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VARIATION AMONG FIRST GENERATION SOMACLONAL AND IRRADIATED TOMATO PROGENY IN RESPONSE TO *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*

By

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ABSTRACT

VARIATION AMONG FIRST GENERATION SOMACLONAL AND IRRADIATED TOMATO PROGENY IN RESPONSE TO *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*

By

Renee M. De Vries-Paterson

A mutation induction program involving susceptible and partially resistant *Lycopersicon esculentum* and *L. pimpinellifolium* cultivars was initiated for the purpose of inducing variation, including resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), causal organism of bacterial canker of tomato. Mutations were induced by either somaclonal variation or gamma (Co^{60}) irradiation. A total of six types of morphological variants were observed. Reduced susceptibility to Cmm was observed in one irradiated line and 49 (25 %) somaclonal progeny, in which susceptibility was found to further vary among clones. Resistance to Cmm is polygenic and partial in nature, with resistant (partially) plants expressing somewhat reduced pathogen populations. When somaclones exhibited increased foliar resistance, 27 % simultaneously expressed reduced pathogen populations. Somaclonal variation was found to be more efficient at increasing resistance to Cmm, compared to radiation induced mutation.

Epidemiology studies indicated that strain virulence and method of inoculation have a significant influence on resistance to Cmm.

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INTRODUCTION AND LITERATURE REVIEW

Bacterial canker was first recognized as a disease of tomato (Lycopersicon esculentum Mill.) in the summer of 1909 in Grand Rapids, Michigan by Erwin F. Smith (Smith, 1910). Since its discovery, bacterial canker has been reported in all tomato growing areas of the world. Incidence of bacterial canker varies from year to year and within a single year in different geographical locations. A number of serious outbreaks have occurred in past years, the most recent of which began in 1984 in the United States and Canada. While such fluctuations suggest the occurrence of different pathotypes (Thyr, 1972), little is known concerning the cause(s) of such epidemic outbreaks. The outbreaks in the mid to late 1980's have in part been traced back to the practice of visual inspection used in the certification of Southern-grown tomato transplants. This practice was found to be inadequate in detecting latent infections of Cmm and will no longer be the single means of detection leading to transplant certification (Gitaitis, 1990).

Bacterial canker takes its name from the discolored longitudinal area or canker which forms on an infected tomato stem or petiole and opens into a sunken cavity. However, many names have been used to describe this disease based on complex symptoms including: bacterial wilt, bacterial canker wilt, stem rot, stem canker, tomato canker and

bird's-eye spot. Characteristic symptoms may include: irregular, one-sided wilting and shriveling of the leaflets, upward rolling of the leaflets, adherence of dead leaflets to the stem, foliar firing, discolored streaking of the stem and petioles both internally and externally, excessive adventitious rooting on stems, yellow ooze secreted from squeezing an infected stem (Strider, 1969), mottled and malformed fruit (Layne and Rainforth, 1966) and stunting of plants (Bryan, 1930). In addition, fruit may become superficially infected with bird's eye spots: small (3-6 mm) white, slightly-raised lesions which can enlarge, crack and become dried out at the center leaving a white halo. Expression of bacterial canker symptoms varies with environmental conditions, age of the plant, strain virulence, inoculum concentration, inoculation method, and if the infection is primary or secondary. The disease can be confused with spray damage in the early stages (Penna, 1982; Strider, 1969). Factors affecting disease development include: moisture (Kendrick and Walker, 1948; Blood, 1931), humidity (Tsiantos, 1987) and temperature (Kendrick and Walker, 1948), predisposition at low light (Kendrick and Walker, 1948), and application of highly concentrated nutrient solutions (Walker and Kendrick, 1948). Other researchers have confirmed this nutritional influence: Forster and Enchandi (1975) found that disease severity was inversely correlated with calcium concentration, while Madumadu (1985; Berry et al., 1988) reported a reciprocal relationship between the effect of calcium and nitrogen and tomato canker severity. Kuniyasu and Kuriyama (1972) found an increased level of disease development when nutritional concentration was

increased, with tolerant cultivars being more affected than susceptible ones.

Bacterial canker is one of the most dreaded and destructive diseases of tomato. While it has been most severe in the past decade during the drier (drought) growing seasons (discussion, Fifth Bacterial Canker Workshop, 1989), it has been previously reported that the disease can be equally damaging in cool (Chupp & Sherff, 1960), wet (Bryan, 1930) seasons. Yield is generally reduced in mature, infected plants and fruit blemished with bird's-eye lesions are unmarketable as fresh produce. Bacterial canker can also lead to death if plants are severely infected or if infection occurred at an early stage. Tomato seed is considered the most important source of inoculum (Thyr, 1969; Strider, 1969; Bryan, 1930; Uematsu et al., 1977), with transmission ranging from 0.25 to 100 % (Blood, 1933; Bryan, 1930; Fenner, 1931; Grogan and Kendrick, 1953; Nattrass and Ciccarone, 1946; Thyr, 1969). Additional pathogen carriers include Solanaceous (Strider, 1969; Baines, 1947; Thyr, 1971; Thyr et al., 1975; Ark and Thompson, 1960) and other alternate weed hosts (Chang et al., 1988; Ricker, 1989). Pepper and eggplant can also be infected artifically (Hassan et al., 1968), yet these non-host species all harbour relatively low (10^3-10^4) epiphytic populations, compared to tomato (10^7-10^9) (Chang et al., 1988; Gleason and Braun, 1988). Reports, sometimes conflicting, have been made of the pathogen's ability to survive, resist desiccation and overwinter in soil (Bryan, 1930; Strider, 1967; Van Hatern, 1935; Ciccarone and Carilli, 1948; Wakimoto et al., 1968; Basu 1970; Grogan and Kendrick, 1953;

Rangaswami and Rajagopalan, 1973; Echandi, 1971; Moffett and Wood, 1984; Stebbins et al., 1988; Gleason and Braun, 1988; Louws and Stephens, 1989) and on plant debris (Farley, 1971; Strider 1967; Grogan and Kendrick, 1953; Gorlenko, 1961; Gitaitis, 1989; Gleason et al., 1989). The variability reported in longevity is likely due to differences in locality and region (Bryan, 1930; Ciccarone and Carilli, 1948; Grogan and Kendrick, 1953; Strider, 1967), and the effects of temperature, moisture, pH, microorganism antagonism and other possible factors (Basu, 1970). However, Cmm has been shown to survive in water (Rangaswami and Rajagopalan, 1973; Strider, 1967), on wooden implements (Strider, 1967; Wakimoto et al., 1968), metal and other greenhouse surfaces (Bryan, 1930; Fulbright and Stephens, 1987; Strider, 1967), and clothing and cotton fibers (Basu, 1970; Fulbright and Stephens, 1987; Strider, 1967).

Bacterial canker is highly contagious. Normal production practices such as transplanting and handling, pruning, tying, and clipping have also been demonstrated to be effective in the spread of the disease within a field (Farley and Miller, 1973; Chang et al., 1988; Gitaitis, 1989; Thomas, 1930; Orth, 1937; Mullers, 1937; Strider, 1968, 1969; Ark, 1944; Gleason et al., 1989). It has been demonstrated (Ark, 1944; Orth, 1937; Bryan, 1930; Chupp and Scherf, 1960; Chang et al., 1988) that the introduction of a few artifically infected plants into a field can result in a high number of diseased plants. For example, Chang and co-workers (1988) recently found that the introduction of 50 inoculated plants into a field of 10,000 plants could result in 83% infection. Dissemination is also possible

by wind, rain, and fungicide spraying during wet weather. Spread by insects, although never confirmed, is also probable (Thomas, 1930; Smith, 1920).

Bacterial canker is caused by Clavibacter michiganensis subsp. michiganensis (Smith) Davis, Gillaspie, Vidaver and Harris, (Cmm), a gram-positive, motile, aerobic rod (1 x 0.5 um) bacterium. Since its discovery, several taxonomic changes have occurred and the organism has been listed under six genera: Bacterium, Pseudomonas, Aplanobacter, Phytomonas, Corynebacterium, and currently Clavibacter. The bacterium is pleomorphic, with rods arranged as the letters V, L, or N. Cmm is naturally yellow in pigmentation due to carotenoids. However, colony characteristics, such as pigmentation, vary depending on the growth conditions, media components, mutations and age of the strain. Both variation in color and in virulence have been reported and may be associated (Strider and Lucas, 1970; Thyr, 1972; Bryan, 1930, 1931; Ark, 1944; Fawcet and Bryan, 1934; Jensen, 1934; Berry et al., 1989; Thyr, 1968; Strider, 1969; Baines, 1947). Differences in strain virulence should be considered when comparing inoculation results with those previously reported on resistant cultivars. The bacterium has been found to produce high-molecular weight polysaccarides and it has been postulated that certain of its components, for example fucose, are active in blocking the xylem vessels and causing wilting (Patino-Mendez, 1966). The year after Rai and Strobel (1967) characterized the substance and identified it as a glycopeptide, Rai (1968) suggested that this 'toxin' acted by damaging the cell membranes, playing a major role in Cmm's wilting

mechanism. These authors reported no apparent plugging of the vessels. However, Romeiro and Miura (1989) have recently reported that in-vivo toxin production did not occur in artifically-inoculated plants until after the plants were showing severe disease symptoms. Earlier, Wallis (1977) thought it unlikely that the wall destruction associated with Cmm was due to the action of a toxin. He proposed that pathogenesis in Cmm was due to a very active enzyme system by which the pathogen is able to completely degrade most, if not all, plant cell wall components. It is conceivable that the combined action of cellulases, hemicellulases, ligninolytic and pectinolytic enzymes are responsible for the complete vascular tissue breakdown and consequential wilting characteristic of the advanced stage of this disease. Cmm is a xylem invader and when introduced into a plant the bacterium first moves downward then upward (Pine et al., 1955), invading the phloem elements in the advanced stages of the disease (Wallis, 1977). Natural openings such as leaf trichomes (Kontaxis, 1962), stomates (Smith, 1914) and wounds (Thomas, 1930; Strider, 1968, 1969; Grogan and Kendrick, 1953; Kendrick and Walker, 1948; Orth, 1937; Pine et al., 1955) are the sites of infection.

Prevention is the most effective control of bacterial canker, and includes sanitation, disinfesting equipment, avoidance of over-head irrigation and splashing, staying out of dew-laden fields, fall plowing, crop rotation with non-solanaceous crops, minimizing plant wounds, rouging out infected plants and solanaceous and other alternate weed hosts, using clean, disinfested seed and certified transplants. Copper containing fungicides have also been shown to

significantly reduce the foliar symptoms (marginal firing) of bacterial canker and the percentage of spotted fruit (Shoemaker and Enchandi, 1988). Crop rotation (3 yr.) has been recommended in fields in which the disease was present the previous year.

Methods of diagnosing Cmm include the use of serology, cell wall composition, phospholipid composition, metaquinone analysis, phage and bacteriocin sensitivity, and DNA:DNA hybridization (Vidaver and Starr, 1981). Traditional physiological and biochemical tests have limitations in detecting the pathogen from its associated microflora. More recently, serology (Stevens and Tsiantos, 1979), semi-selective media (Fatmi and Schaad, 1988), stem stamping onto semi-selective media (Gitaitis and Leben, 1988), hypersensitivity reactions (Gitaitis, 1990), gas chromatography of fatty acids (Gitaitis and Beaver, 1988, 1989) and DNA-probe (Thompson et al., 1989) procedures have been used to make detection of Cmm more reliable, rapid, and practical. Detection, however, remains a major limiting factor in the control of bacterial canker.

Resistant varieties are often another viable means of disease control. A vast amount of genetic variation in resistance (to bacterial canker, among others) has been demonstrated to exist in the wild Lycopersicon and Solanaceous species (Table 1). These genes have been exploited in conventional breeding programs to provide the resistances currently available in cultivated tomato. As of 1987, 16 out of 30 such detected disease resistances have been bred into commerical tomato cultivars (Rick, 1987). Interestingly, resistance to Cmm has been shown to be linked to resistance for bacterial wilt,

caused by Pseudomonas solanacearum, in some cases (Laterrot and Kaan, 1978).

However, useful Cmm-resistant tomato varieties are not available to the commercial grower today. High levels ("immunity") of bacterial canker resistance have never been reported in Lycopersicon and levels at best remain partial. Traditionally, only backcross breeding has been used to improve resistance to Cmm but has proven to be difficult and time consuming. There are, however, at least four other avenues available for this purpose, two of which will be considered in this study: mutation breeding by ionizing radiation and somaclonal manipulations. As tomato is very amenable to manipulation (Chapter 2), both of these techniques have been successfully used by a number of researchers to improve other tomato characters, in addition to resistances to viral and fungal pathogens.

No standardization of evaluation techniques nor agreement on criteria for Cmm resistance is found in the literature to date. Nor has the nature of resistance to Cmm been clearly elucidated, although it appears due partially to a somewhat reduced pathogen level. Resistance has been defined by some researchers as immunity, or the lack of foliar symptoms, wilting or death. Other criteria used to assess resistance include yield reductions, stunting, canker size, systemic concentration or vascular movement, presence and extent of vascular discoloration. So, depending on the definition of Cmm resistance, what in one report is classified as resistant, might easily be considered susceptible in a second report. In addition, partially resistant plants inoculated at 4 to 5-weeks of age have

sometimes shown the ability to have the tips of their wilted leaflets dry off, afterwhich the plants grow out of the previous symptoms and develop normally (Basu, 1964; Emmatty and John, 1973). Another consideration in defining resistance to Cmm is that 'resistant' Lycopersicon spp. can respond differently to different strains of Cmm, depending on the strain virulence, plant genotype and age, and method of inoculation (unpublished data). De Jong (1975) observed in inheritance studies that resistance to Cmm is not absolute but rather needs to be quantified with each isolate, inoculation method and plant age.

Resistance to bacterial canker was first reported to be inherited as a dominant trait by Elenkov in 1965. Seven years later, Thyr (1972) suggested that the inheritance of canker resistance was polygenic. Laterrot (1974) confirmed the dominant nature of Cmm resistance when he noted that progeny from crosses with 'Bulgaria 12' and susceptible tomato varieties were equally resistant to 'Bulgaria 12'. Later, based on backcross breeding in 'Utah 20' and 'Utah 737', Thyr (1976) estimated that the gene number for Cmm resistance ranged from 4 - 11 and involved more than one major gene, while crosses involving 'Bulgaria 12' pointed to 1-2 incompletely dominant major genes, accompanied by modifiers. In 1976, de Jong and Honma proposed a 4 gene model for Cmm resistance after inoculating with a mild Cmm isolate. For example, a combination of one recessive gene (a) and 3 dominant genes (B,C,D) was proposed as the model for 'Bulgaria 12'. Resistance in L. pimpinellifolium 'Utah 20' and 'Utah 737' was enhanced by a dominant allele (D^2). This resistance is different from

L. esculentum and studies suggest different genes or alleles of the same gene are involved in its resistance. Resistance in L. hirsutum is different than that found in L. esculentum or L. pimpinellifolium, being controlled by the genes (AAbbccdd), an additional recessive gene (x), and a modifier gene (F). Madumadu (1985), using a highly aggressive strain of Cmm, proposed that L. esculentum 'Plovdiv 8/12' possessed four resistance genes like 'Bulgaria 12'. Likewise, 3 or 4 genes were thought to control resistance in 'Irat L3', but with different alleles at the (B,C,D) loci. Alleles at the C and D loci appeared to be better equipped at conferring resistance to Cmm. Three or four genes were proposed to control resistance in 'Heinz 2990' and 'Cm VF 232' while 'Okitsu Sozai No 1-20' was shown to possess alleles at genes (C,D). In summary, breeding for Cmm resistance is complicated due to its multigenic nature with hybrids possibly exhibiting a continuous range of resistance, explained by the addition of minor genes which act independently to modify the major gene action (Thyr, 1976).

In the past century, plant breeders have been dependent on the selection of natural, spontaneously generated mutations occuring in existing crop varieties as a source of new traits. Such mutations were one in a million events and more often would go unrecognized. Later, in the 1950s, induction of mutations by chemical and physical means became a popular approach by which existing varieties were improved for disease resistance and other characters. Then mutation breeding research in potato (Van Hatern and Bouter, 1973; Van Hatern et al., 1981) led to the suggestion that in vitro techniques would

probably replace mutagenic treatments as the preferred method for inducing variation (Reisch, 1983). This same idea was proposed in 1980 by other researchers regenerating somaclones from potato protoplasts and suspension cultures (Shepard et al., 1980; Behnke, 1980). Somaclonal variation is an extension of the mutation breeding process (Reisch, 1983), resulting in the induction of genetic variation, including disease resistance, in specific crop species. Somaclonal variation can be more appealing than physical or chemically-induced mutations as it will often occur at a greater frequency. In 1987, Gavazzi and co-workers compared the type and frequency of mutational events resulting from chemical application and somaclonal variation (R_1 progeny) in tomato. Several mutants were recovered, including disease resistance to Verticillium wilt. The authors concluded that these two mutation methods differed in the spectrum, frequency and pattern of segregation of mutations, and that while both treatments yielded mutants, somaclonal variation proved to be more efficient both quantitatively and qualitatively.

OBJECTIVE: Although bacterial canker resistance is available in Lycopersicon to a limited extent, the selection of resistance in L. esculentum has proven quite difficult and time consuming, and is often associated with undesirable agronomic characters, such as poor fruit size. A logical and reasonable alternative is to develop a mutation induction program involving popular, high quality tomato cultivars. Both ionizing mutations and somaclonal variants have been reported to result in differing degrees of susceptibility against one or several pathotypes or races of a pathogen, including increases and

decreases from the original resistance level (IAEA, 1983). The main objective of this project was to induce variation in partially resistant and susceptible tomato cultivars for the purpose of creating greater Cmm resistance. Specific goals were to: 1. create somaclonal variants of popular fresh market, processing and breeding L. esculentum cultivars and in breeding cultivars of L. pimpinellifolium; 2. create mutants of a susceptible, fresh market L. esculentum cultivar and a susceptible processing cultivar by gamma (cobalt-60) radiation; 3. assess resistance to two Cmm strains in first generation somaclonal and irradiated seed progeny by two methods: rating of foliar disease symptoms and quantification of systemic Cmm populations in order to determine the nature of resistance.

