GERANIUM TISSUE CULTURE FOR THE DEVELOPMENT OF BACTERIAL BLIGHT RESISTANCE

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ABSTRACT

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In vitro procedures were evaluated to facilitate the introduction of bacterial blight resistance in *Pelargonium* X *hortorum* (seed-propagated geranium). Protocols were developed for adventitious shoot formation and plant regeneration from primary callus cultures of seed-propagated geranium, regal geranium (*P.* X *domesticum*), and several wild *Pelargonium* species. A protocol also was developed to regenerate *P.* X *domesticum* plants from callus-derived protoplasts. Partially purified culture filtrates of *Xanthomonas campestris* pv. *pelargonii* were evaluated and found to be ineffective for use in evaluating *Pelargonium* germ plasm for bacterial blight resistance. Therefore an *in vitro* assay using cell suspensions of *X. c.* pv. *pelargonii* was developed to screen plantlets and seedlings. This assay was used to screen 2,100 tissue culture plantlets from primary callus cultures of seed-propagated geranium cultivars. Two somaclones that survived the initial screen were micropropagated, and when rescreened *in vitro* were found to have a higher resistance to bacterial blight than controls. The two rescreened somaclones, plus two somaclones that survived *in vitro* screening and direct transfer to soil, were propagated by

cuttings and screened in the greenhouse. None of these somaclones had a higher level of bacterial blight resistance than controls in the greenhouse test. The *in vitro* assay also was used to screen 24 Geraniaceae species as seedlings or plantlets and resistance to bacterial blight was found in *P. hispidum*, *P. multicaule*, *P. grandiflorum*, *P. betulinum*, *P. scabrum*, *P. cordifolium*, *P. reniforme*, *G. nodosum*, *G. napalense*, *G. sylvaticum*, *G. richardsonii*, and *G. ibericum*.

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INTRODUCTION

The genus Pelargonium contains approximately 300 species, most of which are native to South Africa (40). Pelargonium zonale was first introduced into Europe in 1609 (24). Currently, P. X hortorum L. H. Bailey (garden geranium), P. X domesticum L. H. Bailey (regal geranium), and P. peltatum (ivy geranium) are the most economically important Pelargonium species. Records of the crosses that produced the two hybrid species were not kept. Pelargonium X hortorum is thought to have originated from four wild species: P. zonale, P. inquinans, P. frutetorum, and P. scandens. Pelargonium X domesticum is thought to have originated from seven wild species: P. angulosum, P. betulinum, P. capitatum, P. cordifolium, P. cucullatum, P. fulgidum, and P. grandiflorum (24).

Traditionally P. X hortorum was propagated by cuttings. The majority of the cutting-propagated garden geranium cultivars were tetraploid (2N=4X=36). Commercial production of P. X hortorum from seeds became possible in 1965 with the development of the first true breeding F-1 hybrid seed-propagated cultivars (51). Seed-propagated geraniums (2N=2X=18) have the advantages of vigorous growth, ease of production, reduced problems with vascular pathogens, and a wide selection of colors (51). Cutting-propagated geraniums have the advantages of large showy plants and flowers, and variegated varieties. Since their introduction, seed-propagated geraniums have secured a significant portion of the geranium market (21).

Bacterial blight, caused by Xanthomonas campestris pv. pelargonii, is considered the most serious disease of geranium (44). This disease affects both greenhouse and field grown geraniums around the world (42,57).

Brown (10) completed Koch's Postulates on this bacterium and Pelargonium, and suggested Bacterium pelargoni as the name of this pathogen. Other scientific names include Phytomonas pelargonii, P. geranii and Xanthomonas pelargonii. Two species names have been used for this organism because Burkholder (12) was unable to infect Pelargonium spp. with bacteria isolated from Geranium species. However, a later study by Star et al. (54) with 13 isolates from P. X hortorum and four isolates from Geranium spp. showed that these isolates were morphologically and biochemically the same and had similar virulence. Common names used for this disease include bacterial blight, bacterial leaf spot, bacterial stem rot, and bacterial leaf spot and stem rot.

Xanthomonas campestris pv. pelargonii is a gram-negative, aerobic, medium-sized rod-shaped bacterium. It is catalase positive, hydrogen sulfide positive, oxidase negative, motile by means of one polar flagellum, and produces yellow colonies on nutrient agar. This organism can liquify gelatin, reduce nitrate, and does not produce indole. Xanthomonads can be easily identified when isolated from infected plant tissue by the presence of a yellow non-water-soluble pigment (xanthan). Pathogenicity tests are required to differentiate X. c. pv. pelargonii from other X. campestris pathovars (23). Isoenzyme analysis (8), plasmid digest patterns (39) and monoclonal

antibodies (5) have shown potential for use in differentiation of X. campestris pathovars.

There are two types of leaf symptoms associated with bacterial blight of geraniums. Lesions may first appear as small water-soaked spots on the undersides of leaves. These lesions become slightly sunken, enlarged and surrounded by a yellow halo, followed by a wilting and death of the leaf. V-shaped lesions often associated with this disease are necrotic angular areas bounded by veins (32,55).

Plants with leaf lesions may develop the stem rot phase of bacterial blight. The stem turns gray to dull black and eventually will develop a dry rot. Cuttings from infected plants fail to root, wilt, and eventually rot (32,55).

Temperature and nutrient levels play an important role in the development of this disease. Stem rot progresses faster as temperatures increase from 1Ø C to 27 C. Growing plants at high temperatures can be used to stimulate symptom development for the detection of plants with latent infections. High-nitrogen or high-phosphorous fertilizers also increase the rate at which symptoms develop (37).

Xanthomonas campestris pv. pelargonii enters the plant through wounds or stomata. Wainwright and Nelson (59) made histochemical observations of both susceptible and resistant Pelargonium spp. inoculated with the bacterium through wounds in the stem. In the susceptible response, the bacterium initially spread through the plants in the xylem, then moved laterally into the xylem parenchyma. Later the pathogen entered the vascular cambium and rapidly moved into the

phloem cortex and epidermis. As the pathogen spread it caused cellular collapse and decomposition. In the resistant response the pathogen had limited proliferation in the xylem and reduced lateral spread out of the xylem (58).

The most important control for bacterial blight of geraniums is to prevent the introduction of the bacterium into the greenhouse. This can be done with the use of culture-indexed stock plants. Xanthomonas campestris pv. pelargonii may be introduced into a greenhouse through symptomless diseased plants or may survive in the greenhouse in symptomless infected stock plants. When cuttings are rooted close together in a common rooting bench, the bacterium can spread through the propagation medium. Similarly, when geraniums are closely spaced on greenhouse benches, X. c. pv. pelargonii can readily spread from plant to plant by physical contact, and by splashing from overhead watering. Xanthomonas campestris pv. pelargonii has been isolated from symptomless regal geranium (P. X domesticum) plants, demonstrating the potential for regal geraniums as a source of inoculum for other geraniums in the greenhouse (38). Therefore, different types of geraniums should not be grown near each other (32,55). Strict sanitation practices should be used to prevent the spread of the bacteria through the greenhouse. Whiteflies that have fed on infected geraniums for 24 hours have been shown to vector this bacterium. Xanthomonas campestris pv. pelargonii has been isolated from whiteflies and from previously healthy plants inoculated by infested whitefly feeding (12). This bacterium can survive in soil on undecayed plant

material for up to 3 months. The survival of X. c. pv. pelargonii in soil is dependent on the rate of decay of infected plant parts (42).

Useful resistance to bacterial blight has not been observed in cutting-propagated geranium (38,56), seed-propagated geranium (57,22) or ivy geranium cultivars (57). Pelargonium inquinans, P. zonale and P. scandens (the major contributors to P. X hortorum) are all highly susceptible to X. c. pv. pelargonii (38). Resistance has been observed in P. X domesticum, P. acerifolium, P. tomentosum, P. scarboroviae, P. scabrum, P. betulinum, P. grandiflorum, P. multicaule, and P. hispidum (22,38).

Differences in tannin-like materials have been observed between susceptible and resistant Pelargonium species. Wainwright and Nelson (59) suggested that differences in tannin-like materials may be responsible, in part, for differences in resistance to X. c. pv. pelargonii. Tylose formation and deposition of suberin-like material have been observed in plants infected with X. c. pv. pelargonii and also may play a role in restricting the spread of the bacteria within the plant (59).

