA 1526 grown from seed and regenerated from micropropagated shoot tips, cotyledon calli and thin cell layers.

Treatment	Phenotype								χ^2
	+ +		+ are		wv +		wv are		
	obs	exp	obs	exp	obs	exp	obs	exp	
Seed (n = 4106)									
Red Alert	652	640.4†	139	131.1	131	131.1	621	640.4	3.63 NS
Large Red Cherry	1112	1063.6	192	217.9	201	217.9	860	952.1	
Heterogeneity									
Micropropagation (n = 5146)									
Red Alert	918	952.1††	246	181.9	244	181.9	860	952.1	2.63 NS
Large Red Cherry	1159	1208.1	283	230.9	332	230.9	1102	1208.1	
Heterogeneity									
Cotyledon calli (n = 3556)									
Red Alert	573	597.7††	152	110.8	129	110.8	527	579.7	4.41 NS
Large Red Cherry	878	913.1	204	153.3	211	153.3	859	913.1	
Heterogeneity									
Thin cell layers (n = 3169)									
Red Alert	768	802.2††	204	153.3	211	153.3	728	802.2	2.14 NS
Large Red Cherry	513	528.1	126	100.9	126	100.9	496	528.1	
Heterogeneity									

†Expected values were calculated from map distances provided by the Tomato Genetics Cooperative.

††Expected values were calculated from observed frequencies of plants grown from seed.

NS = nonsignificant.

The totals represent the sums of 12, 18, 17, and 14 individually backcrossed plants derived from seed, micropropagation, cotyledon calli, and thin cell layers, respectively.

Appendix C. Chemical names and abbreviations

Table 30. List of chemical names and abbreviations.

Chemical name	Abbreviation
Arginine decarboxylase	ADC
N ⁶ -benzyladenine	BA
α -(3-chloro- <i>o</i> -tolyl)acetic acid	CTA
4-cyclobutylamino-2-methylpyrrolo[2,3- <i>d</i>]pyrimidine	CB-P
2,4-dichlorophenoxyacetic acid	2,4-D
DL- α -difluoromethylarginine	DFMA
DL- α -difluoromethylornithine	DFMO
6-furfurylaminopurine	kinetin
Gibberellic acid	GA ₃
7- β -D-glucopyranosyl-BA	7G-BA
6-[4-hydroxy-3-methylbut-2-enylamino]purine	zeatin
Indole-3-acetic acid	IAA
Indole-3-butyric acid	IBA
N ⁶ -[Δ^2 -isopentyl]adenine	2iP
Naphthaleneacetic acid	NAA
1-naphthylphthalamic acid	NPA
Ornithine decarboxylase	ODC
2,4,5-trichlorophenoxyacetic acid	2,4,5-T
2,4,6-trichlorophenoxyacetic acid	2,4,6-T
2,3,5-triiodobenzoic acid	TIBA

Vita

Michael E. Compton

Education:

- Ph.D.** Virginia Polytechnic Institute and State University. Horticulture with the plant physiology option. May, 1990. Specialty: Plant Tissue Culture.
- M.S.** Southern Illinois University at Carbondale. Plant and Soil Science. August, 1986. Specialty: Plant Tissue Culture.
- B.S.** Southern Illinois University at Carbondale. Plant and Soil Science. May, 1983. Major: General Horticulture
- A.A.S.** Danville Area Community College, Danville, Illinois. Horticulture. May, 1981.

Personal Information:

Born May 13, 1961

Graduate student representative on the Department of Horticulture seminar committee, Virginia Polytechnic Institute and State University, 1988-1989.

Honors and Awards:

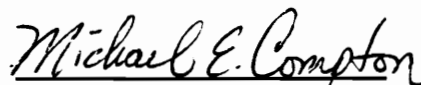
Student travel award to the 86th annual ASHS meeting at Tulsa, OK, 1989.

Research grant from the Virginia Polytechnic Institute and State University Graduate Student Association, 1988.

Student travel award to the 85th annual ASHS meeting at Michigan State University, 1988.

Pi Alpha Xi, 1986

Alpha Zeta, 1982.


Michael E. Compton