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Assessment of somaclonal variation in apple. I. Resistance to the fire blight pathogen, *Erwinia amylovora*

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SUMMARY

After inoculation of glasshouse-grown somaclones regenerated from apple leaf discs (cv. Greensleeves) 33% of 270 showed an increase in resistance to the fire blight pathogen *Erwinia amylovora*. In contrast only 21% produced less severe symptoms than parental material after inoculation of plants held in culture *in vitro*. Sixteen somaclones which showed the highest levels of fire blight resistance were tested intensively as both glasshouse-grown plants and as micropropagated plants in culture. In tests conducted with glasshouse-grown plants up to 60% of plants of the most promising somaclones exhibited minimum symptoms after inoculation compared with 4% of 'Greensleeves' parental plants. The comparable figures for inoculations of *in vitro* cultured plants were 70% and 8%. The growth of *E. amylovora* was reduced in resistant somaclones compared with parental plants. Pretreating leaf discs with virulent *E. amylovora* cells prior to somaclone regeneration had no effect on the frequency of regenerated somaclones exhibiting increased resistance to the pathogen.

VARIATION observed in plants regenerated from tissue cultures, termed somaclonal variation (Larkin and Scowcroft, 1981) is now a well recognized phenomenon of possible use in crop improvement (Evans and Sharp, 1986). Somaclonal variation has been reported to occur for a range of agronomic traits such as yield, protein content, salt tolerance and herbicide and disease resistance (Daub, 1986; Larkin, 1987).

Much effort has been spent on determining the possible causes and origins of somaclonal variation (Larkin and Scowcroft, 1981; Karp and Bright, 1985; Lee and Phillips, 1988). The genotypic and phenotypic variation found indicates that many factors are involved (Karp and Bright, 1985). Somaclonal variation may result from pre-existing genetic variation expressed in regenerated plants or may be induced by the tissue culture process itself (Orton, 1984).

Tissue and cell–culture techniques have potential for tree improvement where long generation times pose constraints on traditional breeding methods (James, 1987). New and desirable traits, not otherwise available, might be introduced to a crop through the exploitation of somaclonal variation. Increased disease resistance resulting from somaclonal variation has been recorded for a number of tree species. Ostry and Skilling (1988), regenerated clones of *Populus* with increased resistance to the foliar pathogen *Septoria musiva,* whilst Hammerschlag (1988), reported increased resistance to *Xanthomonas campestris* pv *pruni* in peach plants regenerated from callus cultures subjected to selection with pathogen culture filtrate. Plants of eastern cottonwood (*Populus deltoides*) with improved resistance to leaf rust have also recently been obtained (Prakash and Thielges, 1989).

This study was initiated to determine whether somaclonal variation could be used to increase levels of fire blight resistance in apple. Fire blight, caused by *Erwinia amylovora* (Burril) Winslow *et al.*, is a serious disease of Rosa-

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ceous species (van der Zwet and Keil, 1979). Most commercial apple cultivars are susceptible to fire blight which can result in serious crop losses (Paulin and Lespinasse, 1987). The use of resistant varieties will probably provide the most effective control of fire blight, in the long term, and somaclonal variation was investigated as a means of increasing fire blight resistance in the susceptible apple cv. Greensleeves.

MATERIALS AND METHODS

Clone production

An axenic shoot culture of the apple cv. Greensleeves was used as the source material of somaclones. Leaf discs (7 mm diameter) were cultured in multiwell dishes (Sterilin) on medium C81 containing 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} BAP (James *et al.*, 1988). Filter sterilized cefotaxime ('Claforan' Roussel Laboratories Ltd) at 200 μg ml^{-1} was added to the medium after autoclaving. Leaf material was cultured in the dark at 25°C. After 6-12 weeks in culture, the regenerated shoots (somaclones) obtained were transferred to shoot proliferation medium A17 (Jones and Hopgood, 1979) and maintained at 25°C. Shoot cultures regenerated in this way were routinely subcultured at 4-6 week intervals.