Many researchers have investigated Pelargonium spp. tissue culture (1,21). Pelargonium spp. have been regenerated from apical meristems (16,19,26,47,49,56), shoot tips (21,25,33,35,46,50), callus tissue (6,9,14,18,34,46,48), anthers (2,3,4,), and protoplasts (36,60). Tissue culture protocols for Pelargonium spp. have been developed to regenerate disease-free stock plants (19,26,33,50,56), regenerate haploid plants from anthers (2,3,4), and rescue zygotic embryos (52). Cultures of Pelargonium tissue also have been used to study plastid

inheritance (17,36,60), transmission of beneficial virus-like infections (17), changes in chromosome number of cultured tissue (6,7), somaclonal variation (15,34,53), and the effects of toxic metabolites at the cellular level (29). Most plants derived from Pelargonium tissue culture have been regenerated by apical and axillary bud proliferation (16,19,33,35,47,49) or adventitious bud formation (9,15,18,21,34,48). Somatic embryogenesis has been indicated only once for Pelargonium, with a single section of an embryoid-like structure as evidence (14). The majority of this tissue culture research deals with the economically important cutting garden geranium. Plant regeneration has also been reported from tissue cultures of regal geranium (15,16,22,27), ivy geranium (16,27), seed-propagated geranium (21,28,33) and scented Pelargonium spp. (9,34).

The production of culture virus-indexed Pelargonium species involves a number of steps designed to eliminate vascular pathogens from stock plants (45). These disease-free stock plants make growing Pelargonium profitable (45). Meristem culture plays an important role in producing culture virus-index Pelargonium plants (45).

The tissue culture techniques of in vitro disease screening (22,29), somaclonal variation (15,22,29) and protoplast fusions (57) may play an important role in development of bacterial blight resistance in P. X hortorum. The purpose of this investigation was to develop and evaluate the potential of tissue culture techniques for introduction of bacterial blight resistance into P. X hortorum. This investigation encompasses: 1) development and histochemical evaluation of plant regeneration from primary callus of seed-propagated geranium, 2)

evaluation of toxic culture filtrates from X. c. pv. pelargonii for use in disease screening, 3) development and use of an in vitro assay for detecting bacterial blight resistance in geranium seedlings and somaclones, and 4) development of protoplast isolation and regeneration protocols for P. X domesticum.

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CHAPTER I

Resistance in Seedlings of the Family Geraniaceae to Bacterial Blight of Geranium.

ABSTRACT

Five-week-old seedlings of Geranium and Pelargonium species growing in culture tubes containing 15 ml of Hoagland's solution solidified with 0.7% agar were inoculated with a 10^7 cfu/ml suspension of Xanthomonas campestris pv. pelargonii cells. Eight weeks after inoculation all seedlings from susceptible P. X hortorum cultivars were dead or severely blighted. Seedlings of P. zonale, P. frutetorum, P. fulgidum, P. fruticosum, P. alchemilloides, P. inquinans, P. acraeum and G. viscosissimum were not significantly different from P. X hortorum in reaction to X. c. pv. pelargonii. However, P. reniforme, P. cordifolium, G. nodosum, G. napalense, G. sylvaticum, G. richardsonii, and G. ibericum seedlings were significantly more resistant than P. X hortorum. Pelargonium cordifolium and G. ibericum seedlings had the lowest levels of tissue blighted 25 days after inoculation. In addition, culture filtrates of X. c. pv. pelargonii were evaluated in a callus bioassay for use in selecting bacterial blight-resistant germ plasm. The growth of geranium callus treated with culture filtrates from X. c. pv. pelargonii was significantly less than callus treated with culture filtrates from X. c. pv. campestris. The growth of callus from Pelargonium genotypes resistant and

susceptible to bacterial blight was not significantly different after treatment with culture filtrates from X. c. pv. pelargonii cultures. An attached leaf assay was used to compare symptomology of bacterial blight to that caused by culture filtrates in greenhouse-grown Pelargonium plants. Culture filtrates from strain X-1 caused leaf damage similar to the symptoms of bacterial blight, but filtrates from strain X-7 of X. c. pv. pelargonii caused significantly less leaf damage than filtrates from strain X-1. The results from this investigation indicate that using culture filtrates from X. c. pv. pelargonii would not be the best approach for evaluation and selection of bacterial blight-resistant germ plasm.

INTRODUCTION

Bacterial blight of geranium is caused by Xanthomonas campestris pv. pelargonii and is the most serious disease of the garden geranium (Pelargonium X hortorum L. H. Bailey). Resistance to bacterial blight has not been reported for cutting- (18,19,34) or seed-propagated garden geranium (7,32). Xanthomonas campestris pv. pelargonii infects both Pelargonium and Geranium species (29). Only a small portion of the more than 300 species from these genera have been evaluated for bacterial blight resistance (7,19,29). Some of these species may have bacterial blight resistance that can be transferred to horticulturally important Pelargonium species. Regal geranium (P. X domesticum L. H. Bailey) cultivars are resistant to bacterial blight, but cannot be sexually crossed with P. X hortorum (8).

An in vitro assay could be useful in screening Geraniaceae species

for bacterial blight resistance (7). In vitro screening protocols using plant pathogens have been developed to evaluate disease resistance of plants to bacteria (1,7,10,27,28), fungi (4,13,20,21,22,24,29,30,35), and nematodes (14,15) for disease resistance. In vitro disease screening has been used to evaluate seedlings of asparagus (4,30), alfalfa (13), and wheat (21,22); plantlets of aspen (20), potato (27,28), geranium (7), and peach (15); shoots of papaya (26), larch (24), and peach (10); and root explants of soybean (14). Such protocols have been suggested as a way to rapidly screen large amounts of germ plasm in a small controlled environment (4,7,10,26,30). In vitro disease screening has the potential to evaluate the effect of environmental factors on disease more easily than in the field or greenhouse (1,35) in some cases.

An in vitro assay has been developed that detects resistance to bacterial blight in Pelargonium plantlets (7). Plantlets screened with this assay developed symptoms similar to plants grown in the greenhouse. With this assay eight Pelargonium species were evaluated for resistance to bacterial blight (7). This in vitro assay holds promise for detecting bacterial blight in seedlings of the Geraniaceae.

Toxic metabolites from culture filtrates of plant pathogens have been used to evaluate disease resistance of plant germ plasm (16,33) and to select for toxin resistance from in vitro plant cell populations (3,11). Daub (3) cited 19 investigations where toxins or toxic culture filtrates were used to select plant cells from which disease-resistant plants were regenerated. In the majority of these studies an increase in resistance to toxins or culture filtrates was correlated with an

increase in disease resistance (3,11). These investigations demonstrate the possibility of using toxic culture filtrates for selection and regeneration of disease-resistant plants in some cases.

Culture filtrates of X. campestris pathovars have been shown to contain toxic metabolites active at the cellular level (9,12). Some observations indicate that these toxic metabolites may play a role in bacterial blight of geranium (12) and bacterial spot of peach (9,11). The level of virulence of X. c. pv. pruni strains has been associated with the production of a toxic culture filtrate (9), and resistance of peach cultivars to bacterial spot has been correlated with their sensitivity to culture filtrates of X. c. pv. pruni (9,11). Peach plants resistant to bacterial spot were regenerated from callus cultures selected for insensitivity to culture filtrates of X. c. pv. pruni (11).

Partially purified culture filtrates of X. c. pv. pelargonii cultures have been shown to be toxic to callus culture tissue and seedlings of geranium (12). The regeneration of plants from callus cultures selected for insensitivity to toxic metabolites from X. c. pv. pelargonii has been suggested as a way to generate novel resistance to bacterial blight in P. X hortorum (12). Daub (3) stated that the use of culture filtrates for selection of disease resistance is not the best approach, but can be used if precautions are taken to avoid selecting for resistance to a nonspecific substance found in the filtrates (3). This investigation evaluates in vitro bacterial blight screening using cell suspensions or partially purified culture

filtrates of X. c. pv. pelargonii, and uses an in vitro assay to evaluate disease resistance in seedlings of Geraniaceae species.

MATERIALS AND METHODS

Seedling screen. Seeds from Pelargonium and Geranium species, and from Brassica oleracea var. capitata were surface disinfested, germinated, transferred to Hoagland's solution (5) solidified with agar (HSS) (7) and cultured as described by Dunbar and Stephens (7). Inoculum was prepared using aggressive strains X-1 and X-7 of X. c. pv. pelargonii and a strain of X. c. pv. campestris (7).

Seedlings were inoculated 30-35 days after being placed onto HSS medium in culture tubes. Upper surfaces of individual leaves were gently rubbed with a sterile cotton swab that had been moistened with inoculum. Then the tubes were sealed with Parafilm M (American National Can, Greenwich, CT) for 2 days. Twenty five days later the plants were rated for tissue blighted using a rating scale of 1-6, where 1 = no symptoms; 2 = 20% tissue blighted; 3 = 20-50% tissue blighted; 4 = 51-75% tissue blighted; 5 = >75% tissue blighted; 6 = plant death. Percentage of surviving seedlings was recorded 8 wk after inoculation.

To determine the effect of inoculum concentration on disease development in seedlings growing in tubes, seedlings of geranium cultivar 'White Orbit' (Ball Seed Co., West Chicago, IL) were inoculated as described above with a cell suspensions of X. c. pv. pelargonii at concentrations of 0, 10^3, 10^5, 10^7, and 10^9 colony forming units (cfu) per ml. This experiment had a completely random

design with six single-plant replicates (one plant per culture tube) per treatment, and was repeated once.