Other research included: A. Evaluation of Lycopersicon accessions using two strains of Cmm, comparing two inoculation techniques, clipping and cotyledon excision, a technique newly devised by the author; B. Evaluation of F12-F14 generation hybrids bred at Michigan State University for Cmm-resistance inheritance studies by Dr. Jan de Jong in the 1970's and since selected by Dr. Shigemi Honma (Department of Horticulture) for Cmm resistance and horticultural characters; C. An 8-week study comparing systemic Cmm multiplication in a partially-resistant and a susceptible cultivar after inoculation using two different methods and two strains of Cmm; D. Studies comparing (1) lower and upper plant parts of partially resistant and susceptible tomato varieties for Cmm populations, (2) 'Bulgaria 12' somaclones, classified as possessing increased levels of foliar

resistance after cotyledon excision inoculation, were left to further survive in a greenhouse for a period of 7 - 10 months in order to assess Cmm persistance, and (3) to compare foliar ratings and pathogen populations resulting from clip inoculation to those resulting from cotyledon excision inoculation, in somaclonal lines (5 cultivars) classified as possessing increased foliar resistance to Cmm.

Table 1. Tomato germplasm reported as resistant (partially) to Clavibacter michiganensis subsp. michiganensis.

Cultivar	Source
L. esculentum	
Bulgaria 12 (P.I.324707, 324708, 330727)	Elenkov, 1965
Heinz 2990	Emmatty and John, 1972 and 1973
MR4	Forster and Echandi, (Laterrot and Kaan, 1978)
Monense	Laterrot et al., 1978
CmVF 232	(Berry et al., 1989)
Okitsu Sozai 1-20	Kuniyasu and Kuriyama, 1974
Flora Dade	Laterrot, 1987
Plovdiv 8/12	Elenkov, 1965
IRAT-L3	Laterrot, 1980
Cerasiforme (Utah 659)	Thyr, 1968 and 1969
NC 409-1-1	Gardner, Shoemaker and Echandi,1989
Carette	Kaan (Laterrot and Kaan, 1978)
53 RC	Kaan (Laterrot and Kaan,1978)
72 TR 4.4	Henderson and Jenkins, (Laterrot and Kaan, 1978)
74 TR 10	Henderson and Jenkins, (Laterrot and Kaan, 1978)
Hawaii 7996	Gilbert,(Laterrot and Kaan, 1978)
Hawaii 7998	(Poysa, 1988)
Saturn	Henderson and Jenkins, (Laterrot and Kaan, 1978)
Venus	Henderson and Jenkins, (Laterrot and Kaan, 1978)
Cocabul	(Poysa, 1988)
L.pimpinellifolium	
	Yatzynina, 1941; Orth, 1937; Ark, 1944
Cervena Kapha (P.I.340905)	Thyr, 1968 and 1969
Red Current 1149K	(Berry et al., 1989)
Corina	Stamova and Yordanov,1986
Utah 20 (P.I.344102)	Thyr, 1968 and 1969
Utah 737 (P.I.344103)	Thyr, 1968 and 1969
LA 12156L	Thyr, 1968 and 1969
L. hirsutum (P.I. 251305)	Hassan et al., 1968
L. peruvianum humifusum	Alexander et al., 1942
(L.esculentum x L. chilense) x L. peruvianum var. humifusum	Valkova-Achkova and Sotirova, 1981; Sotirova and Vulkova-Atchkova, 1988

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CHAPTER 1

Inoculation and Quantification of *Clavibacter michiganensis* subsp. *michiganensis* Populations in *Lycopersicon* Accessions.

INTRODUCTION

Cmm Inoculation. A large number of different techniques have been used for inoculation of *Lycopersicon* with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), and have included all tomato plant parts: buds, cotyledons, fruit, leaves, petioles, roots, and stems (see review by Strider, 1969). Methods of introducing the pathogen have either been of a direct, systemic nature, such as needle injection or excision with contaminated scissors and knives, or non-systemic nature, such as by spraying flowers and foliage.

While the threshold required for infection depends on the method of inoculation, it has been noted that as few as five cells of Cmm systemically introduced can result in infection (Thyr, 1968). It has been further demonstrated that concentration levels of 10^7-10^9 cells/ml produce similar responses in resistant and susceptible tomato cultivars (Foster and Echandi, 1973). In contrast, a 500-fold difference in dilutions of two Cmm isolates had little effect on the reaction of resistant cultivars (de Jong and Honma, 1976). With any type of artificial inoculation however, it should be remembered that under natural, field conditions bacterial populations would not tend to be highly concentrated ($\geq 10^6$). When too great a concentration is used for inoculation, a number of concerns should be considered: the limited number of susceptible sites in the plant,

competition/antagonism between bacterial cells, nutritional limitations or other factors. In addition, expression of Cmm resistance can be influenced by inoculum concentration, resulting in the susceptible reaction of resistant genotypes. For example, inoculation with virulent strains can kill L. pimpinellifolium 'Utah 20' or 'Utah 737' (de Jong, 1975). Inoculation with low numbers of bacterial cells or with a mildly aggressive isolate would be less likely to mask moderate levels of resistance (Strider, 1970) and valuable sources of resistance would not likely be eliminated (Forster and Echandi, 1973).

Variation in virulence of Cmm isolates has been noted regularly (Strider and Lucas, 1978; Berry et al., 1989; Baines, 1947; Fawcett and Bryan, 1934; Smith, 1914; Strider, 1969; de Jong, 1975; Thyr, 1972, 1968) and should be considered when reporting Cmm resistance. A number of researchers have used 'highly virulent' strains of Cmm to evaluate breeding selections while others have used 'mildly virulent' strains and this discrepancy has resulted in confusion and contradictory results. Among the factors which influence the reaction of tomato seedlings to infection by Cmm, strain differences appeared to be the most important in at least two previously published reports (Strider and Lucas, 1970; Thyr, 1972).

Different methods of inoculation can also result in contradictory results. For example, in L. hirsutum, (Forster and Echandi, 1973; Hassen et al., 1968) differences in inoculation methods led to opposite conclusions about this species inherent level of Cmm resistance. Secondly, L. esculentum 'Heinz 2990' is considered

resistant by petiole inoculation (Emmatty and John, 1972, 1973) but susceptible by clip inoculation (D. Emmatty, personal communication).

Plant age is another factor influencing resistance to Cmm. De Jong and Honma (1976) compared four L. esculentum cultivars as seedlings and as mature plants and found that if resistance was expressed at the younger stage it remained or increased at maturity. 'Bulgaria 12', for example, was resistant when inoculated either as a seedling or a mature plant. Two susceptible cultivars, 'Earliana' and 'Saturn', were susceptible and somewhat tolerant, respectfully, as seedlings, but both were considered to have low tolerance as mature plants. A more susceptible cultivar, MH 1, however, was found to be susceptible at either stage of plant development. Strider (1970) compared inoculation of 14 and 28 day-old tomato plants and found disease development to be rapid and uniform in the younger plant material. Likewise, in a comparison of 3 and 30 day-old inoculated tomato seedlings, the youngest plant material exhibited more rapid disease development and plant death, while the latter resulted in slower disease development and rarely died (Basu, 1964).

Although researchers tend to inoculate tomato plants 4 to 5-weeks old, inoculations with Cmm have sometimes been made on seedlings less than 4-weeks-old (Kendrick and Walker, 1948; Basu, 1964, 1966; Strider, 1970; Thyr, 1967, 1969; Dick and Mac Neill, 1981; Hassan et al., 1968; Strider and Konsler, 1965). It has been suggested that the inoculation of young plants could be used as a reliable technique and criterion for selecting Cmm resistance (de Jong and Honma, 1976).

There have been two related reports on the use of cotyledons as

the site of inoculation. Strider and Konsler (1965) and later Hassan and co-workers (1968) described the use of a cotyledonary spray technique for evaluating resistance to Cmm: Five-day-old seedlings are sprayed with a Cmm suspension and after evaluation for number of lesions, were classified as immune, resistant or susceptible. There are however important drawbacks to this particular inoculation technique including (i) the inability to accurately judge the number of lesions on the small surface area of cotyledons, (ii) resulting infection is non-systemic and therefore uncharactersitic of the pathogen. For example, Thyr and co-workers (1975) compared inoculation by stem wounding (vascular inoculation) to cotyledon spraying and found no uniform correlation between the two methods: A host could develop cotyledonary spots but fail to develop subsequent vascular infection or the reverse could also occur; (iii) the inoculum concentration applied to the cotyledons could be high enough to result in a susceptible reaction with a resistant accession (Thyr, 1969), (iv) thrip infection also can complicate the rating evaluation.

The rational for testing very young plant material has been that Cmm-infected tomato seed, the primary source of inoculum, results in infection of the seedling during seed germination, with the development of the infection occuring along with the seedling's growth (Rangaswami and Rajagopalan, 1973), although plants may become infected during any stage of growth and development, from seedling to mature plant (Bryan, 1930). Bryan (1930) reported that cotyledons of infected seedlings are the first plant parts to show

symptoms of wilting, followed by browning and shriveling of the tissue, with symptoms on true leaves appearing later. Two-week-old plants have been found to exhibit symptoms as severe as 4-week-old plants (Strider, 1970). Likewise, an additional comparison of young and mature plant material indicated there was no difference in susceptibility to Cmm (Van Steeklenburg, 1985). Another advantage of younger seedlings is that a more rapid susceptibility response is produced (Kendrick and Walker, 1948). In other words, symptoms take longer to develop as the age of the plant material which is to be inoculated is increased. It can be expected that if a tomato accession is found significantly resistant when inoculated at 1-2 weeks, for example, it should be found significantly more resistant when inoculated by a similar method at 5-weeks of age (Boelema, 1976).

Quantification of Cmm in inoculated *Lycopersicon* accessions. It has been generally accepted that plant disease symptoms due to bacterial infection are correlated rather closely with bacterial multiplication in the intercellular spaces (Allington and Chamberlain, 1949). However, this is not always the case and indeed can be quite the opposite, depending on the bacteria. For example, Allington and Chamberlain (1949) found that in inoculation studies with Pseudomonas glycinea and bean (non-host), the plants exhibited no disease symptoms, however the pathogen was found to have persisted and multiplied appreciably. Similar results have been reported with Cmm, and other C. michiganensis subspecies and Clavibacter species. For example, De Boer and McCann (1989) reported that resistant potato

cultivars never or very seldom expressed disease symptoms yet high population densities of C.michiganensis subsp. sepedonicum could be systemically detected (De Boer and Mc Naughton, 1986). Davis and co-workers (1988) found that population densities of C. xyli subsp. xyli were lower in resistant than in susceptible sugarcane cultivars. Like C.m. subsp. sepedonicum, the nature of resistance for Cmm appears to be partially based on a diminished pathogen population, with no inhibition of the pathogen's ability to multiply (Thry, 1971; Wakimoto et al., 1968; Van Steekelenburg, 1985).

Prior to 1988, estimation of population densities for Clavibacter species was determined by manually chopping or grinding infected tomato tissue. Two new techniques have been recently described: stomaching (Fatmi and Schaad, 1988) and immunofluorescence (Davis et al., 1988; De Boer and McCann, 1989), in addition to ELISA. The stomacher technique was used in this research.

OBJECTIVES: The main objectives of these studies were: (1) to develop and evaluate an inoculation technique and screening procedure by which 10 to 12-day-old tomato seedlings could be screened for resistance to Cmm; (2) to explore how the two strains (Cm-103, Cm-63) of Cmm used in this thesis reacted with a particular method of inoculation; and (3) to determine the reaction of Cmm-susceptible and resistant tomato accessions inoculated by the newly developed cotyledon excision method with the two Cmm strains.

MATERIALS AND METHODS

Inoculum. Two strains of Cmm, Cm-63 and Cm-103, were included in each experiment. These two strains were previously identified as Cmm by cultural characteristics (pigmentation, Gram stain, colony morphology) and pathogenicity on tomato, as determined by J. Chamot (Michigan State University) and M. Ricker (CIRT, Napoleon, OH), respectfully. Strain Cm-63 originated from a Cmm-infected field in southwest Michigan and is typically light yellow. Strain Cm-103 is from the collection (Cm 156-2) of A. Vidaver (Univ. of Nebraska, Lincoln) and no record of its exact origin is available. This strain is typically golden-yellow and unlike Cm-63, has the ability to produce cankers on tomato stems and petioles.

Inoculum was prepared by inoculating 10-ml nutrient broth (Difco, Detroit, MI) with a loopful of either bacterial strain and placing it on a shaker (Lab-Line Orbit Environ-Shaker, Melrose Park, IL) at 25 C. After 48 hrs, the suspension was centrifuged (IEC Clinical Centrifuge) at 2400 RPM for 20 min. and the pellet was resuspended in 10-ml sterile water, recentrifuged and resuspended. After dilutions (10-fold) were made in sterile distilled water, the percent transmission of the 10^{0} to 10^{-3} dilutions was determined (Bausch and Laumb Spectronic 20) and compared to a best-fit curve (cfu/ml versus percentage transmission). In addition, the 10^{-8} and 10^{-9} dilutions were plated onto nutrient agar (Difco) ammended with 5 % glucose/liter to further enumerate the exact concentration used in each experiment. The final concentration of inoculum used to make all inoculations ranged from 10^{6}- 10^{8} cfu/ml, as this range results

in similar foliar disease reactions.

Inoculation methods. A new method of inoculation was devised where by 10-12 day-old seedlings could be evaluated. This method, herein called cotyledon excision, involved the excision of a single cotyledon from a 10 to 12-day-old tomato seedling, at its point of attachment to the hypocotyl. Clip inoculation, a previously reported technique, was used for comparison and involves excising the stem and apical portion of a 2 to 5-week old plant, $\geq$1 cm above the cotyledonary leaves.

Excisions for both methods were made using a pair of scissors dipped in either strain of inoculum. After each excision, a droplet of the bacterial suspension could be seen visibly adhering to the cut plant surface. It was determined that approximately 0.01 ml was applied per excision.

Disease evaluation. Inoculated plants were assessed for their reaction to Cmm in two ways: (A) rating the amount of foliar disease and (B) quantification of systemic pathogen populations.

(A) Foliar disease rating. Plants were individually rated for foliar disease symptoms, 8 weeks after inoculation. A scale of 1-6 (Fiqure 1), based on visual foliar symptoms, was devised, where 1 = > 9 symptomless leaves present; 2 = 7 - 9 symptomless leaves present; 3 = 5 - 6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant is stunted and/or cankered; 5 = canker(s) may be present, plant may be stunted; 2 - 3 symptomless leaves present; 6 = only the apical leaf is symptomless or the plant was

dead. A leaf was considered symptomless if all its leaflets lacked the following symptoms: necrosis, chlorosis associated with wilting, black veins, marginal firing, wilting and curling of the leaflets. A plant was determined to be stunted if it was 8.5 cm less than the mean height of that particular line inoculated with that particular Cmm strain. Chlorosis and necrosis generally began marginally with the chlorosis being distinguishable from that resulting from poor plant nutrition.

(B) Quantification of pathogen populations. After the 8-week incubation period, selected plants were excised at the soil line. Pathogen populations were determined using a commercial laboratory blender (Stomaker model no. STO-400, Tekmar Co., Cincinnati, OH). A plant sample was placed in a heavy-duty plastic bag containing liquid buffer, and then locked into place in front of a pair of internal paddles. When the machine was turned on, the sample was paddled against the metal door causing the tissue to split open and the bacteria to escape into the liquid buffer. Plants were stomached (stem and leaves, but no roots) for 1-2 min. using a phosphate buffer (0.05 M, pH 7.45, 100 ml/plant) ammended (0.02 %) with Tween-20 detergent, as recommended by Fatmi and Schaad (1988). Samples from each bag were serially diluted (10-fold) and 0.1-ml samples of the 10^{-3} and 10^{-4} solutions were plated onto nutrient agar ammended with 5 % glucose/liter. After 7 days incubation at 25 C, the number of colony forming units (cfu) per gram fresh weight was determined and a $\log_{10}$ transformation was made. Generally three plants per line per strain were assayed.

Fiqure 1. Foliar rating scale of 1 - 6, where: 1 = > 9 symptomless leaves present; 2 = 7 - 9 symptomless leaves present; 3 = 5 - 6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant is stunted and/or cankered; 5 = 2 - 3 symptomless leaves present; plant may be stunted and/or cankered; 6 = only apical leaf is symptomless or plant is dead.

Four separate studies were performed:

1.) Accession study. Seeds of 9 Cmm-resistant Lycopersicon accessions: L. hirsutum, L. peruvianum, L. pimpinellifolium cv. Utah 20, and L. hirsutum cv. Bulgaria 12,, P.I. 324707 (Bulgaria), P.I. 324708 (Bulgaria) P.I. 358815 (Philippines), Heinz 2990, Flora Dade, and 4 Cmm-susceptible L. esculentum cultivars: Campbell 1320, Easy Winner, Pik-Red and Sunny, were germinated and transplanted at 7 days into 9-cm plastic pots containing Baccto planting medium (Michigan Peat Co., Houston, TX). Eleven experiments involving combinations of these accessions were arranged in completely randomized designs (CRD) with 6-12 pots (2 plants each) per strain per line. Seedlings were inoculated (10^5 to 10^6 cfu/ml) by cotyledon excision or clip inoculation. The clip inoculations involved removal of the apex and upper 2nd and 3rd true leaves of a 3-week-old seedling. Plastic saucers (10.5 cm) were placed beneath each pot to reduce cross contamination of the two strains. Seedlings were then placed into a controlled environmental chamber, 25-27 C, 16 hr photoperiod (200 $uEm^{-2}s^{-1}$ cool-white and incandescent lamps). Data were analyzed by analysis of variance (ANOVA) for each experiment; LSD tests were used to separate the treatment means (Steel and Torrie, 1980).