Single shoots were cut from proliferating cultures and rooted using standard procedures (James and Thurbon, 1979). Three to five weeks after transfer to rooting medium, rooted shoots were transferred to a peat based compost and grown on in propagators. Shoots were weaned from propagators over a 2-3 week period and transferred to controlled environment (CE) cabinets (16 h photoperiod, 250 μE m^{-2} s^{-1}, 80% r.h., 22°C day, 18°C night temperature). Plants were grown on in propagators for 2-3 weeks prior to transfer to a glasshouse. As many plants became woody within seven days of being placed in the glasshouse some modifications were made. To maintain conditions of high humidity favourable for fleshy shoot growth a plastic tent was contructed within the glasshouse. Capillary matting (Lantour), which was kept moist, was placed on the floor and the glasshouse was maintained at ca. 25°C.

Bacterial cultures

Wild type *E. amylovora* strain T was cultured on yeast-peptone agar (YPA) containing 3 g l^{-1} yeast extract, 5 g l^{-1} bacteriological peptone (Oxoid) and 15 g l^{-1} agar (Oxoid) for 48 h at 25°C. Broth cultures were obtained by inoculating yeast peptone broth (YP) (100 ml) with single colonies of *E. amylovora* and incubating overnight at 25°C on an orbital shaker (100 rpm). The bacterial concentration, determined by plate dilution, was adjusted to 10^9 colony forming units (cfu) ml^{-1}.

Leaf pretreatment

Five treatments, A-E, were given to leaf discs (Table I). In most experiments no pretreatment (A) was given. In treatments B-D leaf discs were vacuum infiltrated with 10^9 cfu ml^{-1} *E. amylovora* strain T cells prior to regeneration. This was an attempt to select any potentially variant cells in the leaf disc population possessing pre-existing resistance to the pathogen. The concentration of inoculum chosen was the highest possible that caused no bacterial overgrowth in subsequent leaf disc culture. A short, 2 min (pretreatments B and D), and a prolonged (1 h) infiltration treatment (pretreatment C) were tested. In pretreatment B, leaf discs were vacuum infiltrated with 200 μg ml^{-1} cefotaxime immediately after infiltration with *E. amylovora* whilst in treatment D the antibiotic was omitted. In a control pretreatment (E) leaf discs were subjected to a 2 min infiltration with sterile distilled water only.

All leaf material was washed with sterile distilled water following infiltration and then cultured and grown-on as described above.

Screening for somaclonal variants

Preparation of inoculum: E. amylovora stain T was cultured on slopes of YPA for 48 hours at 25°C. Inoculum was prepared by washing cells from slopes with sterile distilled water and diluting with water to give a final concentration of 10^8 cfu ml^{-1}.

TABLE I

Treatments of 'Greensleeves' leaf discs as duration of vacuum infiltration (min) used prior to somaclone regeneration

Treatment code	10^9 cfu ml^{-1} *E. amylovora* strain T	Sterile distilled H_2O	200 μg ml^{-1} cefotaxime
A	—	—	—
B	2	—	2
C	60	—	—
D	2	—	—
E	—	2	—

In vitro *shoot inoculation:* Three to four weeks after transfer to rooting medium, rooted shoots growing *in vitro* were inoculated by the cut leaf method described by Norelli *et al.* (1988). Scissors dipped in bacterial suspension were used to cut an apical leaf of each plant. Plants were incubated at 25°C under a 16 h photoperiod. Disease symptoms were recorded usually on a scale of 0 to 10, 14 d after inoculation as defined by Donovan (1991). According to this definition plants were classed as showing a 'resistant' reaction (0–5 on the symptom scale) when necrosis was limited to the inoculated leaf and petiole. Plants were considered to show a 'susceptible' reaction (6–10 on the symptom scale) if necrosis spread systemically in the plant through the stem to uninoculated leaves. Micropropagated rooted plants of non-regenerated cv. Greensleeves growing *in vitro* were inoculated as controls.

Glasshouse inoculation: Plants (15-20 cm in height) with vigorously growing shoots were inoculated in the glasshouse by inserting a hyperdermic needle through the stem above the youngest unfolded leaf (Norelli *et al.*, 1984). Sufficient inoculum (10^8 cfu ml^{-1}) was introduced to fill the wound and leave visible drops at both ends. Micropropagated plants of cv. Greensleeves were inoculated as controls.