To test the reaction of seedlings growing in tubes to another plant pathogenic bacterium, geranium cv. 'White Orbit' and cabbage seedlings were inoculated with suspensions of either 10^7 cfu/ml of strain X-1 of X. c. pv. pelargonii, X. c. pv. campestris, or sterile water. The experimental design was random and factorial with species and inoculum as independent variables. There were eight single-plant replicates per treatment and the test was repeated once.

To determine if different species of Geraniaceae varied in susceptibility to X. c. pv. pelargonii, seedlings from P. X hortorum, P. capitatum, P. zonale, P. frutetorum, P. fulgidum, P. fruticosum, P. alchemilloides, P. inquinans, P. reniforme, P. cordifolium, P. acraeum, G. nodosum, G. napalense, G. sylvaticum, G. richardsonii, G. viscosissimum, and G. ibericum were inoculated with a 10^7 cfu/ml of strain X-1 or X-7 of X. c. pv. pelargonii. The experiment had a completely random design with six single-plant replicates per treatment, and was repeated at least once for each species.

Plant tissue culture. Seed-propagated geranium (cv. 'Orbit Red' and 'Orbit Scarlet', Ball Seed Co., West Chicago, IL) callus cultures were initiated from shoot tip explants and regal geranium (cv. 'Melissa', Oglevee Associates Inc., Connellsville, PA) callus cultures were initiated from leaf explants using the protocols described by Dunbar and Stephens (6). Seed-propagated and regal geranium callus was transferred to two Murashige and Skoog (MS) (23) media supplemented with 2.0 mg/l NAA, 2% sucrose, solidified with 0.9% agar (Sigma

Chemical Co., St. Louis, MO), adjusted to pH 5.8, and poured into sterile 100 X 15 mm polystyrene Petri dishes. Medium A also was supplemented with 2.0 mg/l 6-BAP, and medium B with 2.0 mg/l kinetin. All cultures were maintained at 25 C with 40 $uEm^{-2}s^{-1}$ light and a 16 hr photoperiod provided by cool white fluorescent lamps.

Preparation of culture filtrates. Partially purified culture filtrates were prepared using a modification of procedures developed by Hammerschlag (12). The three X. c. pv. pelargonii strains (X-1, X-5, and X-7) and the X. c. pv. campestris strain used in this investigation have been described (7). Broth cultures of these strains were grown as described by Dunbar and Stephens (7) in 25 ml of a modified Lederberg's complete medium (7). After 48 hr of incubation bacteria was pelleted by centrifugation at 1000 g for 20 min. The supernatants were passed through a 0.22 um filter. The culture filtrates and filtered medium were adjusted to pH 3.0 with 1.0 N HCl, and were partitioned with equal volumes of ethyl acetate. After the aqueous phase was rotary evaporated for 10 min at 40 C, the filtrates were adjusted to pH 8.0 with 1.0 N KOH, and returned to their original volume with distilled water.

Callus bioassay. Seed-propagated and regal geranium callus tissues were treated using the methods of Hammerschlag et. al (12) by placing approximately 50 mg pieces of tissue onto Whatman No. 1 filter paper in 100 X 25 mm Petri dishes containing 10 ml of partially purified culture filtrate or partially purified filtered medium for 3 hr, before subculturing onto fresh callus medium (12). Plates were incubated for 21 days as described above, and callus fresh weight was

measured. The percentage of initial weight was used for the analysis of variance.

To determine the effects of plant genotype, X. campestris pathovar, and plant tissue culture medium on the callus bioassay, 'Orbit Red' and 'Melissa' callus cultured on medium A and B were treated with filtered medium, or culture filtrates from X. c. pv. pelargonii, or X. c. pv. campestris. This experiment was random and factorial with plant species, tissue culture medium, and filtrate as variables. The data from medium A of this experiment also was analyzed alone as a factorial with plant species and filtrate as independent variables. There were six replicates and the experiment was repeated once.

To determine the effect of strain of X. c. pv. pelargonii on toxicity of culture filtrates to callus, culture filtrates from strains X-1, X-5, and X-7 were used to treat 'Orbit Red' and 'Orbit Scarlet' callus tissue in the callus bioassay. These cultures were maintained and subcultured on medium A. Culture filtrates from strain X-7 also were assayed after a heat treatment (100 C for 15 min) to test the heat stability of the toxic metabolites in the culture filtrates. This experiment had a completely random design with six replicates per treatment and the experiment was repeated once.

Attached leaf bioassay. 'Orbit Red' seeds were germinated in moist Bacto Professional Planting Mix (Michigan Peat Co., Houston, TX) in 20 X 13 X 6.4 cm trays, 20 seeds per tray, by watering as needed to keep soil from drying. Seedlings were transferred to 10 cm diameter clay pots 30 days after germination. The cultivar 'Melissa' was

propagated by vegetative cutting of lateral branches. Cuttings were placed into moist perlite in 20 X 13 X 6.4 cm trays, eight cuttings per tray, and misted at 10 min intervals. After 30 days rooted cuttings were transplanted to 10 cm diameter clay pots and fertilized every 2 wk by watering with N=180 ppm (20-20-20). Plants were used in the attached leaf bioassay 60 days after transplanting to 10 cm pots. Partially purified culture filtrates (0.1 ml) from X. c. pv. pelargonii and X. c. pv. campestris, and filtered modified Lederberg's medium were infiltrated through stomata at the base of geranium leaves by pressing a syringe firmly to the underside of a leaf between leaf veins and delivering 0.1 ml volume. Leaves were inspected visually for tissue damage at 24 and 96 hr after infiltration. A leaf tissue damage rating scale was used to evaluate the treatments: 0= no symptoms, 1= necrotic spot only at the infiltration site, 2= necrotic area spread beyond the infiltration site, 3= necrotic area spread beyond the infiltration site with surrounding chlorotic area.

To determine the effects of the filtrates on geranium leaves in the greenhouse, leaves of 'Orbit Red' plants were treated with culture filtrates from X. c. pv. pelargonii and X. c. pv. campestris, and filtered medium. This experiment had a completely random design with eight single-leaf replicates and the experiment was repeated once.

To determine the effect of culture filtrates of X. c. pv. pelargonii on the leaves of resistant and susceptible Pelargonium genotypes, culture filtrates from strain X-1 and X-7 were infiltrated into leaves of 'Orbit Red' and 'Melissa' plants. This experiment had a

completely random design with eight single-leaf replicates, and the experiment was repeated once.

Greenhouse inoculations. Cell suspensions of strain X-1 and X-7 of X. c. pv. pelargonii at a concentration of 10^7 cfu/ml were infiltrated into 'Orbit Red', 'Orbit Scarlet', P. cordifolium, and 'Melissa' plants as described above for culture filtrates. These plants were inspected every 4 days for 21 days. 'Orbit Red' and 'Orbit Scarlet' were propagated from seeds and P. cordifolium and 'Melissa' were propagated from cuttings as described above.

RESULTS

Seedling screen. Symptoms of bacterial blight on seedlings of the Geraniaceae inoculated in vitro included circular water-soaked lesions, leaf blight, leaf wilt while petiole remained erect, dry rot and plant death. Water-soaked lesions were observed within the first week after inoculation of susceptible seedlings with 10^7 cfu/ml of X. c. pv. pelargonii. Leaves with lesions often became completely blighted within 1-2 wk. Small circular red-brown necrotic lesions were observed on seedlings of P. cordifolium and G. ibericum within the first week after inoculation. These symptoms were similar to bacterial blight symptoms observed on greenhouse-grown geraniums and in vitro grown plantlets regenerated from tissue culture (7).