2.) de Jong/ Honma study. Seeds of 24 partially-resistant, F-13 and F-14 generation L. esculentum lines ('Earliana' x 'Bulgaria 12', intercrossed with 'Rapids') were germinated. These lines were previously cross-bred at Michigan State University (Department of Horticulture) by Dr. Jan de Jong (1975) for Cmm resistance inheritance studies and selected for horticultural characters and

Cmm-resistance by Dr. Shigemi Honma. A partially resistant control, 'Bulgaria 12', was included. Seeds were transplanted at 7 days of age into 12.5 cm clay pots, 3 plants per pot. The experiment was arranged as a randomized complete block in the greenhouse (September - November) with three replications of three plants per strain. Ten-day-old seedlings were inoculated by cotyledon excision with initial inoculum concentrations of 10^5 for strain Cm-63 and 10^8 for Cm-103. Data were analyzed by analysis of variance (ANOVA); LSD tests were used to separate treatment means (Steel and Torrie, 1980).

3.) 8-week pathogen monitering study. Seeds of 'Pik-Rite' (susceptible) and 'Bulgaria 12' (partially resistant) were germinated and transplanted into 9-cm plastic pots (2 plants per pot) at 7 days of age. Seedlings were inoculated (10^6 to 10^7 cfu/ml) by cotyledon excision or clipping (5-week-old plants). Systemic pathogen populations were monitered by stomaching one replication at 3-days post inoculation, at 7-days, and weekly thereafter for a total period of 56 days (8-weeks). The experiment was arranged in a controlled environmental chamber as a randomized complete block with three replications of one plant each, split over the inoculation method. Data were analyzed by analysis of variance (ANOVA) (Steel and Torrie, 1980).

4.) Study of plant portions for pathogen populations. Seeds of three partially resistant L. esculentum cultivars (Bulgaria 12, Flora Dade and Heinz 2990) and three Cmm-susceptible cultivars (Ohio 7880, PSR 77084 and Marglobe) were germinated. Seedlings were transplanted as described above and inoculated by cotyledon excision (12-days-old)

and/or clip inoculated (3-weeks-old). After an 8-week incubation period, plant height was measured and the plants were excised at the soil line and halfway up the stem, thereby dividing individual plants into lower and upper-half plant portions (roots not included). Each portion was then stomached separately, as previously desribed.

RESULTS

1. Accession study. Seven out of 11 experiments produced significant ANOVA's (Table 1) and mean disease ratings were further separated by strain.

When the experiments included both inoculation techniques (Experiments 1, 4, 6), clip inoculation generally produced higher disease ratings than inoculation by cotyledon excision.

Strain virulence influenced the development of foliar disease symptoms. Regardless of inoculation method, strain Cm-103 inoculations produced somewhat higher levels of foliar disease than did Cm-63.

Of the four experiments (Experiments 8-11) with nonsignificant ANOVA strain factors (Table 2), Experiment 9 resulted in significant disease differences among the two Cmm-susceptible cultivars and the PR cultivar.

Cmm-susceptible cultivars reacted similarly to PR cultivars in four experiments (Experiments 1, 8, 10-11) however, four other experiments (Experiments 2, 3, 5, 9) produced significant genotypic differences.

2. de Jong/ Honma study. None of the 24 F-13 and F-14 L. esculentum hybrid lines were more resistant than the 'Bulgaria 12' control after

cotyledon excision inoculation with Cm-63 or Cm-103 (Table 3). Cmm strain and the line x strain interaction were both highly significant. Pathogen populations were 100-fold greater in lines inoculated with strain Cm-103 (mean 8.4 cfu/g f.w.) compared to those inoculated with Cm-63 (mean 6.5 cfu/g f.w.). In addition, reduced pathogen populations were found in the 'Bulgaria 12' control, compared to 21 of the 24 hybrid lines.

Table 1. Comparison by experiment of foliar mean disease ratings for Lycopersicon esculentum accessions inoculated with Clavibacter michiganensis subsp. michiganensis (Cmm) by cotyledon excision or clip inoculation.

Experiment accession	Type	Cotyledon inoculation		Clip inoculation		Significance		
		Cmm-103	Cm-63	Cmm-103	Cm-63	Inoc method	Acce-ssion	Cmm strain
Experiment 1						**	**	*
L. esculentum								
Bulgaria 12	PR	3.9 b	3.5 b	5.0 a	4.6 b			
Flora Dade	PR	4.6 a	4.6 a	5.4 a	5.3 a			
Ohio 7880	S	3.9 ab	3.8 b	4.9 a	3.9 b			
Experiment 2						n.s.	**	*
L. esculentum								
Mountian Pride	S			5.5 a	4.6 ab			
L. pimpinellifolium								
Utah 20	PR			4.7 b	4.1 b			
Experiment 3						n.s.	*	**
L. esculentum								
Campbell 1320	S	6.0 a	6.0 a					
Bulgaria 12	PR	5.5 b	5.8 a					
L. pimpinellifolium								
Utah 20	PR	5.3 b	5.8 a					
Experiment 4						n.s.	*	**
L. esculentum								
Heinz 2990	PR	5.7 a	5.0 b	5.5 a	4.3 b			
Experiment 5						n.s.	*	n.s.
L. esculentum								
Marglobe	S		5.4					
PSR 77084	S		5.7					
Experiment 6						*	n.s.	n.s.
L. esculentum								
Flora Dade	PR		2.8		4.7			
Experiment 7						--	--	--
L. hirsutum	PR		2.6					

** significant difference at p 0.01; * significant difference at p 0.05.

Means in a column followed by the same letter are not significantly different by LSD (p= 0.05).

Table 2. Comparison of accessions by average foliar disease rating in experiments where a nonsignificant ANOVA strain factor was observed.

Experiment accession	Type	Avg. dis rating[z]	Signifigance of disease rating
Experiment 8			n.s.
L. esculentum			
PI 358815	PR	3.3	
PI 324707	PR	4.0	
PI 324708	PR	3.5	
L. peruvianum			
PI 128653	PR	3.4	
L. pimpinellifolium			
PI 340905	PR	3.7	
Experiment 9			**
L. esculentum			
Mountain Pride	S	5.5 a	
Pik Red	S	5.6 a	
L. pimpinellifolium			
Utah 20	PR	4.7 b	
Experiment 10			n.s.
L. esculentum			
Bulgaria 12	PR	3.9	
Sunny	S	4.3	
Experiment 11			n.s.
L. esculentum			
Bulgaria 12	PR	5.9	
Easy Winner	S	5.8	
Sunny	S	5.8	
L. pimpinellifolium			
Utah 20	PR	6.0	

[z]Averaged disease rating from both strains of Cmm. Rated on a scale of 1 to 6 where 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant is stunted and/or cankered; 5 = 2-3 symptomless leaves present, plant may be stunted and/or cankered; 6 = only apical leaf is symptomless or plant is dead.

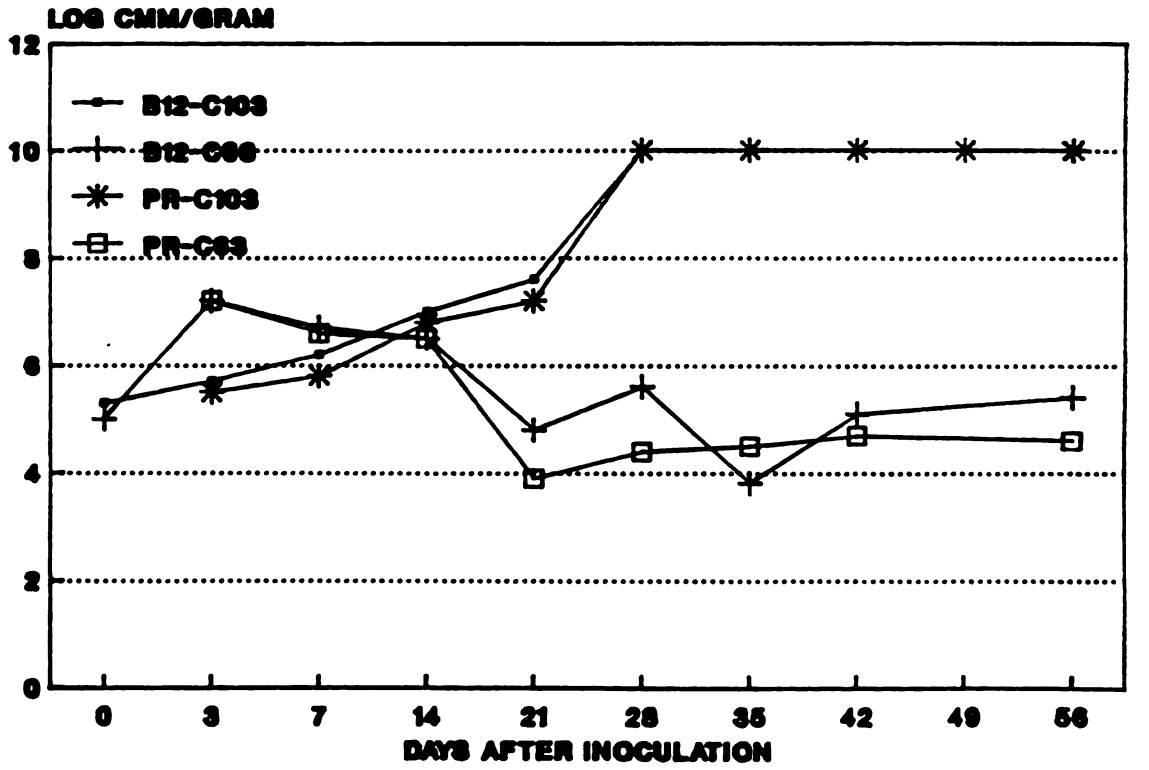
** significant line affect at p 0.01. Means in a column followed by the same letter are not significantly different by LSD (p = 0.05).

Table 3. Comparison of mean foliar disease ratings and pathogen populations in 24 F-13 and F-14 generation MSU hybrids, after cotyledon excision inoculation with two strains of Clavibacter michiganensis subsp. michiganensis.

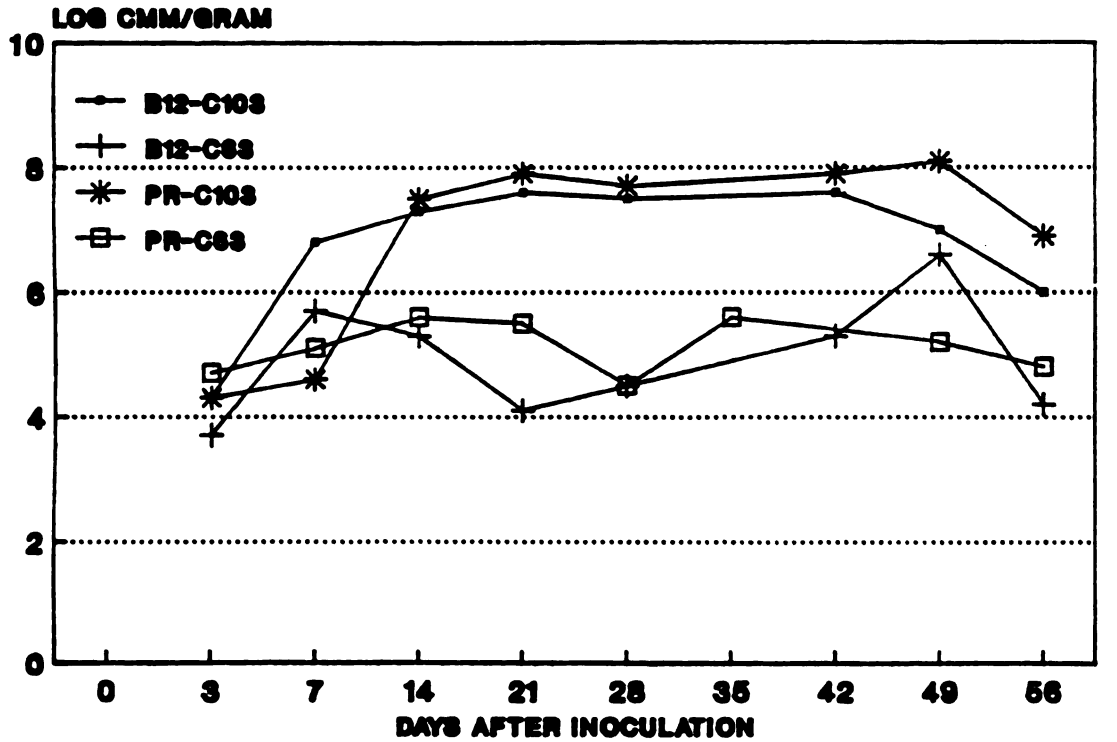
Line	Strain Cm-103[v]		Strain Cm-63	
	Mean[z] foliar disease rating	Mean[y] pathogen population (log 10)	Mean[z] foliar disease rating	Mean[y] pathogen population (log 10)
Bulgaria 12[x]	5.2	6.6	3.2	5.9
2004-2	4.7[w]	8.4	4.3	5.3
2012-1	4.9	8.5	3.2	6.3
2013-3	5.0		3.7	
2024-3	5.4	8.5	4.6	6.7
2026-1	4.9		3.4	
2027-1	5.1		3.7	
2028-2	4.6	8.4	3.4	5.9
2031-2	4.4	8.3	4.2	6.9
2036-1	5.2	8.0	3.1	5.5
2037-1	5.0		3.1	
2040-2	5.3		3.3	
2041-2	4.9		2.7	
2041-3	4.7		3.7	
2042-3	4.9		3.1	
2048-3	4.5		2.7	
2048-4	5.5		3.8	
2048-6a	4.4		3.1	
2049-1	4.6	8.3	3.3	6.6
2054-2	4.8		3.3	
2059-2	5.5	8.4	3.5	7.2
2064	5.3		3.6	
2066-2	5.2	8.9	3.5	7.8
2067-2	4.8	8.2	3.3	6.5
2068-1	4.8		3.4	

[v] Strain difference was highly significant (p 0.01).
[w] None of the hybrid lines rated significantly better than the 'Bulgaria 12' control, for either Cmm strain, based on mean separation by LSD (.85; p 0.05).
[x] Partially resistant control.
[y] Mean of three plants.
[z] Mean of 9 plants, rated on a scale of 1 - 6, where 1 = > 9 symptomless leaves present 2 = 7 - 9 symptomless leaves present; 3 = 5 - 6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant is stunted and/or cankered; 5 = 2 - 3 symptomless leaves present, plant may be stunted and/or cankered; 6 = only apical leaf is symptomless or plant is dead.

3. 8-week pathogen population monitering study. Population trends of Cm-103 and Cm-63 are depicted in Fiq. 2 and 3. Pathogen levels in the clip inoculated plants dropped from 5.1 - 5.7 cfu/g to 3.5 - 4.5 after three days. Conversely, pathogen levels in the cotyledon excised plants tended to remain more stable within the first few days after inoculation. After 3-7 days, the growth rates of the two strains began to differ significantly, with the level of Cm-103 rising drastically and that of Cm-63 declining. Populations of both strains began to level off 14 (Fiq. 3) to 28 days (Fiq. 2) after inoculation. For strain Cm-103, the cotyledon inoculation method resulted in at least a 1000-fold greater systemic multiplication than did inoculation by clipping. (Systemic concentrations greater than log 10 were not determined.) Overall, strain Cm-63 resulted in lower (log 4.6 - 5.3) population levels for either inoculation method. The method of inoculation, time (sampling date) and Cmm strain each produced a significant effect (Table 4).



Fiqure 2. Log of Clavibacter michiganensis subsp. michiganensis (Cmm) per gram fresh weight in Lycopersicon esculentum B12 (Bulgaria 12, partially resistant) and PR (Pik-Rite, susceptible) over a 56-day incubation period after cotyledon excision inoculation with two strains of Cmm (C103, C63).



Fiqure 3. Log of Clavibacter michiganensis subsp. michiganensis (Cmm) per gram fresh weight in Lycopersicon esculentum B12 (Bulgaria 12, partially resistant) and PR (Pik-Rite, susceptible) over a 56-day incubation period after clip inoculation with two strains of Cmm (C103, C63).

Table 4. Analysis of variance for the 8-week study on *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) populations in a susceptible and a partially-resistant *L. esculentum* cultivar, inoculated by cotyledon excision or clip inoculation with two strains of Cmm.

Source	Degrees of freedom	F-value	Probability
Replication	2	.25	n.s.
Inoc. Method (M)	1	24.78	**
Day (D)	8	3.7	***
M x D	8	3.54	***
Cultivar (C)	1	.19	n.s.
M x C	1	5.39	*
D x C	8	1.77	*
M x D x C	8	2.95	**
Cmm strain (S)	1	235.98	***
S x M	1	1.47	n.s.
D x S	8	19.77	***
M x D x S	8	5.28	***
C x S	1	.11	n.s.
M x C x S	1	.12	n.s.
D x C x S	8	1.77	*
M x D x C x S	8	1.46	*

* = significantly different at $p = 0.1$, ** = significantly different at $p = 0.05$, *** = significantly different at $p = \leq 0.001$.

4. Plant portion/pathogen population study. When strain Cm-103 was used for cotyledon inoculations (Table 5), greater pathogen levels were detected in both the upper and lower plant portions of 'Heinz 2990', compared to the other PR cultivar, Flora Dade. Inoculations with strain Cm-63 produced similar pathogen levels in the lower plant portion of all four cultivars. As for the two PR cultivars, systemic pathogen populations in the upper plant portions of 'Heinz 2990' were half that found in the respective lower plant portions, whereas, Flora Dade had similar systemic populations in either portion. 'PSR 77084' did not respond similar to the susceptible cultivar Marglobe, having a reduced pathogen level in the upper plant portions. 'Marglobe' however, exhibited similar pathogen populations in both plant portions.

When clip inoculated (Table 6), the greatest systemic populations were found in the upper plant portions of the susceptible cultivar 'Ohio 7880'. In contrast, the three PR cultivars, Bulgaria 12, Heinz 2990 and Flora Dade contained the greatest pathogen populations in the lower plant portions. Overall, the three PR cultivars exhibited reduced pathogen populations in the upper plant portions compared to 'Ohio 7880'. Of the three PR cultivars, Heinz 2990 had the highest pathogen populations for either plant portion.

Table 5. Log of numbers of colony-forming units of Clavibacter michiganesis subsp. michiganensis (Cmm) per gram fresh weight recovered from 2 susceptible and 2 partially resistant Lycopersicon esculentum cultivars inoculated by cotyledon excision using two strains of Cmm.