Plants were examined 14 d after inoculation and those which developed typical fire blight symptoms (apex wilt and browning of the apex and leaves) were classed as 'susceptible' to fire blight (Donovan, 1991).

Identification of somaclones with increased resistance to fire blight: All 270 regenerated somaclones were tested for resistance to fire blight using the *in vitro* and glasshouse tests. Initially one plant of each somaclone was used in each test. Any somaclone which gave a 'resistant' reaction (Donovan, 1991) in either test was re-tested. Between one and five replicate plants of each somaclone re-tested were inoculated in each test and the symptoms recorded.

Sixteen somaclones which showed the highest levels of fire blight resistance in these preliminary tests were subsequently re-tested intensively using 10-15 replicate plants of each somaclone.

Growth of E. amylovora *in inoculated plant material*

In vitro rooted shoots of somaclones and 'Greensleeves' were inoculated with 10^8 cfu ml^{-1} *E. amylovora* strain T as described above. The bacterial populations were determined in eight replicate shoots 0, 3, 7, 10 and 14 d after inoculation. Symptoms at the time of harvest were recorded.

Inoculated shoots from which the roots had been removed were homogenized in 1 ml of sterile 50 mM potassium phosphate buffer (pH 6.5) in a sterile mortar. An additional 9 ml of phosphate buffer was added and the suspension serially diluted. Dilutions of the suspension (0.1 ml) were spread in Petri dishes containing the medium of Miller and Schroth (1972). Dishes were incubated at 25°C for 2 d when the number of colonies per plate was recorded. Regression analysis was used to compare rates of bacterial growth in shoots of somaclones and 'Greensleeves'.

Statistical analysis

The fire blight symptom rating of 10-15 replicate plants of each of the 16 intensively screened somaclones in response to inoculation with 10^8 cfu ml^{-1} *E. amylovora* was recorded. From this, the percentage of plants of each somaclone displaying a resistant reaction in *in vitro* and glasshouse screens was determined. The percentage of resistance reactions recorded for these 16 somaclones was compared with that obtained for control 'Greensleeves' plants using analysis of deviance (McCullagh and Nelder, 1989).

Analysis of deviance was also used to assess the effect of leaf pretreatment prior to somaclone regeneration both on the proportion of somaclones that showed resistance in the initial screening of 270 somaclones and on the proportion of plants that showed resistance in the subsequent intensive screening of 16 somaclones.

RESULTS

Effect of leaf pretreatment on resistance

Various leaf pretreatments referred to as A-E in Table I had no significant effect on the percentage of somaclones which showed more resistance than cv. Greensleeves to fire blight (Table II). Of the 270 somaclones tested for response to fire blight, 58 (21%) showed some increase in resistance *in vitro* compared with parental cv. Greensleeves in that they produced less severe disease symptoms (mean symptom

TABLE II

Percentage of somaclones regenerated from 'Greensleeves' leaf discs which showed less severe symptoms of fire blight compared with parental 'Greensleeves' tissue in in vitro *shoot and glasshouse inoculations*

Leaf* pretreatment	*In vitro*	Glass-house	Total no. of soma-clones tested	No. of plants tested
A:none	23	32	190	904
B:2'vac+2'cefo	17	33	24	111
C:60'vac	16	32	25	119
D:2'vac	23	38	13	69
E:2'vacH_2O	22	39	18	119
Total	21	33	270	1322

*Treatments are as described in Table I. Results are based on average of 5 replicates for each somaclone.

score <6.5, compared with 8.1 for 'Greensleeves'). After inoculation of glasshouse-grown plants, 89 (33%) of the somaclones developed less severe symptoms than 'Greensleeves' with a mean symptom score <1.6, compared with 1.9 for 'Greensleeves'.