An inoculum level of 10^3 cfu/ml of X. c. pv. pelargonii was too low to insure consistent disease development, while cell suspensions of 10^7 and 10^9 cfu/ml led to death of all seedlings within 3 wk (Figure 1.1). In contrast, with 10^5 cfu/ml, 60% of the seedlings survived for 3 wk, but all were dead within 5 wk. Symptoms were not observed on seedlings

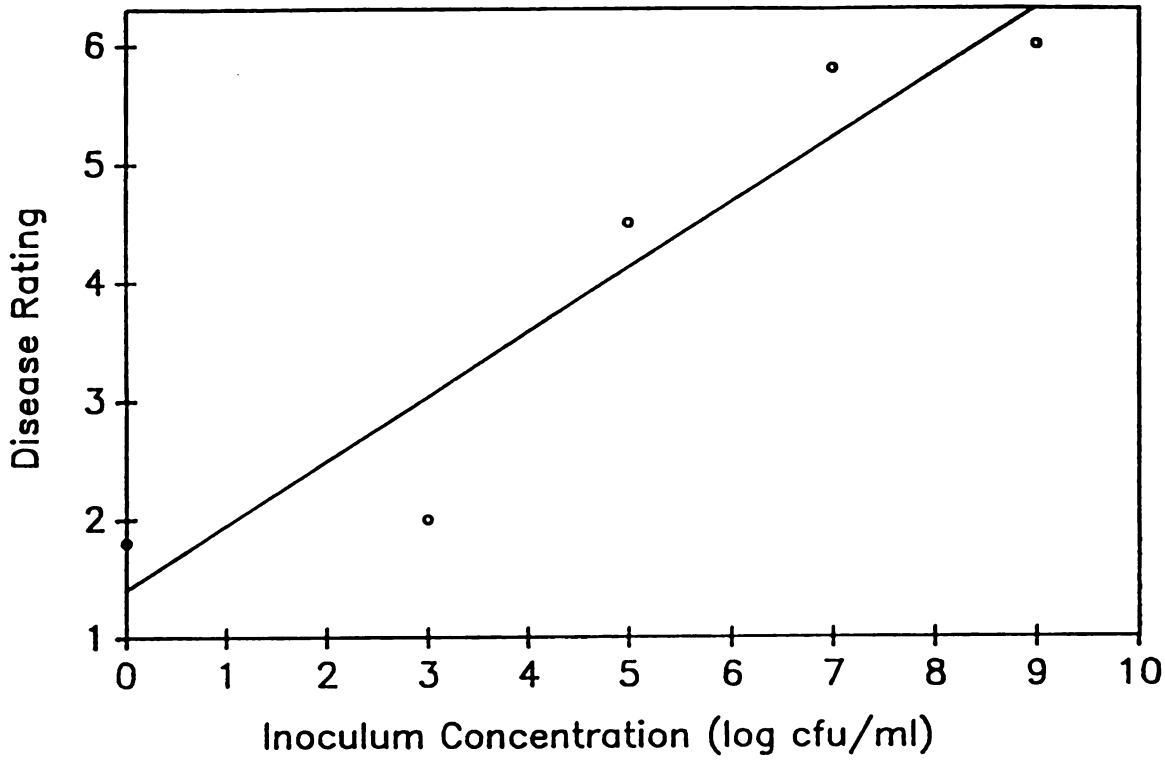

Figure 1.1. Effect of concentration of Xanthomonas campestris pv. pelargonii on disease in seedlings of geranium cultivar 'White Orbit' rated 3 wk after inoculation in vitro. 1 = no symptoms; 2 = <20% tissue blighted; 3 = 20-50% tissue blighted; 4 = 51-75% tissue blighted; 5 = >75% tissue blighted; 6 = plant death. r value = 0.832 ($P<0.001$).

inoculated with sterile water. An inoculum level of 10^7 cfu/ml was chosen for successive experiments since it was the lowest level that would allow rapid screening of seedlings for resistance to bacterial blight.

While the environment in the tubes during the 25 day incubation period was conducive for disease development, seedlings of P. X hortorum inoculated with X. c. pv. campestris (mean disease rating {MDR} = 2.0) or sterile water (MDR=2.3) were free of disease. In contrast, many plants inoculated with X. c. pv. pelargonii (MDR=4.0) were dead. All cabbage seedlings were dead 25 days after inoculation with X. c. pv. campestris (MDR=6.0). The MDR of cabbage seedlings was 2.9 after inoculation with X. c. pv. pelargonii and 1.0 after treatment with sterile water. The effect of inoculum, and the inoculum/host interaction on disease was highly significant (F-test P<0.001) while host species was not significant.

Seedlings of P. cordifolium were significantly more resistant to bacterial blight than the other species of Geraniaceae evaluated (Table 1.1). Seedlings from P. reniforme, G. nodosum, G. napalense, G. sylvaticum, G. richardsonii, and G. ibericum were significantly more resistant to bacterial blight than P. X hortorum (the susceptible control) in reaction to X. c. pv. pelargonii. Seedlings of P. zonale, P. frutetorum, P. fulgidum, P. fruticosum, P. alchemilloides, P. inquinans, P. acraeum and G. viscosissimum were not significantly different than P. X hortorum in resistance to X. c. pv. pelargonii (Table 1.1).

Table 1. 1 Bacterial blight in seedlings of Geraniaceae species

species/cultivar	MDR	SE	% surviving
Pelargonium X hortorum			
'Red Orbit'	4.74	0.37	33
'White Orbit'	4.79	0.45	44
P. capitatum a	4.30	0.28	47
P. zonale a	5.28	0.25	26
P. frutetorum a	4.74	0.37	25
P. fulgidum a	4.21	0.40	37
P. fruticosum a	4.74	0.37	17
P. reniforme a	2.57	0.52	64
P. cordifolium a	1.56	0.23	100
P. alchemilloides d	4.25	0.51	0
P. inquinans a	5.35	0.23	4
P. acraeum a	4.75	0.54	17
Geranium viscosissimum b	3.75	0.54	50
G. ibericum d	1.91	0.08	100
G. nodosum c	2.67	0.50	82
G. napalense c	2.58	0.19	100
G. sylvaticum b	2.67	0.45	83
G. richardsonii b	2.25	0.28	83

Seedlings were rated for percentage of blighted tissue 25 days after inoculation with 10^7 cfu/ml of Xanthomonas campestris pv. pelargonii. 1 = no symptoms; 2 = <20% tissue blighted; 3 = 20-50% tissue blighted; 4 = 51-75% tissue blighted; 5 = >75% tissue blighted; 6 = plant death. MDR = Mean Disease Rating. SE = standard error. The percentage of surviving seedlings was recorded 8 wk after inoculation. a) Dr. L. C. Ewart, Horticultural Department, Michigan State University, East Lansing Michigan b) Devoniam Botanic Garden in Edmonton, Alberta, Canada c) Botanic Garden of the University of Leiden, Netherlands d) Alpengarten IM Belvedere Vienna, Austria.

Callus bioassay. The analysis of variance showed the effect of filtrate in the callus bioassay was significant (F-test P<Ø.Ø5), and the effect of cultivar, medium, and the cultivar/medium interaction were highly significant (F-test P<Ø.ØØ1). The filtrate/cultivar, filtrate/medium, and filtrate/cultivar/medium interactions were not significant. Seed-propagated geranium callus growth was friable and undifferentiated on medium A and B. However, regal geranium callus growth was friable and undifferentiated on medium A, but was hard and contained shoot primordia (6) when grown on medium B. To evaluate the effect of these culture filtrates on geranium at the cellular level the experiments were analyzed using the data from medium A, on which both regal and seed-propagated geranium callus was friable. On medium A alone, the effect of filtrate was significant (F-test P<Ø.Ø5), but the effect of cultivar and filtrate/cultivar interaction were not significant. Growth of geranium callus treated for 3 hr with culture filtrates from X. c. pv. pelargonii was significantly less than callus treated with filtered medium (Table 1.2). However, growth of callus treated with culture filtrates of X. c. pv. campestris was not significantly different from callus treated with filtered medium. Growth of callus treated with culture filtrates of X. c. pv. pelargonii was significantly different from callus treated with culture filtrates of X. c. pv. campestris (Table 1.2). Significant differences were not observed between seed-propagated geranium callus treated with culture filtrates from strains X-1, X-5, and X-7 of X. c. pv. pelargonii. Heat treatment of culture filtrate from strain X-7 did not significantly change its effect on callus growth.

Table 1.2. The effect of culture filtrates on geranium callus.

pathovar	% initial weight	SE
X. c. pv. *pelargonii*	86.3	9.3
X. c. pv. *campestris*	111.8	9.8
filtered medium	131.8	17.1

Analysis of Variance

source	df	mean square	F-test
Pelargonium genotype	1	6242	NS
inoculum	2	6346	*
genotype/inoculum interaction	2	44	NS
error	30		
total	35		

Callus tissue of cultivar 'Melissa' and 'Orbit Red' were soaked for 3 hr in culture filtrates and then were subcultured onto MS medium A. After 21 days fresh weights were measured and used to calculate percentage of initial weight. SE = standard error. NS = F-test not significant. * = significant F-test ($P<0.05$).

Table 1.3. Effect of culture filtrates on geranium leaf tissue.

strain of filtrate	tissue damage rating	SE
X. c. pv. *pelargonii* strain X-1	2.6	0.13
X. c. pv. *pelargonii* strain X-7	1.4	0.26
X. c. pv. *campestris*	1.1	0.11
filtered medium	1.1	0.06

Leaves from 90 day-old 'Orbit Red' plants with 0.1 ml of partially purified culture filtrate infiltrated through the stomata of the underside of the leaf. Leaf tissue damage was rated 96 hr after infiltration. The rating scale was 0= no symptoms, 1= necrotic spot only at the infiltration site, 2= necrotic area spread beyond the infiltration site, 3= necrotic area spread beyond the infiltration site with surrounding chlorotic area. SE = standard error.