Cultivar	Type	Plant portion	Cm-103	Cm-63
H-2990	PR	lower half	9.0	5.2
		upper half	7.5	1.8
Flora Dade	PR	lower half	4.6	4.6
		upper half	---	5.1
PSR 77084	S	lower half	ND	5.5
		upper half	ND	2.9
Marglobe	S	lower half	ND	5.2
		upper half	ND	4.9

[z]Mean pathogen population of three inoculated plants.

--- = not detectable, ND = not determined.

Table 6. Log of numbers of colony-forming units of Clavibacter michiganesis subsp. michiganensis (Cmm) per gram fresh weight recovered from 3 susceptible and 1 partially resistant Lycopersicon esculentum cultivars inoculated by clip inoculation using two strains of Cmm.

Cultivar	Type	Plant portion	Cm-103	Cm-63
Bulgaria 12	PR	lower half	6.2[z]	2.0
		upper half	4.2	1.0
H-2990	PR	lower half	8.5	5.4
		upper half	6.7	1.4
Flora Dade	PR	lower half	5.0	2.5
		upper half	1.0	1.0
Ohio 7880	S	lower half	7.3	4.2
		upper half	8.7	3.4

[z]Mean pathogen population of three inoculated plants.

DISCUSSION

Based on the studies presented here and elsewhere in this thesis, inoculation by the cotyledon excision method produced canker disease reactions consistent with those occurring from other vascular inoculation techniques. Cotyledon excision inoculation permits rapid evaluation of a large number of individual plants or seedlings, either in a greenhouse or controlled environmental chamber, thereby reducing the amount of required field work. It also reduces the amount of time required to carry out inoculation experiments by several weeks, depending on the length of the incubation period. After the 8-week incubation period, resistant plants can be transplanted and grown on for further evaluation of horticultural characters. Inoculation by cotyledon excision also allowed for the systemic introduction of the pathogen and can readily differentiate Cmm-susceptible or resistant plants. This method gave consistent results when similar enviromental conditions were met, was reliable, and produced the characteristic symptoms usually associated with inoculation of older plants: cankering, unilateral wilting and withering, and firing, as well as the younger seedling symptoms of wilting and death. Foliar disease symptoms were visible by 4 weeks after inoculation in susceptible plants. Resistant plants may have been rated more susceptible than they would have with other published rating scales.

Cotyledon excision inoculation can be of use in determining differences in pathogenicity of previously untested Cmm strains and can also be used to measure differences in pathogenicity of two or

more strains by comparing pathogen growth rates.

There were no significant differences in the 24 tomato hybrid lines, produced in the 1970's by de Jong, when inoculated with Cmm strains Cm-103 and Cm-63 by the cotyledon inoculation method. When inoculated by de Jong, 4 to 5-week old plants were clipped 1 cm above the cotyledonary leaves with what was described as a mildly virulent strain of Cmm. This may explain why the hybrids appeared resistant to de Jong's study but not in our study. Lower foliar disease ratings resulted in our study when the less virulent Cmm strain (Cm-63) was used, whereas, strain Cm-103 produced greater amounts of disease. Pathogen populations were lower in the plants inoculated with Cm-63, as compared to plants inoculated with strain Cm-103. Bacterial populations within the 24 lines were significantly different than the 'Bulgaria 12' control. Thus, the difference in disease reactions expressed in this study were associated with inoculation method and strain virulence.

The less virulent strain, Cm-63, had a lower rate of multiplication compared to that of the more virulent strain Cm-103. Surprisingly, within a method/strain combination, the genotypic and inherent differences in Cmm resistance which exist between 'Pik-Rite' and 'Bulgaria 12' had little ($\leq$ 10 fold) influence on the pathogen's ability to colonize, persist and multiply appreciably over the 8-week incubation period. Populations of both strains leveled off within the first four weeks, although some fluctuation was found to occur in strain Cm-63. Resistance did not slow the multiplication of the pathogen: the PR cultivar did not express lower bacterial

populations. These results parallel those of Chang and co-workers (1988) in which genotype resistance was found to have no role in determining the epiphytic populations of a susceptible and 'tolerant' tomato cultivar. The observation of a higher bacterial population in the younger plants (cotyledon excision treatments) supports the findings of Gleason and co-workers (1989), who compared Cmm populations in three different-aged tomato seedlings. These researchers found that stems of the younger (2.5-week-old) seedlings were colonized much more rapidly after inoculation than were the stems of the 6.5-week-old plants. In our study, the two Cmm strains were easily distinguishable from each other. For strain Cm-103, the cotyledon inoculation method resulted in at least 1000-fold greater cfu/gram than did the clip method, log $\geq$ 10 compared to 6.5, respectfully. The younger seedlings used in the cotyledon inoculation treatments were colonized by larger populations than were the older seedlings used in the clip inoculation treatments. Surprisingly, genotype was found to be nonsignificant in estimating the population density of Cmm. The absence of a cultivar x strain interaction suggests that the two cultivars did not differ in their reaction to Cm-103 and Cm-63. However, the (inoculation method x sample day x cultivar) interaction was significant and the cultivars did differ on certain sampling dates.

In the PR cultivars used in the plant portion study, the pathogen was more likely to remain at higher levels near the site of inoculation, which was located in the lower portion of the plant, with reduced upward systemic spread. Conversely, susceptible

cultivars typically possessed a relatively larger pathogen population which was found to be distributed more equally throughout the entire plant. The PR cultivar Heinz 2990 differed from the other two PR cultivars tested by possessing higher pathogen populations. Again strain virulence and the method of inoculation played important roles, influencing the reactions of the cultivars. Higher pathogen populations occured systemically when plants were clip inoculated with strain Cm-103. Results from this study partially support those from a previous study by Van Steekelenburg (1985), where Cmm was detected in the upper portions of susceptible cultivars which showed symptoms of wilting. In her study, Cmm was also detected in symptomless plants, including from some of the upper portions of some resistant plants. The presence of Cmm in the upper portions of resistant plants can relate to the aggressiveness of the pathogenic strain used, as well as to the ability of a resistant plant to inhibit the multiplication.

Studies by Gillaspie and co-workers (1976) and Bailey (1977) on the relative populations of C. xyli subsp. xyli, a species related to Cmm, indicated a positive correlation between population density and the degree of susceptibility to ratoon stunting disease. If a cultivar was susceptible, C. xyli multiplication was found to be comparatively rapid; whereas, in a non-susceptible cultivar, the rate was much reduced. This suggested that measurement of the pathogen population might provide a means for screening for resistance to ratoon stunt. Vegetatively-propagated stalks (stems) of resistant sugarcane cultivars had lower population densities and often did not

contain detectable levels of the pathogen. Further work by Harrison and Davis (1988) found that the reduction in bacterial densities was due to a reduction in the numbers of infected vascular bundles.

Cmm appears to systemically behave more like C. m. subsp. sepedonicum, (Cms), a related subspecies, causal organism of bacterial ring rot of potato, than C. xyli. Evidence, as reported in a study by De Boer and McCann (1989) demonstrates that (i) resistant potato cultivars never to very seldom express disease symptoms; (ii) symptomless plants harbour the pathogen; (iii) a potato cultivar which did not develop bacterial ring rot symptoms also had the lowest stem bacterial densities.

Over the past 20 years, resistance to Cmm has been considered to be partially a matter of bacterial inhibition, with resistance expressed by action directly upon the pathogen itself (Thyr, 1971). Wakimoto and co-workers (1968) were some of the first bacterial canker researchers to detect Cmm populations in the field resistant cultivar 'Sekko'. Although relatively high Cmm populations were detected in 'Sekko', log 8.3 - 12 cfu/stem, they were the lowest of the 4 popular tomato cultivars examined. Thyr (1971) reported that the resistant tomato species L. hirsutum harbored 8 x 10^5 cells/g fresh weight, compared to 1.5 x 10^{11} in the susceptible L. esculentum cultivar Highlander. In a prior study, Thyr (1969) found extensive vascular invasion in lines of L. hirsutum and L. pimpinellifolium, another resistant species. An investigation by Van Steekelenberg (1985) compared the multiplication of Cmm in partially resistant and susceptible L. esculentum cultivars. She found that symptomless

plants of the resistant cultivar 'Okitsu Sozai No 1-20' were carriers of the pathogen. However, 10^6-fold reductions in Cmm were found in this cultivar, compared to wilted plants of the susceptible cultivar 'Moneymaker'.

It appears that tolerance to Cmm may be an important aspect of resistance to Cmm, as in Cms: partially resistant cultivars are more capable of withstanding infection of their vessel elements. Such a tolerance has been previously described by Wakimoto and co-workers (1968) when they accounted the relative resistance of 'Sekko' to the 'rigidity of the tissues'. Boelema (1976) suggested that 'susceptible sites' were associated with the vessel elements, specifically with the composition of the wall of the vessels between the spirals, and the wall of the pathogen. Such sites are where the pathogenic bacterium can attach and multiply. This would suggest that resistance to Cmm involves, like C. xyli, a reduction in the numbers of infected vascular bundles.

In conclusion, it has been shown in our studies that Cmm (strains Cm-103 and Cm-63) multiplies when introduced into tomato cultivars that are considered to be resistant to bacterial canker based on visual foliar symptoms. Populations of Cmm were found to vary quantitatively among tomato species and cultivars, and increased in number, to a point, as the plant material matured after inoculation. It was further evident that there are definite trends in the population of Cmm, depending on the host genotype, but more importantly on Cmm strain or virulence. Multiplication of the two Cmm strains used in this study was not always affected by genotype. For

example, genetic resistance to Cmm generally influenced bacterial populations associated with the lower/upper plant portions, but strain virulence proved as important a factor. Genotype however did not influence the overall population densities and the partially resistant cultivar behaved similarly to the susceptible cultivar within a strain x inoculation method combination. Populations of Cmm are clearly detected in partially resistant genotypes, but at lower levels, at least in certain cultivars, depending on the experimental conditions and design. Comparing Cmm populations will not necessarily indicate the relative foliar resistance of a cultivar. Differences in Cmm strain virulence had the greatest influence on resistance to Cmm, followed by method of inoculation.

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CHAPTER 2.

Somaclonal Variation in *Lycopersicon* to *Clavibacter michiganensis* subsp. *michiganensis*

INTRODUCTION

Although originally advocated as a procedure for the clonal propagation of asexual species, Murashige and Nakano noted in 1967 that the introduction of plant cells into culture resulted in genetic changes. Plant cell and tissue culture is a process that involves (i) the establishment of a dedifferentiated cell or tissue culture, (ii) proliferation for a number of generations, and (iii) the subsequent regeneration of plants, called somaclones (Larkin and Scowcroft, 1981). There are seven processes employed to generate somaclones: a protoplast, callus or long-term culture cycle; the use of explants from specific tissues; the generation of random variation simultaneously with the selection of a specific nutrient medium or hormone formulation; and the use of certain genotypes that tend to produce increased amounts of variation (Reisch, 1983). Somaclonal variation may be viewed as tissue culture mutagenesis and has been described as any variation, phenotypic or genotypic, which occurs in plants regenerated from a cell or tissue culture (Larkin and Scowcroft, 1981). The value of somaclonal variation was first recognized in the asexually propagated crop of sugarcane (Heinz and Mee, 1971) when somaclones regenerated from susceptible cultivars expressed variation in chromosome number, morphology, sucrose yield (Heinz et al., 1977) and stable resistance to several diseases (Evans et al., 1984). When Larkin and Scowcroft urged plant breeders

and geneticists in 1983 to exploit somaclonal variation for the purpose of improving plant stocks, the idea was received with skepticism. Researchers believed that this manipulation might prove more difficult than other mutation breeding techniques, and that variants might not breed true (Fox, 1986). Evans and Sharp (1983) were the first to prove that somaclonal variation was a controllable and feasible avenue in plant breeding when they discovered 13 single gene mutations occuring at a frequency of 1 in 18 regenerants, in 230 regenerated tomato plants. This discovery opened the door for using cell and tissue culture as a tool for the introduction of variation into seed propagated crop species (Evans et al., 1984). Tissue culture methods have been suggested to be a powerful mutagen, capable of inducing novel genetic variation for plant improvement (Lorz, 1984). They can also be used to transfer and manipulate disease resistance genes, whether already present in the crop, related genera, or, if intended to be mutagenized during the culture process (Earle, 1989).

Somaclonal variation has been shown to exist in tomato plants derived from explant-generated callus (Merideth, 1979; Padmanabhan et al., 1974; Behki and Lesley, 1976; Montagno, 1986; Kut and Evans, 1982; Locy, 1983; Kurtz and Lineberger, 1983; Kartha, et al. 1976; DeLanghe and de Bruijne, 1976; Thomas and Pratt, 1982; Evans and Sharp, 1983; Buiatti et al., 1985; Gunay and Rao, 1980; Tal et al., 1977; Ohki et al., 1978; Herman and Haas, 1978; Barden et al., 1986; Zagorska et al., 1986; Yu and Masiunas, 1989), protoplasts (Zapata et al., 1981; Neidz et al., 1985; Morgan and Cocking, 1982; Merideth,

1978; Muhlbach, 1980; Shahin and Spivey, 1986; Mulback, 1980; Shahin, 1984, 1985; Wijbrandi et al., 1988; Imanishi and Hiura, 1982; Hassanpour-Estahbanati and Demarly, 1986; Tan et al., 1987), or cell suspensions (Merdith, 1979). Regeneration of tomato variants from haploid tissues has also been successfully reported (Gresshoff and Doy, 1972; Zamir et al., 1981; Ziv et al., 1982, 1984; Varghese and Yadar, 1986; Cappadocia and Sree Ramula, 1980; Devreux et al., 1976).

While the causes of somaclonal variation are not completely known, a combination of several factors are believed responsible. Some of the variability is due to preexisting genetic changes (Evans et al., 1984) in the donor tissue which can be amplified when the somaclones are regenerated. Other mechanisms proposed for somaclonal variation include single gene (dominant, semi-dominant or recessive) changes, chromosome and gene rearrangements, cytoplasmic gene changes, mitotic crossing over, activation of transposable elements, gene amplification and deletion, cryptic-virus elimination and changes in copy number of DNA (Larkin and Scowcroft, 1981; Evans, 1989). Transient or epigenetic variation may also result from somaclonal variation but is not sexually transmissible (Meins, 1983). Lee and Phillips (1988) recently reviewed somaclonal variation and concluded that chromosomal rearrangement has emerged as the prevalent type of variation.

Somaclonal variation has now been reported in a large number of crop species, occurs at a relatively high frequency, is genetically stable and heritable, and occurs for both mono- and polygenic traits (Daub and Jenns, 1989). Somaclonal variation in response to plant

pathogens has been reported in many species with resistant plants, R_0 or progeny, obtained from both selected and unselected tissue cultures, having been screened in vitro and/or in vivo.

During its remarkably short history, somaclonal variation has been used to improve crop maturity, yield, herbicide tolerance and disease resistance, whether viral, fungal or bacterial. Such variation has resulted in new cultivars of geranium (Skirvan and Janick, 1976), pepper (Morrison and Evans, 1988), sugarcane (Krishnamurthi and Tlaskal, 1974; Daub, 1986) and sweet potato (Moyer and Collins, 1983) and two cultivars of tomato: DNAP-9 (high solids) and DNAP-17 (Fusarium oxysporum lycopersici race 2 resistant), (Evans, 1987, 1989). Somaclonal variation reduces the time required for new variety development to four years minimum, compared to mutation and backcross breeding, which take a minimum of 6 or 7-8 years, respectively. This occurs because the technique uses existing varieties for the development of new breeding lines (Evans et al., 1984).

Besides contributing to plant breeding, tissue culture may also be used in plant pathology. Its most significant contribution to date has been to function as a tool to elucidate basic mechanisms of pathogen virulence and host defense, including resistance responses of plant cells infected with fungal and bacterial pathogens (Daub, 1986; Helgeson and Deverall, 1983). In the 1970s, researchers utilized tissue cultures of tomato, tobacco and cotton to examine resistant and susceptible responses to the pathogens Phytophthora and Xanthomonas (Warren and Routly, 1970; Helgeson and Deverall, 1983;

Haberlach et al., 1978; Carlson, 1973; Evans et al., 1984). A common observation from these researchers was that plants derived from resistant lines expressed resistance to the pathogen while those from susceptible lines remained susceptible.

There have been a number of successful reports of introducing agriculturally useful, genetic variability into tomato by somaclonal variation, including paraquat tolerance (Thomas and Pratt, 1982), increased solid density (Evans, 1987), and resistances to Fusarium wilt caused by F.oxysporum f.sp. lycopersicii race 2, ((FOL), Miller et al. 1985; Shahin and Spivey, 1986; Evans, 1989)) and to a tomato strain of tobacco mosaic virus ((To/TMV), Barden et al., 1986, Smith and Murakishi, 1987, 1988)). Susceptible tomato cultivars were used as the parent material for the development of both types of disease-resistant somaclones, with identification of increased disease resistance occurring both with and without selection pressure. The resistance to FOL was found to be controlled by a single dominant gene (Shahin and Spivey, 1986), while the resistance to To/TMV appeared to be encoded by a single, incompletely dominant gene; also involving a maternally transmitted factor (Smith and Murakishi, 1988; Smith, 1989).

OBJECTIVE: The objective of this study was to determine whether somaclonal variation, without selection pressure, might be useful in the generation of tomato lines with increased levels of resistance to bacterial canker, caused by Clavibacter michiganensis subsp. michiganensis (Cmm).