In preliminary tests 16 somaclones were selected from the pool of 270 as showing the highest level of resistance (see Materials and Methods). Upon more detailed testing these also showed significantly increased resistance to the pathogen compared with parental 'Greensleeves' after inoculations of *in vitro* and glasshouse-grown plants ($P<0.001$) (Table III). There was variation between somaclones in the proportion of plants which developed systemic symptoms indicative of a susceptible reaction but differences between pre-treatments (Table IV) were not statistically significant. For Greensleeves 8% (6/75) of plants *in vitro* and 4% (3/75) of plants in glasshouse tests developed no or limited symptoms whilst in the most resistant somaclones the comparable figures were up to 70% and 60% (Figure 1). The combined results from the *in vitro* and glasshouse tests indicated that the most resistant somaclones were R20 63, R46 3, R47 11 and R78 3. The growth of *E. amylovora* strain T in inoculated *in vitro* grown plants was assessed in these somaclones.

Growth of E. amylovora *in resistant somaclones and 'Greensleeves'*

Bacterial numbers recovered from inoculated shoots increased linearly (on a logarithmic scale) over the 14 d period (Figure 2). The

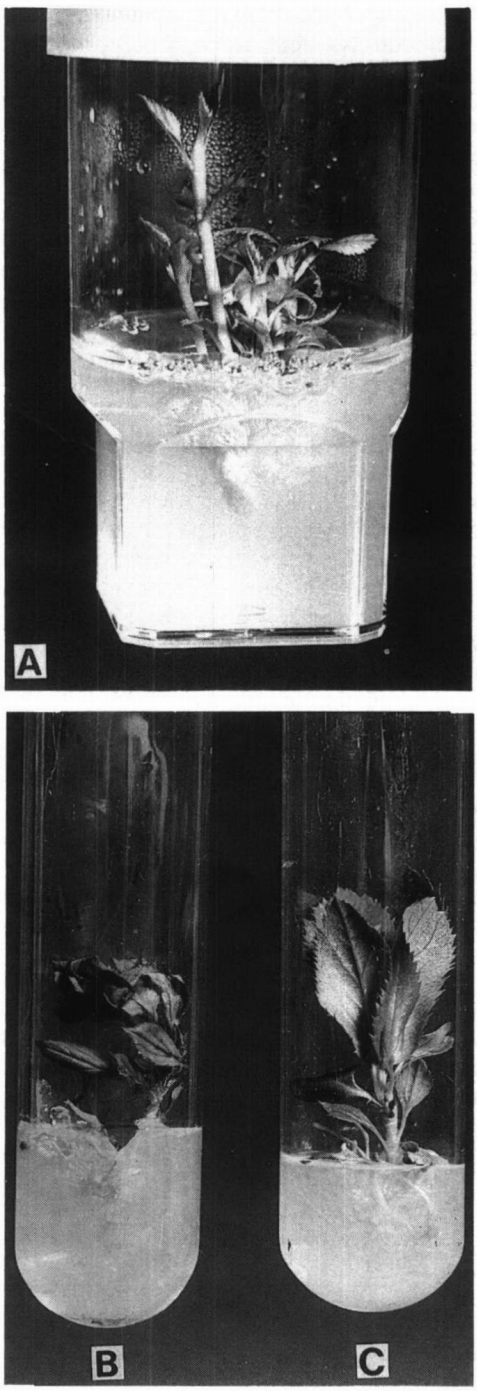

FIG. 1

Shoot culture of somaclone R47 11 on shoot proliferation medium A17 (A); rooted plants of (B) 'Greensleeves' and (C) somaclone R47 11, two weeks after *in vitro* inoculation with 10^8 cfu ml^{-1} *E. amylovora* strain T.

TABLE III
Effect of leaf pretreatment on the percentage of plants showing resistant reactions in response to inoculation in intensive screening of 16 somaclones

	Leaf* pretreatment	
	In vitro	Glasshouse
A:none	53	41
B:2'vac+2'cefo	38	30
C:60'vac	70	40
D:2'vac	70	40
E:2'vacH_2O	40	40

*Treatments are defined in Table I.
Results are based on 10-15 replicates of each somaclone with 10 somaclones regenerated from Treatment A, 2 from each of treatments B and C and 1 from each of treatments D and E.

rate of increase differed for different sources of plant material ($P<0.001$), being greatest for 'Greensleeves' and least for the somaclone R46 3. Bacterial growth in the somaclones R47 11, R20 63 and R78 3 was intermediate between R46 3 and 'Greensleeves' with R47 11 allowing the least and R78 3 the most. These differences in bacterial growth rate were reflected in patterns of symptom severity (Figure 3).