Attached leaf bioassay. Seed-propagated geranium leaf tissue treated with culture filtrates from strain X-1 of X. c. pv. pelargonii had significantly more damage 4 days after infiltration than leaf tissue treated with culture filtrates of strain X-7 of X. c. pv. pelargonii, X. c. pv. campestris, and filtered medium (Table 1.3). Culture filtrates of strain X-1 caused necrotic V-shaped lesions surrounded by a chlorotic halo to 'Orbit Red' leaves. Leaf tissue damage caused by culture filtrates of strain X-7 of X. c. pv. pelargonii, X. c. pv. campestris, and filtered medium was not significantly different (Table 1.3). Culture filtrates from X. c. pv. pelargonii caused significantly more leaf damage to seed-propagated geranium than to regal geranium (F-test $P<0.001$) data not shown. The interaction between Pelargonium species/strain of X. c. pv. pelargonii culture filtrate was not significant.

Greenhouse inoculations. Four days after inoculation with cell suspensions of X. c. pv. pelargonii, 'Orbit Red' and 'Orbit Scarlet' plants growing in the greenhouse developed necrotic spots at the site of inoculation. After 6 days, water-soaked lesions had developed around the inoculation site. Eight to 12 days after inoculation a few inoculated leaves were wilted while petioles remained erect, and after 12 days chlorosis surrounded the lesions. After 16 days some inoculated leaves had become completely necrotic, and by day 21 all inoculated leaves were completely necrotic. In contrast, necrotic spots developed within 6 days at the site of inoculation on 'Melissa' and P. cordifolium leaves and no further symptoms developed.

DISCUSSION

A small number of Pelargonium species have been screened and reported to contain significant levels of resistance to bacterial blight (7,19). The results of this investigation have added P. cordifolium and P. reniforme to the list of bacterial blight-resistant Pelargonium species. This investigation also observed bacterial blight resistance in G. nodosum, G. napalense, G. sylvaticum, G. richardsonii, and G. ibericum.

The in vitro assay used in this investigation was originally developed to screen Pelargonium plantlets. This study showed that the seedlings in the in vitro assay had a response similar to tissue culture plantlets (7). Both seedlings and plantlets of P. X hortorum were highly susceptible to X. c. pv. pelargonii, but were resistant to X. c. pv. campestris (7). This in vitro assay can be used to detect differences in bacterial blight resistance similar to that observed in the greenhouse (7). Previous investigations reported results from greenhouse screenings of P. zonale, P. inquinans, P. capitatum, P. fulgidum (19), and P. X hortorum (7,32) and the present investigation reports similar results for these species. We observed P. cordifolium to be resistant both as seedlings and as mature plants screened in the greenhouse.

Knauss and Tammen (19) suggested that bacterial blight resistance could be most easily introduced into popular P. X hortorum cultivars, if it could be found in P. X hortorum (19). However, useful resistance has not been observed in P. X hortorum (1,19,32,34). The major contributors to the hybrid species P. X hortorum are most likely

P. zonale, P. inquinans, P. scandens and P. frutetorum (8). Pelargonium zonale, P. inquinans, and P. scandens have been observed to be susceptible to bacterial blight (19). This investigation confirms the susceptibility of P. zonale and P. inquinans, and also found P. frutetorum to be susceptible.

Seed-propagated geraniums have become a major part of the geranium market (6). Therefore, resistance to bacterial blight that is expressed in the seedling would be desirable. Previous bacterial blight screening procedures have used mature plants (19,32,34), but the procedure in this paper can identify resistance expressed in the seedling. Seedlings of P. cordifolium had a high level of resistance to bacterial blight. Sexual crossing attempts between P. cordifolium and P. X hortorum were unsuccessful. These species are in different sub-genera (2), therefore techniques like embryo (25) or ovule rescue (24) may be required to create hybrid plants.

Using the callus bioassay described by Hammerschlag et al. (12) this investigation confirms an earlier observation that culture filtrates from X. c. pv. pelargonii cultures contain metabolites that are toxic to Pelargonium callus tissue (12). The effect of medium and the medium/cultivar interaction were highly significant (F-test $P<0.001$) in the callus bioassay. This was correlated to a difference in callus differentiation on media A and B. Seed-propagated geranium callus was friable and undifferentiated on both media, but regal geranium callus had more organized growth on medium B. This illustrates the importance of selecting a medium that promotes

undifferentiated and friable growth of callus tissue of all genotypes to be used for evaluation of cell selection protocols.

Hammerschlag (9) suggested that filtrates from cultures of X. c. pv. pruni fulfilled three criteria that indicated these filtrates could be successfully used in a cell selection system for regenerating disease-resistant plants (9). This pathogen's filtrate was toxic at the cellular level, correlated with the aggressiveness of the pathogen, and disease-susceptible genotypes of this host were more sensitive to the culture filtrates than were disease-resistant genotypes (9). Culture filtrates from X. c. pv. pelargonii cultures fit only one of these criteria. Culture filtrates from two aggressive X. c. pelargonii strains (X-7 and X-1) were not significantly different from a less aggressive strain (X-5) in the callus bioassy. There was no significant difference observed between the callus growth of bacterial blight-resistant (regal geranium) and susceptible (seed-propagated geranium) genotypes in the callus bioassay. However, culture filtrates were toxic at the cellular level. The results from this investigation suggest that the use of culture filtrates from X. c. pv. pelargonii would not be the best approach for evaluation of germ plasm for resistance to bacterial blight of geranium.

Geranium seedlings developed symptoms similar to those of bacterial blight of geranium when grown on culture filtrate saturated filter paper from a single strain of X. c. pv. pelargonii (12). In this investigation, leaves from mature seed-propagated geraniums developed necrotic V-shaped lesions surrounded by a chlorotic halo after treatment with culture filtrates from strain X-1. These lesions

were similar to those found on geranium leaves infected with X. c. pv. pelargonii (31). Culture filtrates from strain X-7 did not cause symptoms similar to filtrates of strain X-1 or the pathogen itself when infiltrated into geranium leaves. Strains X-1 and X-7 have been shown to be similar in aggressiveness when inoculated on seed-propagated geraniums (7). Although toxins and culture filtrates from cultures of some plant pathogens can be used successfully to quickly mass screen germ plasm for disease resistance, the observations of this investigation suggests the importance of testing the effect of culture filtrates from more than one strain of a pathogen before using culture filtrates in a selection system.

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CHAPTER II

Adventitious Shoot Formation from Seed-Propagated Geranium Explants and Variation in Bacterial Blight Resistance of Regenerated Plants.

ABSTRACT

This investigation used histochemistry to show the origin of adventitious shoots from primary callus cultures of seed-propagated geraniums, and used in vitro screening of plantlets to evaluate somaclones for resistance to bacterial blight caused by X. c. pv. pelargonii. Five days after placing hypocotyl and shoot tip explants onto a Murashige and Skoog MS (1962) medium supplemented with 2.0 mg/l zeatin and 1.9 gm/l IAA, small cells with dense cytoplasm and prominent nuclei were present in the epidermis, at the cut edges of stem on hypocotyl and shoot tip, and at the cut edges of cotyledonary petioles on shoot tip explants. These small cells were found in groups with as few as three cells or in multicellular meristemoids. After 10 days in culture, meristemoids had developed into shoot primordia, and in another 5 days shoot promordia were enlarged with expanding leaf primordia. After 20 days in culture, shoot primordia had elongated and developed vascular tissue. Over 2,000 plantlets from such cultures were screened in vitro with a 10^7 cfu/ml cell suspension of X. c. pv. pelargonii. Plantlets that were alive 8 wk after inoculation were either transferred directly to soil, or micropropagated on a MS medium supplemented with 0.2 mg/l zeatin. Two somaclones survived direct

transfer to soil, and clones from two micropropagated survivors had significantly less tissue blighted than seed-propagated geranium control plantlets 2 wk after inoculation *in vitro*. However, when somaclones were propagated by cuttings and screened in the greenhouse, none had a higher level of bacterial blight resistance than the controls.

INTRODUCTION

Seed-propagated geraniums have become an important part of the floriculture industry over the last 35 years because of increased vigor, ease of propagation, reduced problems with vascular pathogens, and a wide variety of flower colors. *Xanthomonas campestris* pv. *pelargonii* causes bacterial blight of geranium resulting in serious losses to garden geranium (*Pelargonium* X *hortorum* L. H. Bailey) (28). Resistance to bacterial blight has not been observed in cutting- (17) or seed-propagated (24) garden geraniums. Regeneration and selection of somaclonal variants has been suggested as a possible source of novel resistance to bacterial blight in geranium (10,11,13). Seed-propagated geraniums can be efficiently regenerated from primary callus culture (10). Protocols for adventitious shoot formation from explants or primary callus cultures have important applications in mutation breeding (3), somaclonal variation (5,18), and *Agrobacterium* transformations (15).