MATERIALS AND METHODS

Somaclone production. A total of eleven fresh market, processing and breeding L. esculentum cultivars and two L. pimpinellifolium cultivars were used as parent material (Table 1). These 13 cultivars included both Cmm-susceptible and partially resistant genotypes. Seedlings were germinated and transplanted into Baccto soil-less planting medium in plastic cell-packs (8 packs/flat) and grown in a controlled environmental chamber. A light intensity of 200 $uEm^{-2}s^{-1}$ (16 hr) was provided by cool-white fluorescent and incandescent lamps at 25±2 C. Somaclones were prepared according to the procedure of Evans and Sharp (1983). Explants were placed on Murashige and Skoog (MS) basal medium (1962) amended with 2 mg/liter BAP (benzylaminopurine) or zeatin, 30 g/liter sucrose, (adjusted to pH 5.8), and solidified with 7.5 g/liter agar (Sigma Chemical Co., St. Louis, MO), dispensed (20 ml) into petri dishes (90 x 15 mm). Thirty days after initial culture, calli with shoot primordia were removed and transferred to MS media containing 0-1 mg/liter of either growth hormone and 7.5 g/liter agar, dispensed (50 ml) in magenta ((3x3x4), Magenta Corporation, Chicago, IL) containers, in order to induce rooting and leaf growth. Additional subcultures were made every 30 days as needed. Three-month-old rooted plantlets were transplanted into cell-packs and placed into sealed plastic bags to acclimate for 7 days. The bags were opened a portion every other day for 7 additional days until fully opened. The cell-packs were then removed and placed into a controlled environmental chamber. After 21-28 days the plantlets were individually transplanted into 12.5 cm

standard clay pots and placed in the greenhouse (natural daylight, 21-27 C). After approximately three months, the original regenerated (R_0, (Chaleff, 1981)) plants flowered and set seed. Ripe fruit was collected and the seeds were removed and soaked (double seed volume) 10 % hydrochloric acid solution for 3-4 hours (Taylor et al., 1961). Treated seeds were then rinsed with tap water in a sieve and allowed to dry overnight. First-generation seed (R_1) packages were stored at 4 C until ready to use.

Second culture cycle. In addition to the first somaclonal cycle, six selected R_0 somaclones from the cultivars Sunny, Ohio 7880, and Bulgaria 12 were taken through a second somaclonal cycle, using the leaf explant somaclone procedure described above. Three of these original somaclones ('Bulgaria 12' 3 (2/2.1) and 8 (1/1), and 'Sunny' 43 (1/1)) were previously rated in this study as having increased levels of foliar resistance to one or both Cmm strains, compared to their parental control, while the other two ('Ohio 7880' 6 (2/4.2) and 'Bulgaria 12' 3 (1/2)) somaclones were rated similar to their parent.

Assessment of Variation. All thirteen cultivars were compared for (1) regenerative abilities and (2) plant and seed production. First generation somaclonal offspring (R_1), hereafter referred to as somaclones, of 12 of the 13 cultivars were assessed for (3) morphological variation and (4) reaction to two strains of Cmm by inoculation with one of two methods: cotyledon excision or clipping. Studies utilizing clip inoculation were performed on a subset of somaclones previously examined by cotyledon excision inoculation. For

either inoculation method, the reaction to Cmm was assessed in two ways: (A) rating the amount of foliar disease, and (B) quantification of systemic pathogen populations.

Cotyledon excision inoculation. A total of 35 cotyledon excision inoculation experiments, carried out with R_1 progeny of 12 cultivars, were conducted in either a controlled environmental chamber or in the greenhouse. The progeny of the resomacloned clones were examined in 5 experiments. Controls in all experiments consisted of the parent seed cultivar. In the controlled environmental experiments, evaluations were conducted over a period of 18 months on the R_1 progeny of cultivars Heinz 2990 (H-2990), Flora Dade, Bulgaria 12, Utah 20 and Utah 737. Seeds were germinated and transplanted at 7 days (2 per pot) into 7.5 cm plastic pots (86 g Baccto). Plastic saucers (10.5 cm) were placed under each pot to reduce cross contamination of the two strains. The number of pots used per strain/somaclone combination varied from 6-20 (mean = 10) between experiments, depending on allowable space. Lighting and temperature conditions were as previously described. All controlled environmental chamber experiments were arranged as completely randomized designs. The greenhouse studies were carried out in two greenhouses between the months of June and February, under natural conditions (minimum 21 $\pm$2 C), on the R_1 progeny of 'Easy Harvest', 'Easy Winner', 'Ohio 7880', 'Ferry Morse', 'Mountain Pride', 'Pik-Red' and 'Sunny'. Seeds were germinated and transplanted at 7 days, three per 12.5 cm standard clay pot or in one instance ('Ohio 7880', Experiment 2), 2 per 10 cm clay pot. The number of pots used per

strain/somaclone combination varied from 2-10 between experiments, depending on the number of somaclones to be tested and allowable space. Greenhouse experiments were generally arranged as completely randomized designs due to space limitations, except for three progeny experiments ('Pik Red', 'Sunny' and 'Ferry Morse 6203'), which were arranged in a randomized complete block design.

All seedlings were inoculated by cotyledon excision, as previously described in Chapter 1. A pair of scissors, dipped between cuts in the bacterial suspension (Cm-103 or Cm-63), was used to make the excisions. Plants were fertilized (150 ppm, Peters 20N-20P-20K) four weeks after inoculation and watered as needed.

Plants were measured for height and scored individually for foliar symptoms on a scale of 1-6 (previously described in Chapter 1), 8 weeks after inoculation.

Data were analyzed by analysis of variance (ANOVA) for each experiment; when the ANOVA's were significant, 'LSD' tests (p 0.05) were used to separate treatment means (Steel and Torrie, 1980). When an inoculation experiment resulted in a nonsignificant somaclone x Cmm strain interaction, an alternative method of evaluation was used: disease response classes. These experiments included those performed on 'Bulgaria 12', 'Mountain Pride' and 'Utah 20' progeny. The three classes of foliar disease response used were: (a) decreased resistance to one or both strains; somaclone's mean disease rating (m.d.r.) = 0.6 to 3.5 times greater than the control's m.d.r.; (b) equally resistant to one or both strains; somaclone's m.d.r. = $\pm$ 0 to 0.5 than the control's m.d.r.; (c) increased resistance to one or

both strains; somaclone's m.d.r. = 0.6 to 1.5 times less than the control's m.d.r.

Three cotyledon-inoculated plants per bacterial strain from 100 selected R_1 somaclones of 9 Lycopersicon cultivars were randomly selected and stomached. Stomaching, as previously described in Chapter 1, involves blending inoculated plants in a stomacher with a 0.05 M phosphate buffer with subsequent dilution plating and colony enumeration. Log_{10} transformations were performed on the population data.

Extended greenhouse study on 'Bulgaria 12'. An additional systemic pathogen population study was conducted on 'Bulgaria 12' R_1 progeny to determine the affects of post-inoculation time and increasing plant age on pathogen populations. Inoculated plants from 12 'Bulgaria 12' somaclones were divided into two groups after the previously described 8-week incubation period. Plants from the first group were stomached immediately while the second group of plants was left in the greenhouse (March - November, natural conditions) for an additional 5-8 months (7-10 months total incubation). The 12 somaclones were previously rated as exhibiting either decreased foliar resistance (class a) or resistance similar to that of the parent control (class b). Twenty-five plants from the second incubation group were brought back to the lab in November and stomached. Ten of the original 'Bulgaria 12' inoculated controls were also included in the second study.

Clip inoculation. After prior evaluation by cotyledon excision inoculation, seed of 24 of the 49 R_1 somaclones (7 cultivars),

exhibiting increased foliar resistance to Cmm, were further evaluated for Cmm resistance. Two separate experiments (19 and 5 somaclones, respectfully), were conducted as randomized complete block designs with 2-3 replicates per strain/somaclone combination, 3 plants per replicate. Seeds were germinated, transplanted three per 12.5 cm standard clay pot at 7 days, and fertilized once as previously described. At 5-weeks of age (5 - 7 true-leaf stage), plants were clip inoculated using the procedure of deJong and Honma (1976), with a pair of scissors dipped in a suspension (10^7 cfu/ml) of either strain. Excisions were made one centimeter above the cotyledons, allowing only the first to second true leaves to remain on each plant. Plants were again fertilized two weeks after inoculation.

Clip-inoculated plants were rated for foliar disease symptoms 8-weeks after incubation, using a modification of the scale designed by de Jong and Honma (1976) where 1 = visually 'healthy' plant, 2 = plant appeared normal size but had some wilting, 3 = extensive wilting, large cankers or stunted growth, 4 = growing point dead. Data were analyzed by analysis of variance (ANOVA) for each experiment; LSD tests were used to separate the treatment means (Steel and Torrie, 1980).

Pathogen populations were determined in 19 of the 24 somaclones using the stomacher procedure previously described.

RESULTS

Ability of cultivars to regenerate somaclones. The 13 tomato cultivars differed significantly in their regeneration ability (Table 1) and the production of primary callus from the leaf explants. Leaf explants of cultivars Flora Dade, H-2990, Utah 20, and Utah 737 exhibited the least capacity for shoot regeneration with < 0.5 shoots regenerating per explant. 'Bulgaria 12', 'Easy Winner', 'Mountain Pride' and 'Pik-Red' were intermediate with 0.5 - 1.0 shoots per explant. Cultivars Bonny Best, Easy Harvest, Ferry-Morse 6203, Ohio 7880 and Sunny had optimum regeneration of more than one shoot per explant.

Plant and seed production. The final number of R_1 progeny seed available for experimentation was lower than the number of R_0 regenerants produced, due to sterility, seedless fruit, or plant death following transplanting the R_0 plants to the greenhouse (Table 2). Meristemless R_0 plants, similar to those described by Young (1955) as "curl leaf" mutant, were very common from 'Flora Dade' and 'H-2990', and somewhat common in 'Bulgaria 12' and 'Sunny'. This variation also resulted in the reduction of normal plants capable of flowering and setting fruit.

Table 1. Lycopersicon cultivars assessed for their capacity to regenerate from leaf-explant primary callus.

Species cultivar	Class[y]	Source	Regenerative[z] capacity
L. esculentum processing cultivars			
Easy Harvest	S	Campbell	3
Easy Winner	S	Campbell	2
Ferry-Morse 6203	S	Ferry-Morse Seed Co.	3
Ohio 7880	S	Capital Seed Co.	3
L. esculentum fresh market cultivars			
Bonny Best	S	LL Olds Seed Co.	3
Flora Dade	PR	Harris-Moran Seed Co.	1
Mountain Pride	S	Asgrow Seed Co.	2
Pik-Red	S	Harris-Moran Seed Co.	2
Sunny	S	Asgrow Seed Co.	3
L. esculentum breeding cultivars			
Bulgaria 12	PR	MSU, Dept. of Horticulture	2
Heinz 2990	PR	Heinz U.S.A.	1
L. pimpinellifolium			
Utah 20	PR	U.S.D.A., B. Thyr	1
Utah 737	PR	U.S.D.A., B. Thyr	1

[y]Scored on a scale of 1-3, where 1 = each explant generated < 0.5 shoots; 2 = each explant generated 0.5 - 1.0 shoots; 3 = each explant generated 1.1 - 2.0 shoots.

[z]S = susceptible, PR = resistance is partial as under certain conditions this cultivar may become susceptible.

Table 2. Production of tomato somaclones (R_0 and R_1), inoculation by cotyledon excision with two strains of Clavibacter michiganensis subsp. michiganensis and evaluation for foliar disease symptoms.

Cultivar	No. of explants that produced callus	Total no. of R_0 plants	No. plants prod. R_1 seed	No. R_1 soma-clones assayed	No. of foliar resistant[y] individuals /no. assayed
L. esculentum processing cultivars					
Easy Harvest	29	61	51	39	11/39
Easy Winner	31	21	16	12	0
Ferry-Morse 6203	56	105	99	49	0
Ohio 7780	64	136	114	17	0
L. esculentum fresh market cultivars					
Bonny Best	28	48	47	0	---
Flora Dade	48	17	13	11	4/11[z]
Mountain Pride	37	18	11	10	3/10
Pik-Red	96	63	51	24	5/24
Sunny	61	67	61	36	4/36
L. esculentum breeding cultivars					
Bulgaria 12	103	68	62	58	19/58[z]
Heinz 2990	118	6	5	4	1/4
L. pimpinellifolium cultivars					
Utah 20	80	17	13	13	2/13
Utah 737	86	13	10	10	0

[y] Foliar resistance to one or both strains of C.m. subsp. michiganensis.

[z] Three somaclones of 'Bulgaria 12' and one somaclone of 'Flora Dade' were resistant to both strains of C.m. subsp. michiganensis.

Morphological variability. Morphological variations were observed in the R_0 somaclones and/or R_1 progeny of each of the 13 L. esculentum cultivars and to a lesser extent in the 2 L. pimpinellifolium cultivars. These variations included 1) plant height and internode length, 2) leaf shape, texture, angle of the petioles, number and splitting of cotyledons, 3) amount of chlorophyll (excesses, variegations or deficiencies--albinism), 4) growth habit, 5) sterility, and 6) tomato fruit size and color, and seed size and number. A "wiry" mutant (Fiq.1), first described by Lesley and Lesley (1928), was found to occur in some R_1 progeny of one 'Flora Dade' somaclone. These plants have now produced a few raspberry-shaped fruit, which probably will be seedless, based on previous experiences of Lesley and Lesley. Leaf texture variations, similar to blistering or 2,4-D injury, were noted in two 'Sunny' somaclones. Two somaclones of 'Bulgaria 12' (Fiq.2) regressed to the growth habit of one of the original parents of 'Bulgaria 12', L. pimpinellifolium (Elenkov, 1965). A total of 5 R_0 albino variants occurred in the cultivars Bonny Best, Bulgaria 12 and Ohio 7880. One somaclone of 'Flora Dade' produced fruit with mottled coloration. Fruit size was reduced to currant or cherry types in 'Bulgaria 12', 'H-2990', and 'Ohio 7880'. Seedless fruits or fruits with seed size smaller than the wire-mesh sieve used to rinse the acid treated seeds in, were noted in 9 of the 11 L. esculentum cultivars. Variation in flower morphology was not recorded.

Fiqure 1. Somaclonal variation in leaf morphology and fruit type in cultivar 'Flora Dade' R_1 somaclone 2 (1/1.b). This mutant (recessive, n = 24) has previously been desribed as a wiry type by Lesley and Lesley (1928).

Fiqure 2. Somaclonal variation in growth habit in cultivar 'Bulgaria 12' R_1 somaclone 8 (1/1), pictured on the right; 'Bulgaria 12' (parent) pictured on the left.

Reaction to Cmm: Rating foliar disease symptoms.

A. L. esculentum processing cultivars

Eleven 'Easy Harvest' R_1 somaclones had increased levels of foliar resistance compared to their parent control (Tables 2, 3 and 4). Four of these were highly (p 0.01) significant and 8 additional somaclones were significantly (p 0.05) improved for increased resistance to either strain.

None of the 'Easy Winner' (12 somaclones), 'Ferry-Morse 6203' (49 somaclones) or 'Ohio 7880' (17 somaclones) inoculated somaclones (Tables 2, 3 and 4) were rated as having significantly improved levels of foliar resistance to either strain of Cmm, compared to their parent controls.

B. L. esculentum fresh market cultivars

(1) Flora Dade. Of the 11 'Flora Dade' somaclones evaluated (Tables 2, 3 and 5), four were improved: One somaclone was highly (p 0.01) significant and a second was significantly (p 0.05) improved for resistance to Cm-103. A third somaclone was significantly improved for resistance to Cm-63. A fourth somaclone was highly significant (p 0.01) for increased resistance to both strains, compared to the parent control.

(2) Mountain Pride. The somaclone x Cmm strain interaction was non-significant, thus, somaclones were separated into the disease response classes. Three of the 10 somaclones evaluated (Tables 2, 3 and 5) were improved for resistance (class c) to Cm-103 and a fourth somaclone was improved for resistance to Cm-63, compared to the parent control.

(3) Pik-Red. Of the 24 somaclones evaluated (Tables 2, 3 and 5), 5 were improved: one somaclone was highly (p 0.01) significant (Figure 3) and three somaclones were significantly (p 0.05) improved for resistance to Cm-103. One somaclone was significantly improved for resistance to Cm-63, compared to the parent control.

(4) Sunny. Of the 36 somaclones evaluated (Tables 2, 3 and 5), 4 were improved: two somaclones exhibited highly (p 0.01) significant levels of resistance to Cm-63 and Cm-103 respectively. Two other somaclones were significantly (p 0.05) improved for resistance to Cm-103, compared to the parent control.

C. L. esculentum breeding cultivars

(1) Bulgaria 12. The somaclone x Cmm strain interaction was not significant for approximately 75 % of the total 22 'Bulgaria 12' progeny inoculation experiments; thus, somaclones were separated into disease response classes.

Of the 58 somaclones evaluated (Tables 2, 3 and 6), nine were determined to be more susceptible (class a) than the control to one strain of Cmm, while three additional somaclones exhibited increased susceptibility to both Cmm strains. Twenty-seven somaclones were classified equally resistant (class b) to the control for both strains. Fifteen somaclones were found to have increased resistance (class c) to one strain of Cmm and four other somaclones were found to have increased resistance to both strains. (For classes a and c, the reaction to the second strain was classified as 'equally resistant', unless otherwise noted.)

(2) Heinz 2990. Of the 4 somaclones evaluated (Tables 2, 3 and

6), one somaclone was significantly (p 0.05) improved for resistance to Cm-103, compared to the parent control.

D. L. pimpinellifolium cultivars

The somaclone x Cmm strain interaction for both 'Utah 20' and 'Utah 737' progeny inoculation experiments was nonsignificant, thus, somaclones were separated into the disease response classes.

(1) Utah 20. Of the 13 somaclones evaluated (Tables 2, 3 and 7), two were improved for resistance (class c) to Cm-103 compared to the parent control.

(2) Utah 737. None of the 10 somaclones evaluated (Tables 2, 3 and 7) exhibited improved levels of Cmm resistance, however, some somaclones did exhibit lower mean disease ratings compared to the parent control.

Second somaclonal cycle. The somaclone x Cmm strain interaction was nonsignificant for all 5 inoculation experiments, thus, somaclones were separated into disease response classes. Forty-four percent of the R_1 progeny from nine somaclones resulting from a second somaclonal cycle possessed increased Cmm resistance (class c) compared to the R_1 seed of their parent (Table 8). Mean foliar disease ratings for 22 % of these re-somacloned clones were more susceptible (class a), while 33 % rated similar (class b) to their parent.