DISCUSSION

Apple somaclones which showed greater resistance to fire blight than parental material were regenerated. The putative resistant clones showed more resistance to fire blight after tests on both glasshouse-grown and *in vitro* grown plants. The basis for this variation was not determined. Variants may be the result of epigenetic or permanent heritable genetic change (Evans *et al.*, 1984). Even a stable epigenetic variant would be of value as apples are normally propagated vegetatively.

A high proportion of the somaclones showed some increase in resistance to fire blight when compared with parental 'Greensleeves' tissue. There are reports in *Populus deltoides* (Prakash and Thielges, 1989), and sugarcane (Heinz *et al.*, 1977) of high frequencies of variation in disease resistance obtained through somaclo-

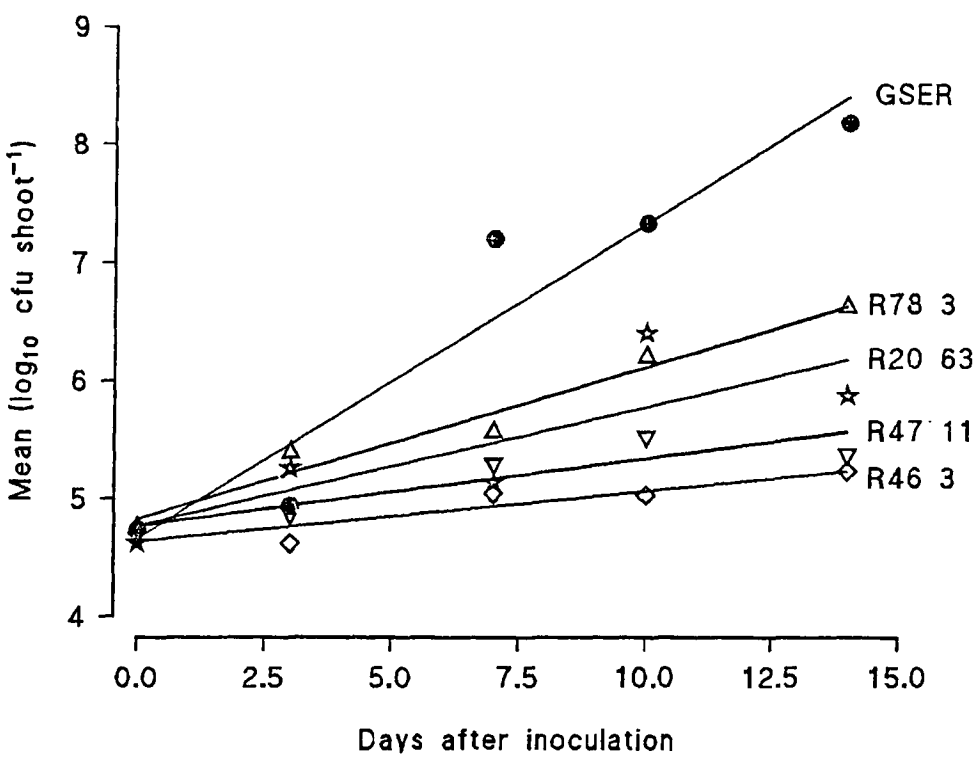

FIG. 2
Populations of *Erwinia amylovora* recovered from *in vitro* grown plants of 'Greensleeves' (GSER) and somaclones R78 3, R20 63, R47 11 and R46 3 following inoculation with 10^8 cfu ml^{-1} *E. amylovora* strain T. Slopes of the fitted regression lines are as follows: 0.27 (GSER), 0.13 (R78 3), 0.10 (R47 11) 0.06 (R46 3), 0.04 (R20 63).