Knowledge of the origin of shoots from the cultured tissue is important in the selection of a tissue culture protocol. Adventitious shoots regenerated from mutated explants have a high frequency of solid mutations which are thought to be due to a single cell origin for most

adventitious shoots (2,3). Axillary buds used to regenerate shoots from mutated explants have a higher frequency of producing chimeras which will impede a mutation breeding program (2,3). The potential for variation is greater among somaclones regenerated via adventitious shoot formation than axillary bud proliferation (5,18). Adventitious shoots regenerated from the wounded areas on explants susceptible to Agrobacterium should prove amenable to Agrobacterium transformations (15).

Efficient in vitro adventitious shoot regeneration and disease screening protocols could facilitate the recovery and evaluation of novel sources of bacterial blight resistance introduced via somaclonal variation, mutation breeding, or Agrobacterium transformations. An in vitro screen for detecting bacterial blight resistance in Pelargonium somaclones soon after shoot regeneration differentiates between resistant and susceptible plant genotypes (11). This investigation used histochemistry to show the origin and development of shoots from primary callus cultures of seed-propagated geraniums, and used in vitro screening of plantlets to select and evaluate somaclones with increased resistance to bacterial blight.

MATERIALS AND METHODS

Histochemistry. Hypocotyl and shoot tip explants from seed-propagated geranium cultivar 'Orbit Scarlet Imp.' (Ball Seed Co., West Chicago, IL) were prepared (10) and cultured on MS-H medium (10). Both hypocotyl and shoot tip explants were fixed immediately after preparation and every 5 days after plating onto MS-H medium. Explants

and tissue removed from culture medium were fixed in a solution of 63% ethyl alcohol, 5.0% propionic acid and 5.0% formalin (FPA) for 16 hr at 23 C (16). Tissue was dehydrated in a tertiary butyl-ethyl alcohol series, embedded in paraffin, sectioned 6 um thick and stained with safranin and fast green (16).

In vitro screening. Seed-propagated geraniums (cv. 'Red Orbit', 'Appleblossom Orbit', 'Scarlet Orbit', 'White Orbit', 'Hollywood Red', and 'Ringo Dolly') were regenerated (10), and plantlets were screened in vitro with a 10^7 colony forming units/ml (cfu/ml) cell suspension of X. c. pv. pelargonii (11). Plantlets surviving 8 wk after inoculation were either transferred directly to soil (11), or micropropagated. For micropropagation, all leaf, petiole, and root tissue was removed from surviving plantlets. The shoot tissue was submerged in 10% (v/v) bleach (5.25% sodium hypochlorite) solution containing 0.5 ml of Tween 20 per liter for 3 min. Shoot tissue was then rinsed three times in sterile distilled water. Shoot explants 0.8-1.0 cm long were placed in 100 X 15 mm polystyrene Petri dishes on a Murashige and Skoog MS (21) medium supplemented with 0.2 mg/l zeatin (trans isomer) (Sigma Chemical Co., St. Louis, MO), 2.0% sucrose, adjusted to pH 5.8, and solidified with 0.9% agar (Sigma Chemical Co., St. Louis, MO). Shoots that developed on this medium were subcultured every 4-7 wk to propagate shoots for rooting to be rescreened and transferred to soil. Shoots were placed into culture tubes (25 X 150 mm) on 15 ml of Hoagland's solution (10) solidified with agar (11), and roots developed within 4 wk.

To determine if the survivors from the initial screenings were resistant to bacterial blight, the reaction of plantlets micropropagated from these survivors was compared to susceptible, previously unscreened seed-propagated geranium plantlets in the *in vitro* screen. These tests had completely random designs with six single-plant replicates (one plant per culture tube). These tests were performed two times with both strain X-1 and strain X-7 (11) for each survivor.

Somaclones from micropropagated survivors that had significantly less disease *in vitro* than controls were transferred to soil and greenhouse conditions (11). The plants transferred to soil were clones of plantlets that had survived an initial screen, but themselves had not been rescreened. After 8 wk plants were transferred to 10 cm clay pots.

Plants that had been transferred to soil after an initial screen and after micropropagation were vegetatively propagated. Cuttings were placed into moist perlite in 20 X 13 X 6.4 cm trays, eight cuttings per tray, and were misted every 10 min. After 30 days rooted cuttings were transplanted to 10 cm clay pots.

Thirty days after transplanting plants were inoculated with 10^7 cfu/ml cell suspension of strain X-7 of *X. c.* pv. *pelargonii* (11). Inoculum was infiltrated through stomata at the base of the first five fully expanded leaves by pressing a 1.0 ml syringe firmly to the underside of each leaf between leaf veins and delivering 0.1 ml. The experiment had a completely random design with five single-plant replicates. The experiment was repeated with strain X-1 (11).

RESULTS

Histochemical observations. Prior to placement on MS-H medium, small densely cytoplasmic cells were not observed at the cut edges of hypocotyl or shoot tip explants. After 5 days on MS-H medium small cells with dense cytoplasm and prominent nuclei were present in the epidermis, at the cut edges of the stem on hypocotyl and shoot tip explants, and at the cut edges of cotyledonary petioles on shoot tip explants (Figure 2.1A and 2.1C). The small densely cytoplasmic cells formed meristemoids (26) which were scattered throughout the surface of the explant tissue (Figure 2.1A, 2.1B, and 2.1C). The development of the meristemoids was not synchronous, and after 5 days in culture, small densely cytoplasmic cells were observed on the same explant in groups that ranged from three cells to multicellular meristemoids (Figure 2.1A and 2.1C). The diameter of the hypocotyl and shoot tip explants had increased from approximately 1.0 mm to 1.5-3.0 mm in the first 5 days of culture. The increase in diameter occurred over the entire length of the explant, but was greatest at the explant's cut ends.

Meristemoids that had developed into shoot primordia (shoot apex with leaf buds) were observed after 10 days on MS-H medium. After 15 days in culture, enlarged shoot primordia with well developed shoot apical meristems and expanded leaf buds were observed (Figure 2.1D). Most explants were approximately 3.0 mm in diameter after 10 days on MS-H medium, and after 15 days most explants were spherical in shape with a diameter of 4-5 mm. Shoot primordia were elongated and had developed vascular tissue (Figure 2.1E) after 20 days in culture.

Figure 2.1A-E. Development of shoot primordia from seed-propagated geranium explants. (A) 'Orbit Scarlet' shoot tip explant with three small cells with prominent nuclei at the cut edge of a cotyledonary petiole. Bar represents 20 um. (B) Longitudinal section through an 'Orbit Scarlet' hypocotyl explant after 15 days in culture with meristemoid attached to epidermis of the hypocotyl. Bar represents 20 um. (C) Meristemoid attached to the cut edge of the cotyledonary petiole of an 'Orbit Scarlet' shoot tip explant after 5 days in culture. Bar represents 50 um. (D) Shoot primordia at the cut edge of the stem of an 'Orbit Scarlet' hypocotyl explant. A = shoot apex, L = leaf primordia, Bar represents 50 um. (E) Longitudinal section of an elongated shoot primordia with vascular tissue (V). Bar represents 100 um.

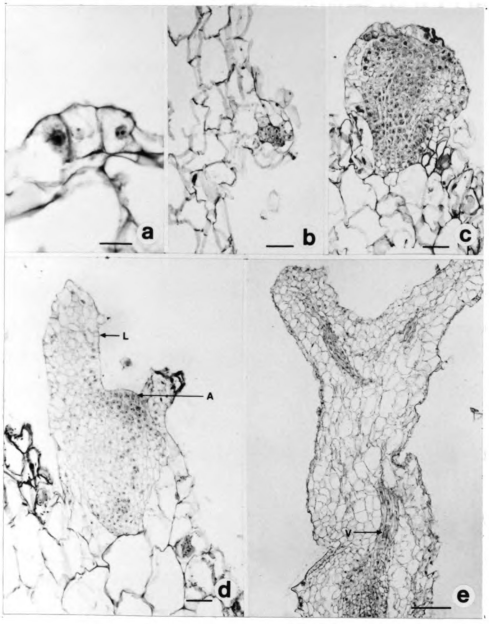

Figure 2.1

After 30 days on MS-H medium there was an average of 43 ± 7 (± standard error = SE) shoot primordia per hypocotyl explant and 36 ± 3 (SE) per shoot tip explant.

Evaluation of somaclones. Of 2100 seed-propagated geranium plantlets screened *in vitro* with *X. c.* pv. *pelargonii*, 91 survived the initial screening. Forty two of these were transferred directly to soil. Two survived and became acclimated to soil and greenhouse conditions (75B and 75H), and the others died with blight symptoms.