Clip inoculation. When clip inoculated with strain Cm-103 (Table 9), three of the 19 somaclones (Experiment 1) were significantly (p 0.01) less susceptible than their respective parent controls: 'Bulgaria 12' 8 (1/1), 'Easy Harvest' 10 (1/1) and 'Mountain Pride' 129 (1/1).

When inoculated with strain Cm-63, one somaclone, ('Sunny' 40 (1/1)), was found to be significantly less resistant than its parent control.

When clip inoculated with strain Cm-103, 4 of the 5 somaclones (Experiment 2) rated equally resistant to their parent, while the fifth somaclone (Utah 20 16 (2/2)) rated significantly worse than its parent control. When inoculated with Cm-63, 3 somaclones rated equally resistant to their parent and the other two somaclones ('Utah 20' 15 (1/1) and 16 (2/2)) rated significantly less resistant than the parent control.

Table 3. Summary of 35 cotyledon excision inoculation experiments conducted on 13 tomato cultivars.

Cultivar	Design[x]	Evaluation[y] conditions	No. plants /strain /somaclone	Total no. somaclones evaluated[z]
L. esculentum processing cultivars				
Easy Winner	CRD	gh, Ap-Jun	20	12
Easy Harvest	CRD	gh, Ap-Jun	20	39
Ohio 7880	RCBD	gh, Jul-Sep	3/rep, 2 reps	17
Ferry-Morse 6203	RCBD	gh, Nov-Jan	3/rep, 2 reps	49
L. esculentum fresh market cultivars				
Flora Dade	CRD	crc	14	11
Mountain Pride	CRD	gh, Ap-Jun	20	10
Pik-Red	RCBD	gh, Nov-Jan	3/rep, 3 reps	24
Sunny	RCBD	gh, Jul-Sep	3/rep, 2 reps	36
L.esculentum breeding cultivars				
Bulgaria 12	CRD	crc	6-20 (avg. 15)	58
H-2990	CRD	crc	12	4
L. pimpinellifolium cultivars				
Utah 20	CRD	crc	10	13
Utah 737	CRD	crc	10	10

[x] CRD = completely randomized design, RCBD = randomized complete block design.

[y] gh = greenhouse, crc = controlled environmental chamber. Greenhouse experiments include months of the year the experiment was conducted: Ap = April, Jan = January, Jul = July, Jun = June, Sep = September, Nov = November.

[z] Only a portion of the R_1 progeny of cultivars Easy Harvest, Pik-Red, Ferry-Morse 6203, Ohio 7880, and Sunny were evaluated. None of the 'Bonny Best' R_1 progeny were evaluated.

Table 4. Disease response of first generation somaclone progeny, originating from four L. esculentum processing tomato cultivars, inoculated by cotyledon excision with two strains of Clavibacter michiganensis subsp. michiganensis.

Disease rating[x]	Frequency distribution of:															
	Easy Harvest				Easy Winner				Ferry-Morse 6203				Ohio 7880			
	ctl[y]		scl[z]		ctl		scl		ctl		scl		ctl		scl	
	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63
1	0	1	10	2	0	1	4	7	0	1	6	78	0	0	0	0
2	2	4	158	206	12	10	59	69	1	1	7	167	0	0	0	1
3	9	9	266	297	4	3	83	88	0	0	8	15	0	1	1	7
4	4	4	167	182	3	5	36	31	2	0	83	10	1	1	22	14
5	3	0	80	45	1	0	14	6	3	0	115	14	4	2	54	53
6	2	2	80	37	0	1	13	11	0	4	62	7	1	2	21	23
n =	20	20	761	769	20	20	209	212	6	6	281	291	6	6	98	98

[x] Disease rating: 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant was stunted and/or cankered; 5 = 2-3 symptomless leaves present, cankers may be present, plant may be stunted; 6 = only apical leaf was symptomless or plant was dead.

[y] Controls (ctls) were seed of the parent cultivar.

[z] Scl = somaclones.

Table 5. Disease response of first generation somaclone progeny, originating from four L. esculentum fresh market tomato cultivars, inoculated by cotyledon excision with two strains of Clavibacter michiganensis subsp. michiganensis.

Disease rating[x]	Frequency distribution of:															
	Flora Dade				Mountain Pride				Pik-Red				Sunny			
	ctl[y]		scl[z]		ctl		scl		ctl		scl		ctl		scl	
	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63
1	0	0	3	0	0	0	3	1	0	0	0	11	0	0	0	0
2	2	4	13	12	1	8	44	72	0	3	6	47	0	2	4	137
3	9	15	42	71	10	4	56	72	0	3	6	47	0	2	4	137
4	5	5	16	40	5	4	44	46	0	0	11	15	3	1	129	18
5	8	3	17	9	3	3	17	3	2	0	85	18	2	0	60	4
6	4	1	44	2	1	1	19	5	7	0	107	4	1	0	22	2
n =	20	20	135	134	20	20	194	199	9	9	216	216	6	6	216	216

[x] Disease rating: 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present, or > 4 symptomless leaves present but plant was stunted and/or cankered; 5 = 2-3 symptomless leaves present, canker(s) may be present, plant may be stunted; 6 = only apical leaf was symptomless or plant was dead.

[y] Controls (ctl) were seed of the parent cultivar.

[z] Scl = total of somaclones..

Fiqure 3. Inoculated somaclones of 'Pik-Red' (right) showing somaclonal variation in reaction to Clavibacter michiganensis subsp. michiganensis: increased foliar resistance. Inoculated 'Pik-Red' parent control plants on the left.

Table 6. Disease responses of first generation somaclone progeny, originating from L. esculentum cv. Bulgaria 12 and Heinz 2990, to inoculation by cotyledon excisions with two strains of Clavibacter michiganensis subsp. michiganensis.

Disease rating[x]	Frequency distribution of: Bulgaria 12 ctl[y] Cmm 103	Cmm 63	Bulgaria 12 scl[z] Cmm 103	Cmm 63	Heinz 2990 ctl Cmm 103	Cmm 63	Heinz 2990 scl Cmm 103	Cmm 63
1	6	8	26	32	0	0	1	0
2	26	71	86	185	0	3	9	9
3	53	118	115	280	5	2	9	18
4	101	52	262	165	1	4	13	3
5	86	60	174	55	4	3	8	7
6	60	27	189	149	2	0	8	11
n =	332	300	852	866	12	12	48	48

[x] Disease rating : 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present, or > 4 symptomless leaves present but plant was stunted and/or cankered; 5 = 2-3 symptomless leaves present, canker(s) may be present, plant may be stunted; 6 = only apical leaf was symptomless or plant was dead.

[y] Controls (ctl) were seed of the parent cultivar.

[z] Scl = total of somaclones.

Table 7. Disease responses of first generation somaclone progeny, originating from L. pimpinellifolium cv. Utah 20 or Utah 737, inoculated by cotyledon excisions with two strains of Clavibacter michiganensis subsp. michiganensis.

Disease rating[x]	Frequency distribution of: Utah 20 scl[y,z]		Utah 20 ctl[y]		Utah 737 scl[z]		Utah 737 ctl	
	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63	Cmm 103	Cmm 63
1	4	2	38	45	7	4	48	59
2	2	6	46	43	1	4	31	16
3	1	1	13	11	0	0	1	2
4	1	1	18	13	1	1	11	20
5	0	0	3	2	0	0	0	0
6	2	0	10	6	1	1	9	3
n =	10	10	128	120	10	10	100	100

[x] Disease rating: 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present, or > 4 symptomless leaves present but plant was stunted and/or cankers were present; 5 = canker(s) may be present, plant may be stunted; 2-3 symptomless leaves present; 6 = only apical leaf was symptomless or plant was dead.

[y] Controls (ctl) were seed of the parent cultivar.

[z] Scl = total of somaclones.

Table 8. Distribution of foliar disease ratings (Clavibacter michiganensis subsp. michiganensis) in regenerants derived from a secondary culture cycle.

Original parent	Mean foliar disease ratings of parent[z]		Total secondary plants recovered and screened[z]	Frequency distribution of secondary plants foliar disease ratings											
				Cm-103						Cm-63					
	Cm-103	Cm-63		1	2	3	4	5	6	1	2	3	4	5	6
'Bulgaria 12'															
8 1/1	2.7	1.9	3	0	0	6	6	2	2	0	0	4	1	1	10
				3	0	0	2	0	0	6	2	0	0	0	0
				3	1	0	0	0	0	5	3	0	1	0	0
3 (1/2.1)	5.2	4.6	2	0	4	2	5	6	2	0	4	6	3	0	2
				1	2	2	6	1	3	0	5	12	2	0	0
3 (2/2.1)	5.2	4.4	1	0	2	2	3	5	2	0	4	3	2	2	3
'Ohio 7880'															
6 (2/4.2)	5.0	4.7	1	0	0	0	1	0	3	0	0	1	0	1	2
'Sunny'															
12 (2/2.1)	4.7	3.1	1	0	0	0	3	2	1	0	2	4	0	0	0
43 (1/1)	4.0	3.0	1	0	0	0	3	2	1	0	0	3	2	1	0

[z] Screened first generation seed was screened by cotyledon excision inoculations with two Cmm strains.

Table 9. Comparison of foliar disease ratings for 24 R_1 somaclones from 7 Lycopersicon cultivars, previously identified by cotyledon excision inoculation as having increased levels of Clavibacter michiganensis subsp. michiganensis (Cmm) foliar resistance, after inoculation (5-weeks of age) by the clipping inoculation method, with two strains of Cmm.

Cultivar somaclone	Mean foliar disease rating[z] Strain Cm-103	Strain Cm-63	LSD mean separation (p 0.05)
Experiment 1			
Bulgaria 12	2.8 abc	1.0 c	.46
3 2/2.1	2.7 abc	1.3 abc	
8 1/1	1.6 d	1.1 bc	
103 2/2.1	2.8 abc	1.7 a	
105 1/1	3.0 ab	1.1 bc	
111 2/2.1	2.8 abc	1.0 bc	
111 2/2.2	2.4 c	1.0 c	
117 1/3	2.5 c	1.0 c	
117 3/3	3.0 ab	1.0 c	
126 3/3	2.6 bc	1.3 abc	
127 1/1.3	2.5 c	1.5 ab	
136 1/1	3.1 a	1.0 bc	
Easy Harvest	3.3 a	1.3 a	.30
1 2/2.1	3.3 a	1.0 a	
4 1/2.2	3.1 a	1.0 a	
10 1/1	2.5 b	1.0 a	
Flora Dade	2.5 ab	1.3 bc	1.2
2 2/2	3.3 a	1.0 c	
Mountain Pride	3.4 a	1.0 a	.54
101 1/1.1a	3.2 a	1.0 a	
104 1/1.1a	3.4 a	1.0 a	
129 1/1	2.5 b	1.0 a	
Sunny	3.3 ab	1.0 a	.59
40 1/1	3.7 a	2.8 b	
Experiment 2			
Flora Dade	3.0 a	2.8 a	.52
15 1/1.a	2.7 a	2.8 a	
27 1/2	2.6 a	2.4 a	
Heinz 2990	2.1 a	2.4 a	.59
15 1/1	2.5 a	2.3 a	
Utah 20	2.4 b	2.3	.32
15 1/1	2.6 b	3.0 a	
16 2/2	3.0 a	3.1 a	

[z] Rated on a scale of 1-4, where 1 = > 9 symptomless leaves present; 2 = 7-9 symptomless leaves present; 3 = 5-6 symptomless leaves present; 4 = canker(s) may be present, plant may be stunted; 4 symptomless leaves present or > 4 symptomless leaves present but plant was cankered and/or stunted; 5 = 2-3 symptomless leaves present; plant may be stunted and/or cankered; 6 = only apical leaf was symptomless or plant was dead.

Values in a column followed by the same letter are not significantly different using LSD (P 0.05).

Reaction to Cmm: Quantification of pathogen populations.

Cotyledon excision inoculation. Cmm was detected in all 100 cotyledon-inoculated R_1 somaclones evaluated (Table 10). Generally, populations in the somaclones inoculated with Cm-103 were greater (150-1000 fold) than those found in somaclones inoculated with strain Cm-63. Some (27 %) somaclones from 'Bulgaria 12' and 'Flora Dade', identified as possessing increased foliar resistance, exhibited a reduction in pathogen populations, 100-fold or 60-100 fold, respectfully. Pathogen populations for 'Easy Harvest', 'Easy Winner' and 'Mountain Pride' somaclones were not determined.

Extended incubation study. In the 'Bulgaria 12' extended incubation study, pathogen populations were found to decrease after 7-10 months incubation if the plants had been inoculated with strain Cm-103 (Table 11). If plants were inoculated with Cm-63, pathogen populations over the 7 - 10 months were found to increase two-thirds of the time, compared to the population levels detected in other plants from the same lines, 8 weeks after inoculation (Table 10). Some plants were able to survive and continue growing while others died back and became like dead, woody, dried out twigs. A decline of plant health to the latter state had no conclusive affect on the pathogen population. Sometimes the populations remained the same or decreased; in three instances the pathogen population increased over the extended period.

Clip inoculation. Similar pathogen (Cm-103) populations occurred in the five parent controls and in 18 of the 19 R_1 somaclones included in the first clip inoculation experiment (Table 12).

However, 'Bulgaria 12' somaclone 8 (1/1) possessed a reduced (130-fold) pathogen (Cm-103) population compared to the 'Bulgaria 12' control. Populations for strain Cm-63 were reduced in the 'Easy Harvest', 'Mountain Pride' and 'Flora Dade' somaclones, compared to the parent cultivars. One 'Sunny' somaclone, 43 (1/1), had a 10-fold greater Cm-63 population, while 36 % of the 'Bulgaria 12' somaclones exhibited 5-100 times greater Cm-63 populations, compared to their respective parent cultivars. The other (64 %) 'Bulgaria 12' somaclones had pathogen (Cm-63) populations approximately the same as the parent control.

Table 10. Logarithum of numbers of colony-forming units of Clavibacter michiganensis subsp. michiganensis (Cmm) per gram fresh weight of randomly selected R_1 somaclones from 7 L. esculentum cultivars and 2 L. pimpinellifolium cultivars.

Cultivar somaclone	Strain Cm-103	Cm-63
L.esculentum processing cultivars		
Ohio 7880	7.9[z]	7.8[z]
7 2/3.1	8.4	7.7
13 1/4.1	8.6	7.7
15 1/4.5	8.1	7.8
Ferry-Morse 6203	ND	6.6
2 3/3.1	7.5	4.4
6 2/2	8.1	5.3
9 2/4.1	7.9	6.0
11 3/4.1	8.6	5.1
12 2/4.1	8.2	7.2
(Easy Harvest)	6.5	5.0
L.esculentum fresh market cultivars		
Sunny	7.5	6.8
1	7.8	5.0
2	7.9	6.0
3	7.8	4.2
40	7.9	6.4
43	7.3	6.5
131 1/1.b	8.1	NA
43111-1 1/1	6.8	NA
Pik-Red	8.5	5.4
3 2/4.3	8.4	5.1
7 1/4.1	8.2	5.0
10 4/4.1	7.6	---
11 3/3.2	8.3	5.6
16 3/4.2	8.5	---
18 2/3.2	9.0	6.2
20 2/3.2	8.8	---
Flora Dade	7.4	5.7
2 1/1.a	7.8	6.0
2 1/1.b	4.8	NA
2 2/2	NA	4.5
6 2/2	7.4	6.0
14	7.3	NA
15 1/1.a	6.8	5.7

TABLE 10 (CONT'D)

15 1/1.b	7.8	5.2
17	6.9	6.0
27 1/2	5.3	5.7
Flora Dade	7.3	5.8
10 1/1	5.3	ND
25 1/1	5.2	7.1
L.esculentum breeding cultivars		
Heinz 2990	8.3	8.0
15 1/2	8.2	6.2
18	7.7	6.4
42	8.2	8.2
43	8.0	8.0
Bulgaria 12	ND	6.0
216 1/1.b	ND	7.2
Bulgaria 12	7.4	5.2
211	7.0	5.0
216 1/1.f	8.4	3.9
Bulgaria 12	6.3	4.5
138	6.5	4.3
Bulgaria 12	6.8	4.0
163	6.7	5.1
169 2/2	6.1	5.2
Bulgaria 12	6.3	5.4
127 1/1.a	6.3	5.0
127 1/1.2	6.6	5.7
127 1/1.3	7.0	5.7
Bulgaria 12	5.5	5.4
106 1/1	8.7	4.3
106 1/3	5.3	5.5
106 3/3	6.1	5.6
126 3/3	6.2	5.6
Bulgaria 12	6.6	3.4
312-5.a	5.8	4.2
312-5.b	5.4	5.2
Bulgaria 12	7.5	5.6
136	6.9	5.1

TABLE 10 (CONT'D)

Bulgaria 12	6.0	5.3
8 1/1	7.4	5.2
Bulgaria 12	7.0	4.5
8 1/1	6.8	7.1
811-1	7.2	6.4
8 1/1	5.2	4.1
811-2	7.3	5.5
811-6	5.1	5.2
Bulgaria 12	7.7	6.6
126 2/3	7.2	7.0
173	6.7	7.6
183	7.7	8.6
Bulgaria 12	7.6	5.5
124 1/1	ND	5.9
164 1/1	5.1	ND
173 1/2.a	8.3	5.3
173 1/2.b	8.1	5.5
174 1/2.c	7.7	ND
174 1/2.a	7.6	ND
174 2/2	8.3	ND
178 1/1.a	5.3	6.8
Bulgaria 12	4.7	4.4
117 1/3	4.9	5.9
117 3/3	6.3	2.9
111 2/2.1	ND	6.0
111 2/2.2	5.0	5.6
125 2/2	ND	3.8
112 2/2	4.7	3.7
Bulgaria 12	7.6	5.5
155 2/2.a	6.8	7.2
155 2/2.b	7.4	6.9
155 1/2.b	7.0	6.7
Bulgaria 12	6.3	4.7
108 1/1.1	8.2	4.6
108 1/2.2	6.6	5.2
Bulgaria 12	4.8	4.8
1 2/2.25	7.3	4.8
7 1/1	5.7	4.6
101 1/1.1	4.3	4.4
101 1/1.b	5.0	4.7

TABLE 10 (CONT'D)

Bulgaria 12	5.5	4.8
105 1/1	4.3	4.4
103 1/2.1	6.2	4.6
103 2/2.1	6.6	4.7
Bulgaria 12	5.4	4.2
3 2/2.1	7.0	3.5
3 2/3.2	4.0	5.6
3 1/2	6.9	4.6
Bulgaria 12	6.0	5.0
139	6.0	4.4
127 1/1.b	4.6	5.2
L. pimpinellifolium cultivars		
Utah 20	4.5	4.8
3 1/1 b.1	4.6	5.6
9	6.2	7.2
11	6.9	5.6
15	5.1	4.9
16 2/2	5.4	5.4
Utah 737	5.3	5.9
1	7.1	ND
4 2/2.1	6.5	5.4
4 2/2.3	5.3	6.6
14	5.3	5.8

ND = not determined.
[z] Mean of three plants, $\log_{10}$ cfu/gram.