The stems from the other 49 surviving plantlets were surface sterilized and placed on micropropagation medium. Thirty of these plants were successfully micropropagated and clones of these plantlets were rescreened *in vitro*. Only two of the plantlets micropropagated from survivors showed significantly less blight than the seed-propagated geranium controls (Table 2.1), and had a smaller percentage of plant death (Table 2.1). However, these rescreened survivors still developed severe blight symptoms 2 wk after inoculation and over 50% of these plantlets were dead after 3 wk. One of these plants originated from an explant (105 W) along with 15 other plantlets that died from their initial inoculation. The other was the only plant screened from its explant (105 A).

Plants 75B, 75H, 105A, and 105W were propagated by cuttings and screened in the greenhouse. The cuttings from these four plants did not have significantly less blighted tissue than controls. Plants from all four clones developed severe blight and eventually died after inoculation.

Table 2.1. In vitro rescreening of surviving clones.

clone	MDR(a)	SE	% death(b)	SE
105A	3.73	0.36	67.5	8
105W	3.71	0.36	54.2	17
control 1	4.92	0.32	85.8	7
control 2	4.71	0.29	78.7	10

Plantlets were inoculated by gently rubbing the upper leaf surface with a cotton swab moistened with 10^7 cfu/ml suspension of X. c. pv. pelargonii cells. Mean disease rating (MDR): 1 = no symptoms, 2 = <20% tissue blighted, 3 = 20-50% tissue blighted, 4 = 51-75% tissue blighted, 5 = >75% tissue blighted, and 6 = plant death. Controls 1 and 2 are populations of seed-propagated geranium plantlets that had not been screened previously. a) data collected 2 wk after inoculation. b) data collected 3 wk after inoculation.

DISCUSSION

Somaclonal variation is a promising source of novel resistance to plant disease. Somaclonal variants with increased disease resistance have been regenerated from tissue cultures of a number of crop species (5,6,20,24,26), and at least 4 cultivars have been developed with disease resistance from somaclonal variation (5,11,13,18). This investigation selected plantlets with slower symptom development than controls when tested in vitro, however, these plants were not resistant to bacterial blight. The plantlets micropropagated from these somaclones still developed blight symptoms and over 50% were dead within 3 wk. When these somaclones were tested under greenhouse conditions, the slowing of symptom development was not significant. Previous studies have observed the apparent loss of somaclonal variation in field trials and have attributed this loss to the transient nature of the variant trait (19) or to differing environmental factors in the field (4). The small difference in susceptibility observed in this investigation, between the controls and plantlets selected and evaluated in vitro, may not have been detectable under greenhouse conditions, or this difference may have been lost during micropropagation and propagation by cutting.

Although this investigation was unsuccessful at finding significant levels of bacterial blight resistance in geranium somaclones, the procedures used in this investigation could be applied to mutation breeding or the development of an Agrobacterium

transformation system. Observations of serial sections indicate single cells give rise to meristemoids and adventitious shoots. A single cell origin for adventitious shoots has been used to explain the high percentage of solid mutant plants recovered from adventitious shoots regenerated from mutated explants (2,3). A single cell origin observed for in vitro adventitious shoots of seed-propagated geraniums suggests that they could be used successfully to regenerate solid mutants from mutated explants with a high frequency. A percentage of the shoots regenerated from shoot tip explants will arise from axillary shoot proliferation from the apical meristem. In contrast, all shoots regenerated from hypocotyl explants would be adventitious. The hypocotyl explants of seed-propagated geraniums should be chosen for mutation breeding programs and somaclonal variation studies to insure the highest frequency of solid mutants.

The protocol described by Dunbar and Stephens (10) could be used in the development of an Agrobacterium transformation system for seed-propagated geranium. The two requirements for Agrobacterium transformations of explants are the origin of adventitious shoots from wounded explant tissue, and a plant species that is a host for Agrobacterium (15). Pelargonium X hortorum is a host for Agrobacterium (8), and this investigation has shown that adventitious shoots originate from wounded cotyledonary petioles and stem of seed-propagated geranium explants.

In vitro screening with a pathogen allows the evaluation of disease resistance soon after plantlet regeneration, but removal of the pathogen may be required prior to acclimation to greenhouse conditions

to insure survival of potentially useful germ plasm. After surviving *in vitro* screening, *Pelargonium* X *domesticum* (resistant to bacterial blight) plantlets have been successfully transferred to soil and established in the greenhouse (3). However, 20% of these plants died while being acclimated. This transfer required placing plants into plastic bags to prevent rapid desiccation of plantlets (11). Even when plants are free from disease some plant death can be expected when transferring *in vitro* grown plantlets to the greenhouse (25). The humid conditions required to transfer plantlets to the greenhouse may increase disease susceptibility adding to the loss of potentially useful germ plasm. This investigation describes procedures to remove *X. c.* pv. *pelargonii* from plants inoculated *in vitro* and to micropropagate these plants for rescreening and then transfer to soil. These protocols may facilitate future studies using *in vitro* techniques to introduce bacterial blight resistance into *P.* X *hortorum*.

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CHAPTER III

Plant Regeneration from Callus-derived Protoplasts of Pelargonium X domesticum

ABSTRACT

A protocol for regenerating plants from callus-derived protoplasts of Pelargonium X domesticum (cv. 'Melissa') has been developed. Protoplasts were isolated from callus tissue 10 to 12 days after subculture on MS medium supplemented with 3.0 mg/l NAA, 2.0 mg/l 6-BAP, and 3.0% sucrose. This callus yielded 2.7 X 10^5 protoplasts/gram of tissue after a 6 hr incubation in an enzyme solution consisting of 2.0% cellulysin, 0.5% macerase, and 0.5 M sucrose. Protoplasts were plated at 1 X 10^5 protoplasts/ml in a mixture (1:1 v/v) of KMP8/KP liquid medium layered on the same medium solidified with 6.0% agarose. Staining with FDA established that 75% of the protoplasts were viable after isolation. Protoplast division was initiated within 2 days, and colonies of 15 to 50 cells developed 8 wk after plating. P-calli 1-2 mm^3 developed 15 wk after plating, and were transferred to MS medium supplemented with 3.0 mg/l NAA, 2.0 mg/l 6-BAP, 3.0% sucrose, and 1.0 g/l casamino acids. Callus tissue was transferred to MS medium supplemented with 0.2 mg/l 6-BAP and 2% sucrose, and shoots regenerated within 30 days. Regenerating calli were subcultured on the same medium. Following elongation, shoots were transferred to one-half

strength MS medium supplemented with 0.1 mg/l NAA, 1.0% sucrose and solidified with 0.9% agar, and roots developed within 3 wk.

INTRODUCTION

The three Pelargonium species most important to the U.S. floriculture industry are P. X hortorum L. H. Bailey (garden geranium), P. X domesticum L. H. Bailey (regal geranium), and P. peltatum (ivy geranium). Pelargonium X domesticum is considered the most beautiful cultivated Pelargonium species; however, problems with cultivation and flowering have prevented it from becoming as popular as P. X hortorum in most parts of the U.S.A. (9). Pelargonium X domesticum requires a long treatment of cool night temperatures to flower, but is resistant to bacterial blight of geranium (4,9). Pelargonium X hortorum does not require a cold treatment to flower, but is susceptible to bacterial blight of geranium (4). Attempts to sexually cross P. X hortorum with P. X domesticum have been unsuccessful (4). Regeneration of plants from protoplasts provides new approaches to traditional plant breeding including the generation of somatic hybrids (11), asymmetric hybrids (7), and cybrids (1). Isolated protoplasts also can be used for direct introduction of DNA or chromosomes by chemical treatment (14), electroporation (13) or microinjection (2). Before these techniques can be utilized for whole plant improvement, protoplast isolation and regeneration procedures for a plant species are required.

Over 45 flowering ornamental plant species have been regenerated from protoplasts; however, the majority of these have been Nicotiana and Petunia species (6). Plant regeneration from protoplasts has been reported for P. X hortorum (10,16), and P. peltatum (16). This report

describes procedures for the regeneration of P. X domesticum from callus-derived protoplasts.

MATERIALS AND METHODS

Callus culture. Leaf explants from regal geranium cv. 'Melissa' were used to initiate green organogenic callus tissue as described by Dunbar and Stephens (3). This callus was transferred (eight to ten 0.5 cm^3 pieces per Petri dish) to a Murashige and Skoog MS (1962) medium supplemented with 3.0 mg/l naphthalene acetic acid (NAA), 2.0 mg/l 6-benzylaminopurine (6-BAP), 3.0% sucrose, solidified with 0.8% agar (Sigma Chemical Co., St. Louis, MO), adjusted to pH 5.8, and poured 20 ml per 100 X 15mm polystyrene Petri dish (C-Medium). After 21 days incubation at 25 C in the dark, white friable callus was selected for subculture. White friable callus was subcultured every 3 wk, and was used for protoplast isolation after the fourth subculture.

Protoplast isolation and culture. White friable callus tissue (7 to 11 grams) was harvested 10 to 12 days after subculture and placed into a 100 X 15 mm polystyrene Petri dish. The tissue was broken into pieces 1 mm^3 with forceps and 15 ml of an enzyme solution consisting of 2.0% cellulysin (Behring Diagnostics, La Jolla, CA), 0.5% macerase (Behring Diagnostics, La Jolla, CA), 0.5 M sucrose, in CPW salts (5) adjusted to pH 6.0 was poured over the tissue. Dishes were incubated for 6 hr at 23 C in the dark on a rotary shaker at 46 rpm. After incubation, protoplasts were released by slowly passing the callus suspended in enzyme solution in and out of a Pasteur pipet two times. Protoplasts were separated from undigested callus tissue by filtration through a 62

um nylon sieve. The protoplasts were centrifuged at 100g for 10 min in 15 X 125 mm test tubes. Protoplasts were collected in 6 ml of W5 solution (11) by layering the W5 solution on top of the enzyme solution and removing the protoplasts from the interface between the two solutions in the W5 solution. An equal volume of W5 solution was added to the protoplast suspension and the protoplasts were centrifuged at 36 g for 10 min. The W5 solution was discarded and the pellet was suspended into 3 ml of filter sterilized KMP8/KP liquid medium (16). The protoplasts were counted on a hemacytometer and diluted to 1 X 10^5 protoplasts/ml. Protoplasts in 4 ml of liquid medium were poured into 60 X 15 mm polystyrene Petri dishes that contained 3 ml of KMP8/KP medium solidified with 0.6% agarose (SeaPlaque, FMC, Rockland, MD). To visualize cell wall material, isolated protoplasts were plated into liquid KMP8/KM medium with 10 mg/l Calcofluor White (CW)(Polysciences, Warrington, PA) (8) and observed immediately after isolation and after 48 hr. The viability of protoplasts was determined immediately after isolation and after 48 hr in culture with fluorescein diacetate (Sigma Chemical Co., St. Louis, MO) (FDA) (15). Cell wall and viability staining were viewed with a Nikon fluorescence microscope equipped with epifluorescence optics (330 to 380 nm exciter, a 420 nm barrier and a 400 nm mirror).

Protoplast cultures were fed every 7 to 10 days by the addition of 0.5 ml of fresh KMP8/KP liquid medium. After 8 wk, the glucose in the feeding medium was reduced to 30 g/l, and after the 12th wk, the glucose was reduced to 15 g/l and the sucrose was raised to 15 g/l. After 14 wk, 1-2 mm^3 protoplast-derived calli were transferred to 100 X

15 mm Petri dishes containing an MS medium supplemented with 3.0 mg/l NAA, 2.0 mg/l 6-BAP, 1.0 g/l casamino acids, 3.0% sucrose, solidified with 0.8% agar (Sigma Chemical Co., St. Louis, MO), adjusted to pH 5.8 (pC-medium). After one month, callus tissue was transferred to a MS medium supplemented with 0.2 mg/l 6-BAP, 2.0% sucrose, solidified with 0.8% agar and adjusted to pH 5.8 (S-medium). Callus with shoot primordia was subcultured onto the same medium for shoots to elongate. Shoots were transferred to culture tubes (25 X 150 mm) containing 15 ml of half strength MS medium supplemented with 0.1 mg/l NAA, 1.0% sucrose, and adjusted to pH 5.8, and solidified with 0.9% agar (R-medium). After 60 days, rooted plantlets were transferred to Bacto Professional Planting Mix (Michigan Peat Co., Houston, Texas) in 8 cm plastic pots and covered with a plastic bag for 1 wk.

Standard error of means was used to evaluate the protoplast isolation and regeneration procedures. Two samples (300-600 protoplast/sample) per isolation were counted with a hemacytometer to determine the averages for protoplasts. The average number of protoplasts isolated per gram of callus was determined from data of seven isolations. The data for viability and cell wall staining represent observations from four isolations. The average plating efficiency represents observations from five isolations. The average number of p-calli surviving the transfer to solid medium represents data from 30 Petri dishes with four p-calli per dish. The average number of p-calli developing shoot primordia represents data from 30 Petri dishes with 10 p-calli per dish.

RESULTS AND DISCUSSION

Callus subcultured on MS-D medium (3) in light, developed numerous shoot primordia on the upper callus surface. When this callus was transferred to medium with additional NAA and sucrose, and incubated in the dark, a white friable callus was produced. This white non-organogenic callus, selected from cultures 10 to 12 days after subculture to C-medium, yielded $2.7 \times 10^5 \pm 6.2 \times 10^4$ protoplasts/gram of callus ($\pm$ standard error = SE) (Figure 3.1a). These protoplasts were buoyant. The sucrose used to provide the osmotic pressure in the enzyme solution caused these protoplasts to float easily when centrifuged. These protoplasts also floated in liquid KMP8/KP medium, therefore W5 solution was used to wash the enzymes from the protoplasts. Immediately after isolation, protoplasts plated in KMP8/KP liquid medium with 10 mg/l CW did not show fluorescence. However, after 48 hr fluorescence was observed on protoplasts. This indicates the isolation procedure was successful at removing the cell walls, and new cell walls were being formed within 48 hr.

Staining with FDA established that 75.5% $\pm$ 3.6 (SE) of recently isolated protoplasts were viable, and 70.0% $\pm$ 3.9 (SE) were viable after 48 hr in culture. First division of protoplasts was observed 3 days after plating (Figure 3.1b). A plating efficiency (% of dividing cells per total protoplasts) of 3.5% $\pm$ 0.5 (SE) was observed 5 wk after isolation. Small colonies of 15-50 cells were observed 8 wk after plating (Figure 3.1c). These colonies formed 12 mm^3 p-calli 7 wk later. These p-calli were transferred onto pC-medium, and within 30 days 93% $\pm$ 2.6 (SE) formed callus tissue (Figure 3.1d). Callus was

Figure 3.1. The development of P. X domesticum cv. 'Melissa' from callus-derived protoplasts to plants (a-f). (a) Callus-derived protoplasts 1 hr after isolation. Bar = 10 um. (b) First division 3 days after isolation. Bar = 5.0 um. (c) Protoplast-derived colony 8 wk after isolation. Bar = 10 um. (d) Protoplast-derived callus 3-4 wk after transfer to pC-medium. (e) Shoot development on callus tissue 30 days after transfer to S-medium. (f) Plantlet 3-4 wk after transfer to R-medium.

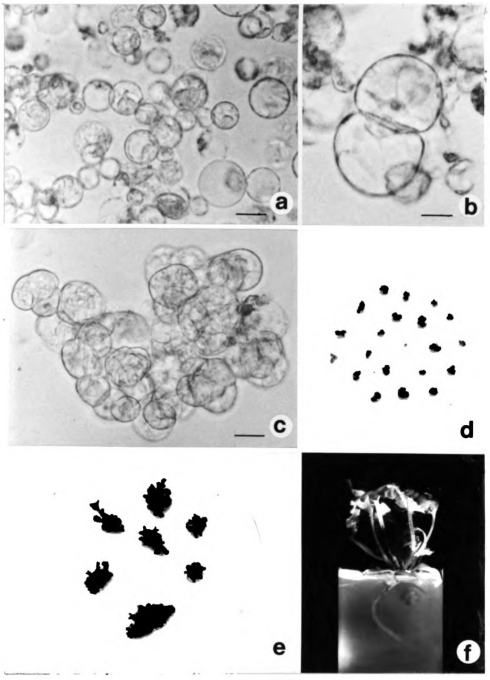

Figure 3.1

transferred to S-medium and within 30 days 63.4% ± 5.3 (SE) had developed shoot primordia (2 mm long shoot tips with leaf primordia). Calli with shoot primordia were subcultured onto the same medium and the shoots elongated (Figure 3.1e). Shoots were transferred to R-medium and roots developed within 3-4 wk (Figure 3.1f). Plants have been successfully transferred and acclimated to greenhouse conditions.

A hybrid between P. X domesticum and P. X hortorum could be used to transfer the bacterial blight resistance of P. X domesticum to P. X hortorum, or the ability to flower without a cold treatment from P. X hortorum to P. X domesticum. Pelargonium X domesticum also has many flower colors not found in P. X hortorum (4). Protoplast fusion technology could be used to make this potentially useful hybrid. Protoplast isolation and regeneration protocols have been reported for P. X hortorum (10,16). With the protoplast isolation protocols for P. X domesticum reported here, regeneration of such a somatic hybrid is feasible.

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APPENDIX A

Cross pollinations between Pelargonium X hortorum and P. cordifolium

Pelargonium X hortorum (cv. 'Orbit Red') and P. cordifolium flowers were emasculated and then pollinated by rubbing the stigma of one species with anthers of the other. Pollinations were done in the greenhouse with natural light from December to February at average temperatures of 21 C day and 14 C night. Over 200 P. cordifolium flowers were pollinated with pollen from P. X hortorum and 100 P. X hortorum flowers were pollinated with pollen from P. cordifolium. Viable hybrid seed was not recovered from these crosses.