Table 11. Log of numbers of colony-forming units of Clavibacter michiganensis subsp. michiganensis (Cmm) per gram fresh weight for 'Bulgaria 12' R_1 somaclones, classified as being more resistant after cotyledon excision inoculation with two strains of Cmm, and incubated for a total of 7-10 months.

Somaclone	Time after inoculation (months)	Strain Cm-103		Strain Cm-63	
		$\log_{10}$ cfu/g	no. dead plants	$\log_{10}$ cfu/g	no. dead plants
Bulgaria 12	8-10	4.8-7.3	1	UD-7.2	0
106 1/1	9	5.1	0	5.8	1
106 3/3	9	6.4	1	5.4-6.3	1
108 1/1.1	10	ND	-	5.0-6.3	0
108 1/1.2	10	UD	0	5.3-7.2	1
108 1/1.2	9	UD	0	5.1-5.7	0
126 3/3	9	UD	0	5.1-5.7	0
127 1/1.3	8.5	6.3	0	ND	-
127 1/1.A	8.5	4.4	1	ND	-
136 1/1	7	UD	1	6.1	0
138 1/1	10	5.6	0	ND	-
163 1/1	9	5.6	0	ND	-
312-5A	8	7.0	0	UD-6.1	1
312-5B	8	ND	-	UD-5.2	0

UD = undetected
ND = not determined

Table 12. Logarithum of numbers of colony-forming units of Clavibacter michiganensis subsp. michiganensis (Cmm) per gram fresh weight determined at 12 weeks post-inoculation in selected resistant R_1 somaclones and their parent controls after clip inoculation (5-weeks-old) using two strains of Cmm.

Cultivar somaclone	Strain Cm-103 (log cfu/gram)[z]	Strain Cm-63 (log cfu/gram)[z]
Easy Harvest (control)	7.8	6.4
1 2/2.1	7.9	6.3
4 1/2.2	7.8	6.0
10 1/1	7.7	5.2
Mountain Pride (control)	7.8	7.1
101 1/1.1a	7.1	5.7
104 1/1.1a	8.1	5.4
129 1/1.1	8.1	4.2
Flora Dade (control)	7.8	6.7
2 2/2	7.9	6.4
Sunny (control)	7.8	6.1
40 1/1.1	7.8	7.4
Bulgaria 12 (control)	8.0	5.7
3 2/2.1	7.9	5.4
8 1/1	6.7	6.9
103 1/2.1	7.6	5.9
105 1/1	7.7	5.7
111 2/2.1	8.0	5.5
111 2/2.2	7.6	6.4
117 1/3	8.0	6.2
117 3/3	8.2	5.3
126 3/3	8.1	5.4
127 1/3.3	7.6	7.7
136 1/1	7.7	5.5

[z]Mean Cmm population of three plants/strain/somaclone.

DISCUSSION

The results of these studies demonstrate that genetic variation was present in somaclones derived from 13 tomato cultivars. Somaclones derived from a single cultivar varied in response to inoculation with Cmm. Significant increases in resistance to one or both strains of Cmm occurred in a total of 49 R_1 somaclones, or 25 % of the total somaclones assayed. Half of these somaclones originated from Cmm-susceptible cultivars and the other half from partially resistant cultivars. Conversely, there was also significant decreases in Cmm resistance (increased susceptibility) for 13 % of the somaclones assayed. Loss of resistance to a pathogen has been previously reported in soybean and celery as a result of somaclonal variation (Olah and Schmitthenner, 1988; Toth, 1989). If the increase in resistance was significant at a probability of (0.01), the plants appeared greatly less diseased, while somewhat less pronounced visual differences occured when the increase was significant at (p 0.05). Interestingly, genotype did not appear to affect the disease response of the somaclones possessing increased foliar resistance to Cmm. Other researchers, however, have observed a cultivar-dependent shift in susceptibilty, with resistant and susceptible cultivars each producing progeny with similar disease reactions (Shoemaker et al., 1985; Behnke, 1980; Helgeson and Deverall, 1983). None of the cultivars in either tomato species used in this study possess the identical number or types of genes and/or alleles which confer Cmm resistance, as evidenced by the numerous combinations observed in previously described (de Jong and Honma, 1976; Madumadu, 1985)

inheritance studies. The presence of preexisting resistance genes did not necessarily create an advantage for obtaining the desired resistance: The five partially-resistant cultivars together produced only 5 % more somaclonal foliar resistance compared to the 8 Cmm-susceptible cultivars.

Somaclonal variation could have affected resistance to the Cm-63 or Cm-103 strains of Cmm by (1) mutating a single gene (recessive, dominant or incompletely dominant) of the 4 major genes identified in resistance to Cmm; and/or (2) amplification of modifiers or minor gene supplements; (3) addition of a new major gene, allele(s) of a gene, or minor gene supplement, which would alter the expression of the major genes; (4) changing the nature of at least one very important dominant allele, which when occupying a B,C, or D loci, would enhance the resistance. Likewise, decreased levels of resistance may have arisen from a single gene mutation, resulting in alleles heterozygous for a homozygously expressed resistance. One of the somaclones with increased foliar resistance and a decreased pathogen population, possessed the wiry mutant morphology. This mutation is known to be recessive, with a normal somatic chromosome number (n= 24) (Lesley and Lesley, 1928).

Variation to Cmm arose both between the calli regenerated from a particular cultivar and among regenerants or 'clones' (Shahin and Spivey, 1986) originating from a single callus. Thus, disease response was independent of the source callus: some 'clones' of 'Bulgaria 12' and 'Flora Dade', for example, exhibited significantly increased foliar resistance and decreased pathogen populations, while

the 'sister' line(s) did not. Other researchers have reported similar observations: For example, careful records were not kept on the origins of Barden's (1985) tomato somaclones that possessed increased To/TMV resistance, but it was believed that at least 2 of the 6 somaclones were produced from separate calli. Similarly, Shahin and Spivey (1987) also reported that while some of their 13 Fusarium-resistant tomato mutants may have been derived from the same callus, variation in reactions to FOL race 3 existed among 'clones' regenerated from the same callus. Assessment of variation in R_1 progeny of tomato somaclones by Buiatti and co-workers (1985) suggested to Smith (1989) that the plants originated from more than one cell in culture. Also, Brown and co-workers (1986) produced more than one regenerant lettuce somaclone was produced from a source callus. Subsequent disease responses of their somaclones to lettuce mosaic virus (LMV) were found to be independent of the source callus; somaclones which were rated as having both high and low LMV resistance were derived from the same callus.

Even though no selection pressure was applied to the R_0 somaclones in this study, the R_1 progeny were the product of two selection steps where-by deleterious R_0 plants could be discarded. First, explants were singled out in culture when incapable of regenerating into plantlets. Secondly, greenhouse selection allowed identification of R_0 plants possessing a normal development and the capacity to flower and fruit (Evans, 1989).

All of the 13 Lycopersicon cultivars assessed for regenerative capacity were able to regenerate from leaf explants. The partially-

resistant cultivars were found to regenerate the poorest; whereas, the Cmm-susceptible cultivars exhibited the highest regeneration rates.

The extent of variation induced using somaclonal techniques was not consistent between the cultivars, even though all somaclones were produced under identical, controlled culture conditions. As previously reported (Liu and Chen, 1976; Skirvin and Janick, 1976; Imanishi and Hiura, 1982; Zagorska et al., 1986; Barden, 1985; Shoemaker et al., 1985; Kurtz and Lineberger, 1983), genetic background of the cultivar can affect the ease of regeneration from leaf explants, in addition to the frequency and type of variation found among the regenerated plants. For example, Zagorska and co-workers (1986) found that callus induction, growth rate and subsequent induction of shoots depended exclusively on the origin of the explant and the tomato genotype.

Somaclonal variation often results in changes in multiple traits. The types of mutations observed morphologically in this study are consistent with those reported by other researchers, as the result of either induced mutations or somaclonal variation (Evans et al., 1984; Yu and Yeager, 1960; Prat, 1983; Toth, 1989; Brown et al., 1986; Kurtz and Lineberger, 1983). For example, Buiatti and co-workers (1985) reported a high percentage of induced variants in tomato, with about 17 % of their R_1 progenies exhibiting chlorophyll and other morphological abnormalities. O'Connell and Hanson (1984) observed growth habit variations in their protoplast-derived tomato somaclones. Evans and Sharp (1983) described 13 morphological

variants in tomato somaclones, some of which were also found in this study. Montagno (1986) described some of his R_0 tomato variants as chlorotic, dwarf, rosetted or meristemless, which he proposed may be the result of auxin inactivation. At least one somaclonal variant, the wiry mutant, was previously described as arising from a natural mutation (Lesley and Lesley, 1928).

Morphogenetic potential has been reported to vary in L. esculentum and other species (Muhlbach, 1980; Tal et al., 1977; Hanson, 1982). Variability of the R_1 progeny in this study appeared, based on a limited and unequal number of evaluations, to be greater in the 11 L. esculentum cultivars than in the 2 L. pimpinellifolium cultivars, although other researchers have demonstrated the opposite to be true (Tal et al., 1977; Kut and Evans, 1982; Zagorska et al., 1986).

Non-genetic factors may also have contributed to the sometimes limited amounts of variation and regeneration. For example, numerous other growth regulators and concentrations have been reported for tomato explant culture. Meanwhile certain tomato cultivars have been reported to require specific hormones in specific concentrations before sufficient shoot production will occur (Montagno, 1986; Uddin et al., 1988). For example, when zeatin was used as the growth regulator, frequent subculturing was required before shoots could be initiated by the explant (Montagno, 1986; Uddin et al., 1988) Likewise, in a study of 18 undescribed tomato cultivars by Tatchell and Binns (1986), a 5 M zeatin concentration was reported to be optimal. Also the type of explant tissue could have played a role in poor regeneration. For example, the use of tomato cotyledons as a

source of explant tissue, instead of other leaf tissues, has been reported (Montagno, 1986) to be three times more efficient and by far the best explant choice for shoot proliferation in a study of five L. esculentum processing cultivars. Other non-genetic factors may have included the length of time in vitro and the donor plant age or condition. Any one of these factors could have effected the frequency and nature of the induced mutations.

There are no apparent successful reports in the literature of further increasing disease resistance in somaclones identified with increased resistance, by means of a secondary tissue culture cycle. In one report, Latunde-Dada and Lucas (1988) found little correlation between the resistant source plant (R1) disease scores and those of the R2 plants recovered from a secondary cycle. The overall reaction of their R2 plants appeared to have reverted to the original parent type. In our study, five (44 %) of the R_1 somaclones resulting from a second somaclonal cycle possessed increased foliar resistance (class c) to 1 or 2 strains of Cmm, compared to their parent. Two of these somaclones were resistant to a single Cmm strain, while the other three were resistant to both strains of Cmm. This trend indicates the potential for additional Cmm resistance, resulting from a second culture cycle.

The somaclonally-induced mutations in resistance to Cmm did not alter the natural host-pathogen interaction, as the bacterium was still capable of multiplication. High foliar disease ratings did not result in higher pathogen populations compared to low foliar disease ratings. It was expected that somaclones exhibiting significantly

improved foliar disease resistance would exhibit a decreased systemic pathogen population, compared to their parent controls. However, just 27 % of such somaclones expressed reduced pathogen populations, and were derived from the partially-resistant L. esculentum cultivars Bulgaria 12 and Flora Dade. It is interesting to note that these same two cultivars possessed the five somaclones identified as expressing increased foliar resistance to both strains of Cmm simultaneously, based on cotyledon excision inoculation. Two other cultivars, Heinz 2990 and Utah 20, also possess resistance to Cmm but their progeny did not express a reduction in pathogen populations.

The extended incubation study on 19 'Bulgaria 12' somaclones identified as possessing increased foliar resistance, demonstrated the effectiveness of desiccated, infected tomato debris for harbouring the bacterium, thus confirming previously mentioned epidemiology studies (see Introduction).

Differences in the disease reactions of the somaclones with increased foliar resistance, inoculated by cotyledon excision or clipping, may have been somewhat influenced by the differing environmental factors found associated with a greenhouse versus controlled environmental chambers. However, experiments which were clip inoculated were inoculated (and therefore rated) at a more advanced (5-week-old) stage of plant development, compared to the cotyledon inoculation experiments, which were inoculated at 10 to 12-days of age. In addition to inoculation method, pathogenic strain and plant age were found to significantly affect foliar resistance to

Cmm. For example, a somaclone from 'Bulgaria 12' or 'Easy Harvest' expressed increased foliar resistance to Cm-63 by cotyledon inoculation but to Cm-103 by clip inoculation. One 'Mountain Pride' somaclone expressed increased foliar resistance to Cm-103 by both inoculation techniques. Thirdly, one 'Utah 20' somaclone exhibited increased foliar resistance when inoculated by cotyledon excision but rated less resistant when inoculated by clip inoculation. Fourthly, 20 of the 24 somaclones which expressed foliar resistance to strain Cm-103 by cotyledon inoculation did not exhibit increased foliar resistance by clip inoculation. Thus, inoculation methods (and therefore plant age) are able to modify resistance to specific strains of Cmm.

A comparison of pathogen populations in the somaclone inoculated by either inoculation technique indicate that clip inoculation can result in 5-1000 times greater pathogen populations, compared to cotyledon excision inoculation. The Cmm strain also played a role: clipping with Cm-103 resulted in approximately 100-fold greater pathogen populations compared to populations in the same somaclones inoculated with Cm-63.

In conclusion, the results presented here demonstrate the existence of novel variation in tomato offspring with a primary-callus origin. Secondly, it presents the possibility of creating a comparitively large number of variants in L. esculentum and L. pimpinellifolium, thus allowing the application of such methods to genetic and plant breeding programs. One of the emerging characters, elevated levels of resistance to one or two strains of Cmm, may be

used to further improve tomato in adjunct to conventional breeding approaches. This approach of generating somaclones from popular fresh market, processing and breeding cultivars, provides material useful for future improvements in tomato resistance. For example, resistance to Cmm can be linked with resistance to bacterial wilt of tomato caused by Pseudomonas solanacearum (Laterrot and Kaan, 1978). Future seed generations will need to be examined in greater detail for yield, fruit characters, and resistances to other tomato pathogens.

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CHAPTER 3

RADIO-INDUCED VARIATION AND REACTION TO *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN THE *L. ESCULENTUM* CULTIVARS OHIO 7880 AND SUNNY.

INTRODUCTION

In 1904, De Vries first suggested the possibility of using radiation for induced mutations in crops (Vose, 1980), and the first article on induced mutations in barley and maize was published in 1928 by Stadler. However, it took until the early 1940's before mutation breeding was truly applied to plant breeding.

Mutation breeding is a technique which has been successfully used to enhance the quality of popular, well-adapted cultivars by improving a specific character while maintaining the basic identity (Reisch, 1983; IAEA, 1977). Quite a number of varieties of crop plants possess useful characters derived from induced mutation, that rarely or never are found in existing reservoirs of genetic variation (Gottschalk and Wolff, 1983). As of 1982, over 581 varieties in 69 crops and ornamental plants, developed with the help of induced mutations, were released IAEA 1972-1982; Ukai, 1982). Fifty-eight (26 %) of the 225 crop species released by 1983 exhibited improved disease resistance to plant pathogens (IAEA, 1983). In many cases, the expenditure in time for mutation induction and selection was considerably less than that necessary for variety development using conventional means. Where other breeding methods have been tried and failed, there is clear evidence that significant improvement can be obtained in certain crops through the use of induced mutations. Plant mutations concern every aspect of plant growth: yield, stem length,

early maturation, nutritional (protein) content, seed characters, hardiness, drought resistance, harvesting qualities and disease resistance and tolerances (Vose, 1980; Micke and Donini, 1982).

There are two basic methods of mutagenesis: chemical and physical (X-rays, gamma rays and neutrons), each of which can be applied to seeds, pollen, whole plants or in vitro plant cultures. While mutation breeding is predominantly used in annual diploid and allopolyploid self-fertilizing food crops, it is of special interest in fully-sterile crops and ornamentals (Gottschalk and Wolff, 1983).

It has been shown that exposure to radiation increases mutation frequency as much as 100,000 times, however, the majority of mutations have been found to behave recessively and are deleterious (Vose, 1980). Frequently desirable and undesirable changes are carried in the same cell and are transmitted together to the mutant offspring (Micki and Donini, 1982). Yet there is a "voluminous collection" of mutants in many different crops: rice, wheat, barley, oats, maize, pearl millet, sugarcane, legumes, mint, and grapes, with many genotypes, proving that induced mutations can be successfully utilized in breeding for disease resistance (Gottschalk and Wolff, 1983).

Ionizing mutations have been reported to result in different degrees of susceptibility against one or several pathotypes or races of a pathogen, including increases and decreases of resistance from the original resistance level. Like somaclonal variation, gamma radiation provides the possibility of chromosomal rearrangement and genome reorganizations, including duplications, inversions, and

reciprocal interchanges. Detection of a new resistance induced by mutagenic treatment may in some instances be the result of the alteration or elimination of such genes (IAEA, 1983).

Induced Mutations in Tomato. Yu and Yeager (1960) quantified ten heritable mutations in tomato when they used X-rays and thermal neutrons to treat seeds. While none of the mutations were believed useful for breeding, the seeds were found to exhibit mutation rates 400 times greater that the non-irradiated controls.

Induced mutations have been successfully reported in tomato for a number of plant characters and disease resistances (Table 1). Khvostova (1967) developed an early-ripening tomato mutant which later became the Russian-released variety, 'Luch 1'. Nuttal and co-workers (1968) found that low dose gamma irradiation of tomato seeds produced increased vigor in rooting and flowering, and also increased fruit size. El-Sayed (1977) identified 5 tomato mutants possessing late blight (Phytophthora infestans) resistance after gamma-irradiation of a susceptible tomato variety. Yamakawa and Nagata (1975) reported on a combined resistance to TMV and Fusarium oxysporum in irradiated lines of Lycopersicon esculentum x L. peruvianum hybrids. Gavazzi and co-workers (1987) identified Verticillium wilt resistance in progeny obtained from chemically mutated tomatoes. Induced mutations, such as brown seed, useful in heterosis breeding, have been reported (IAEA, 1976) by Yakovleva and Shkvarnikov (1969), Dorossiev (1972), Soressi (1967), Soressi and Cravedi (1967) and Monti (1972). Gamma irradiation of tomato has also been successfully used and reported by Sax (1963), Polacek

(1967), Rick and Boynton (1967), Nuttal et al. (1968), Dolgih (1969), Yamakawa (1969), Alexander et al. (1971), Monti (1972), Sidrak and Suess (1973), Butler (1977), Yordanov et al. (1977), and Zagorcheva and Yordanov (1978) (Table 1).

In addition, the affects of tissue culture and ionizing radiation may be successfully combined. For example, Montagno (1986) examined the combined affects of ionizing radiation and somaclonal variation on in-vitro growth of tomato tissue cultures and progeny field performance, fruit quality and yield. This researcher found that increases in shoot number and in the fresh weight of cultured tomato tissues were directly related to increasing levels of gamma irradiation. Treatments of 3000-4500 rads appeared to produce the greatest number of plant mutations and no lethalities resulted from any of the treatments (1500-6500 rads). *OBJECTIVE*: Radiation-induced mutation appeared to be a feasible approach by which existing L. esculentum cultivars might be improved for resistance to Clavibacter michiganensis subsp. michiganensis (Cmm). The purpose of the present study was to use cobalt-60 gamma radiation on tomato seeds in order to induce variation in a Cmm-susceptible fresh market and processing tomato cultivar.

Table 1. Summary of induced mutations in Lycopersicon esculentum.

Mutant type	Author and year
Disease Resistance	
-TMV	Yamakawa and Nagata, 1975
-Fusarium oxysporum	
-Phytophthora infestans	El-Sayed, 1977
-Verticillium wilt	Gavazzi et al., 1987
Early Flowering	
	Khvostova, 1967
	Polacek, 1967
	Yordanov et al., 1977
	Zagorcheva and Yordanov, 1978
Fruit characters	
	Nuttal et al., 1968
	Sidrak and Suess, 1973
	Montagno, 1986
Sterility	
	Rick and Boynton, 1967
	Dolgih, 1969
	Yamakawa, 1969
	Alexander et al., 1971
	Zagorcheva and Yordanov, 1978
	Montagno, 1986
Short-stem	
	Alexander et al., 1971
	Monti, 1972
Seed color	
	Yakovleva and Shkvarnikov, 1969
	Dorossiev, 1972
	Soressi, 1967
	Soressi and Cravedi, 1967
	Monti, 1972

MATERIALS AND METHODS

Seed irradiation. Seeds of two L. esculentum Cmm-susceptible cultivars were used in this study: 'Sunny' (fresh market) and 'Ohio 7880' (processing). Both cultivars are susceptible to bacterial canker. One hundred and twenty seeds from each cultivar were pre-conditioned by imbibing overnight in tapwater (pre-soak seed treatment) and 120 additional seeds were left dry (dry seed treatment). The following day, the seeds were subjected to cobalt-60 (variable flux) gamma radiation (Michigan State University, Chemistry Department) at 20, 25, and 30 krads, using a dose rate of 1100 R/min. Forty seeds of each of the six seed/irradiation treatments per cultivar were irradiated.

Seed production. Immediately after irradiation, the seeds were germinated and after 7-10 days, were transplanted into 12.5 cm clay pots containing Baccto soil-less planting medium (Michigan Peat Co., Houston, TX) and fertilized twice as previously described. After 6-weeks, half the plants were transplanted into the field (Michigan State University, Botany Farm), and the other half were transplanted into 25.5 cm clay pots and grown in the greenhouse. All plants were self fertilized and first generation (M_1) seeds were collected, acid treated, packaged and stored, as previously described in Chapter 2.

Assessment of variation. M_0 and M_1 plants were assessed for (1) seed germination, (2) variation in plant morphology, fertility, survival, and seed production. M_1 plants were also assessed for (3) reaction to 2 strains of Cmm after cotyledon excision inoculation. Reaction to Cmm was assessed in two ways: (A) rating the amount of foliar

disease and (B) quantification of systemic pathogen populations.

Inoculation. Seeds (M_1) were germinated and transplanted at 7-10 days of age, three per 12.5 cm clay pot. In two separate experiments, 35 seed lines (2-7 per treatment) of 'Sunny', plus a 'Sunny' control, and 18 seed lines (2-5 lines/treatment) of 'Ohio 7880', plus a 'Ohio 7880' control were evaluated. Both experiments were arranged as completely randomized designs and were conducted in the greenhouse under natural conditions during the months of July - September. Fertilizer was applied (150 ppm) at 4 weeks. Seedlings were inoculated at 10-12 days of age by excision of a cotyledon, using a pair of scissors dipped in either strain of Cmm (10^6 - 10^7 cfu/ml), as previously described in Chapter 1.

Eight weeks following inoculation, plant height was measured and the plants were rated for foliar symptoms, using a scale of 1-6 as previously described in Chapter 1. Data were analyzed by analysis of variance (ANOVA) for each experiment; when the ANOVA's were significant, LSD tests (p 0.05) were used to separate treatment means (Steel and Torrie, 1980).

Two to three plants of selected lines from each treatment, and of the control, were chosen at random and stomached in order to quantify the systemic pathogen population. Stomaching, as previously described in Chapter 1, involves blending inoculated plants in a stomacher with 0.05 M phosphate buffer and subsequent dilution plating and colony enumeration. Log $_{10}$ transformations were performed on population data.

RESULTS

Germination of M_0 seeds at various dose rates.

Ohio 7880. None of the dry treated seeds irradiated with 25 krads germinated and only 40 % of the pre-soaked seeds germinated. Loss of viability in the 4 other seed/irradiation treatments ranged from 13 to 23 %. In the '20 krad' treatments, dry-treated seeds germinated 8 % better than did the pre-soaked seeds, but in the '30 krad' treatments, pre-soaked seeds germinated 5 % better than did the dry seeds. Overall, seed germination could be ranked 25/dry < 25/pre-soaked << 30 krad < 20 krad. Regardless of treatment, 198 out of 240 seeds germinated and grew into viable plants (Table 2).

Sunny. There was little to no difference in M_0 seed germination between the 6 seed/irradiation treatments. The '20 krad/pre-soaked' treatment resulted in the lowest (67.5 %) rate of germination. Overall, germination could be ranked 20 krad < 30 krad < 25 krad. Regardless of treatment, 147 out of 240 seeds germinated and grew into viable plants (Table 2).

Plant variation. Irradiation resulted in variation in plant morphology and reductions in fruit size and seed number. Variations of both types were most notable in 'Ohio 7880', especially in M_0 plants which received the 30 krad/pre-soak seed treatment. Gamma radiation also reduced the fertility of the M_0 plants (Table 2). Of the 127 'Ohio 7880' M_0 plants which grew and survived to maturity, 75 produced M_1 seed. Of the 143 'Sunny' M_0 plants which grew and survived to maturity, 71 produced M_1 seed.

Table 2. Irradiated tomato (M_0 and M_1) production and inoculation with Clavibacter michiganensis subsp. michiganensis (Cmm).

	Lycopersicon esculentum cv.	
	'Ohio 7880'	'Sunny'
Total no. seeds irradiated	240	240
Total no. M_0 seeds that germinated into viable plants	198	147
Total no. M_0 plants that survived to maturity	127	143
Total no. M_0 plants that produced M_1 seed	75	71
Total no. M_1 seed lines assayed by inoculation with Cmm	18	35
Total no. M_1 lines with significantly increased level of resistance/total assayed	0/18	1/35

Physiology of M_1 seeds. Percent emergence of the M_1 seeds was not recorded. However, seedlings were evaluated for two physiological characters: germination and vigor, by visually comparing the 6 seed/irradiation treatments within each cultivar.

Ohio 7880. M_1 seed derived from the M_0 20 krad treatments exhibited the greatest vigor of the three radiation levels. Pre-soaked seed rated much better than dry seed. Germination of the 25 krad/pre-soaked seeds was similar to the 30 krad/dry treatment, which germinated somewhat better than seeds from the 30 krad/pre-soaked treatment.

Sunny. Pre-soaked seed from the 20 and 25 krad treatments were similar for germination and vigor, with a greater rate of germination arising from the seeds treated with 30 krads. The dry treated seeds germinated somewhat better in the 20 and 25 krad treatments but this rate of germination was similar to that of the 30 krad/pre-soaked treatment.

Reaction to Cmm. Of 53 M_1 lines evaluated (Tables 3 and 4), one line, 'Sunny'-FH (treatment 20krad/dry seed), exhibited a significant increase in foliar resistance to strain Cm-103, compared to the parent control. None of the 'Ohio 7880' lines differed from the parent control.

Pathogen populations in 'Ohio 7880' lines were similar for both strains of Cmm, regardless of the radiation level/seed treatment. Populations of strain Cm-103 ranged from 7.3 - 8.5 log cfu/gram fresh weight (f.w.) and from 7.2 - 8.3 cfu/gram f.w. for Cm-63 (except for line FE). Similar pathogen populations occurred in plants from any

of the six 'Sunny' irradiation/seed treatments, however, this time there was a strain effect. Greater pathogen populations associated with strain Cm-103. Population levels for strain Cm-103 ranged from 7.1 - 7.9 cfu/g f.w., and from 5.4 - 7.1 cfu/g f.w. for Cm-63.

Table 3. Comparison of mean foliar disease ratings and Clavibacter michiganensis subsp. michiganensis (Cmm) populations in M_1 'Ohio 7880' irradiated lines after cotyledon excision inoculation with two strains of Cmm.

Line	Seed treatment[w]	Strain Cm-103 Mean rating[x]	Strain Cm-103 Log cfu/g	Strain Cm-63 Mean rating[x]	Strain Cm-63 Log cfu/g
EXPT.1					
Ohio 7880	control	4.8	7.9[y]	4.5	7.8[y]
FE	20p	5.7	8.1	5.3	5.0
FG	20p	5.5	7.7	5.1	8.3
FC	20d	5.3	8.5	4.9	7.5
FE	20d	5.5		5.6	
FG	20d	5.5	8.0	4.8	7.3
FI	20d	5.5		5.5	
FB	25d	5.3	7.3	5.8	7.3
FD	25d	5.8	8.1	6.0	7.6
#1	30p	5.4	7.8	5.1	8.2
U	30p	5.4	7.8	4.5	
FA	30d	5.3		5.6	
FD	30d	5.6	7.6	5.8	7.2
FG	30d	4.9	8.0	5.7	7.7
EXPT. 2					
Ohio 7880[z]	control	5.6		5.1	
FJ	20p	5.0		5.6	
#3	30p	5.1		5.3	
X	30p	5.0		5.4	
U	30p	5.4		4.5	
#3	30d	5.0		5.3	

[w]Treatment 20p = 20 krad/pre-soaked seed, 20d = 20 krad/dry seed, and so on. Pre-soaked M_1 seed irradiated with 25 krads was not available.

[x]Mean foliar disease rating of six plants. No significant difference occured between values in a column by LSD ($p = 0.05$).

[y]Average Cmm concentration of two plants.

[z]Mean foliar disease rating of four plants.

Table 4. Comparison of mean foliar disease ratings and Clavibacter michiganensis subsp. michiganensis (Cmm) in M_1 'Sunny' irradiated lines after cotyledon excision inoculation with two strains of Cmm.

Line	Seed treatment[x]	Strain Cm-103		Strain Cm-63	
		Mean rating[y]	Log cfu/g	Mean rating[y]	Log cfu/g
Sunny	control	4.6	7.5[z]	3.4	6.8[z]
FF	30d	4.8		3.5	
FB	30d	4.8		3.5	
SSSS	30d	4.6		3.5	
HHHH	30d	4.8		3.0	
KK	30d	4.3		3.2	
Y	30d	5.5	7.8	3.9	5.8
FM	30d	4.8	7.8	3.0	7.1
MM	30p	4.7		3.5	
NNNN	30p	5.3		3.1	
FB	30p	4.1		3.1	
V	30p	4.9	7.3	3.0	5.8
YYYY	30p	4.5		3.7	
ZZZZ	30p	5.1	7.8	5.4	7.1
FA	25d	4.2		3.3	
GGGG	25d	5.3	7.9	4.4	5.4
FE	25d	4.9		3.6	
KKKK	25d	4.9	7.7	5.5	5.4
FB	25d	4.7		3.4	
ZZ	25d	5.2		3.4	
BBB	25d	4.8		3.3	
CC	25p	4.7		3.2	
TTT	25p	4.5		4.2	
XXXX	25p	5.3	7.5	3.1	6.1
VVV	25p	4.6		3.5	
U	25p	4.6	7.8	3.0	NA
FG	25p	4.7		3.3	
LLL	20d	4.4		3.1	
QQQ	20d	4.7		3.2	
RRR	20d	4.4	7.3	3.3	4.4
FC	20d	5.0	7.1	5.9	5.9
FA	20d	4.9		3.7	
FF	20d	4.9		3.2	
FH	20d	4.0 *		4.2	
JJJ	20p	4.8	5.7	2.9	6.1
RR	20w	4.4		3.2	

TABLE 4 (CONT'D)

[x] Treatment 20p = 20 krad/pre-soaked seed, 20d = 20 krad/ dry seed, and so on.

[y] Mean foliar disease rating of six plants.

[z] Average Cmm concentration of three plants.

* = Mean disease rating significantly different at p = 0.05, LSD mean separation (0.57).

DISCUSSION

The results of this study on gamma-induced mutations demonstrates that variation was present in M_0 and M_1 progeny derived from the two Cmm-susceptible, L. esculentum cultivars.

It is essential to use the very best breeding material available when inducing mutations. The two cultivars Ohio 7880 and Sunny, although possessing what are presumed to be ineffective Cmm-resistance genes, were a good choice of parent material since both are popular cultivars with other valuable characters, such as disease resistances. For example, 'Sunny' is resistant to Alternaria solanacerarum, Fusarium oxysporum f.sp. lycopersici race 1, Verticillium wilt, and Septoria lycopersici (Stevenson et al., 1985).

Genotype conditioned differences have been found to affect radiation sensitivity and mutagen rates (Blixt, 1970; Gottschalk and Wolff, 1983). Different cultivars of the same crop can differ markedly from each other for susceptibility against distinct mutagens. For example, Sidrak and Suess (1973) found significant varietal differences when they irradiated tomato seeds of two contrasting cultivars. In this study, L. esculentum cultivar Ohio 7880, a Cmm-susceptible, processing tomato cultivar, was found to be more sensitive to gamma radiation than was 'Sunny', a Cmm-susceptible, fresh market cultivar. Genotypic differences may have also played a role in the frequency of mutations controlling resistance to Cmm. Although 'Ohio 7880' and 'Sunny' exhibit susceptible reactions to Cmm in the field or in greenhouse experiments, there is a strong probability that 'Sunny' possesses a

different set of alleles controlling expression of Cmm resistance, compared to 'Ohio 7880'. Such a difference(s) may have increased the expression of a favorable mutation, one which affected a particular part or section of a important gene, or an allele, capable of conferring additional resistance to Cmm strain Cm-103.

Other researchers (IAEA, 1983) have shown that resistance against several races of the same pathogen can be obtained by induced mutations, as can simultaneous resistance against 2 different pathogens. This did not occur for the M_1 progeny in this study even though two Cmm strains of different virulence were used. Such a resistance may still be possible to identify in other M_1 progeny.

The optimum radiation dosage for inducing mutations varies with the plant material, the variety, and the conditions of radiation. The species L. esculentum has been reported to be somewhat less radiosensitive than other crop species, requiring 2-3 times the average dose of 10-15 krad (Vose, 1980). For 'Ohio 7880', irradiation with 20 krads was less deleterious to M_0 seed germination, plant growth and survival to maturity, whereas, irradiation with 25 krads was the most deleterious for the same characters. As for 'Sunny', irradiation with 20 krads produced the lowest rate of germination and the lowest rate of plant survival to maturity. The 30 krad treatments ranked intermediate, with the least affect resulting from the 25 krad treatments. Increased resistance to Cmm appeared in a 'Sunny' line treated with the lowest radiation level. Thus, irradiation with 20 krads appeared to significantly decrease both pathogen susceptibility and seed germination.

Pre-soaking of seeds has been shown to greatly increase radiosensitivity and reduce oxygen-enhanced damage (Vose, 1980). Comparatively, irradiated seeds possessing low moisture levels are particularly susceptible to oxygen enhanced radiation damage. In this study, pre-conditioning seeds of either cultivar had little effect on M_0 plant physiology, with one exception: capacity of the 20 krad M_0 'Sunny' seeds to survive as plants to maturity. Based on the plant physiological measurements listed in Table 2, the 20 krad irradiation treatment was the most deleterious for 'Sunny', whereas, the 25 krad level was most deleterious for 'Ohio 7880'. This emphasizes the difference in sensitivity which can exist among cultivars (genotypes) of the same species, treated with a physical mutagen.

Gamma-induced mutations did not inhibit the pathogen's ability to multiply in the M_1 progeny of either cultivar, as systemic pathogen populations were similar to those found in non-irradiated parent controls.

In conclusion, induced mutation by gamma radiation has been shown to be of use in creating a mutant seed line of a popular fresh market tomato cultivar, possessing an increased level of foliar resistance to C.m. subsp. michiganensis.

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