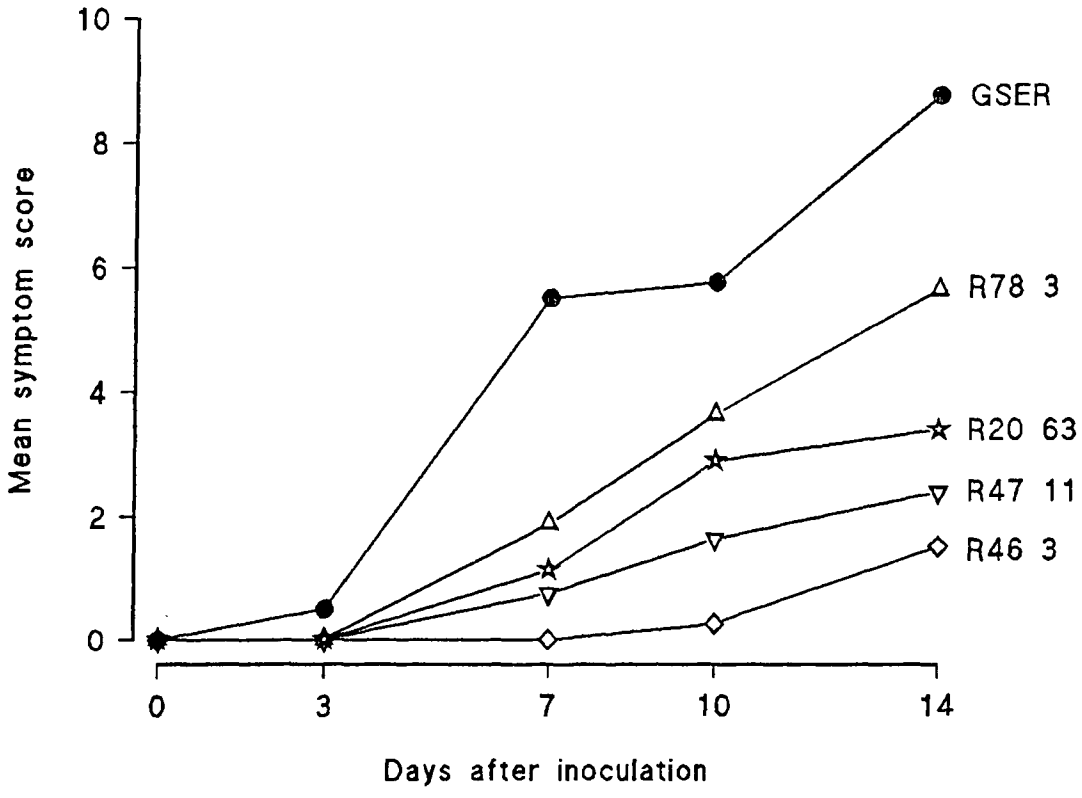

FIG. 3
Development of fire blight symptoms on *in vitro* grown plants of 'Greensleeves' and somaclones following inoculation with 10^8 cfu ml^{-1} *E. amylovora* strain T.

TABLE IV
Percentage of plants of sixteen somaclones showing resistance following inoculation of in vitro *and glasshouse-grown plants*

Clone number	*In vitro*	Glasshouse	Mean
R18 11	64	42	53
R20 10	42	36	39
R20 47	60	30	45
R20 56	58	50	54
R20 61	58	50	54
R20 63	50	60	55
R20 85	56	40	48
R27 7	58	40	49
R27 26	21	20	21
R46 3	70	40	55
R47 11	70	40	55
R76 3	64	30	47
R76 7	10	20	15
R77 7	40	50	45
R78 3	70	40	55
R82 29	40	40	40
GSER	8	4	6

Percentages are based on 10-15 replicates for each somaclone and 75 replicates for 'Greensleeves' control (GSER).

nal variation although many workers (Irvine, 1984; Daub and Jenns, 1989) report frequencies of 1-2% variation for any one character. The frequency of variation generated is affected by plant species, genotype, sourse of explants, culture conditions and the duration of culture employed (Larkin, 1987; Armstrong and Phillips, 1988; Freytag *et al.*, 1989). The nature of the host–pathogen interaction under investigation also affects the frequency of plants showing increased resistance. If resistance to a particular pathogen is dependent on many factors then higher frequencies of somaclones showing altered levels of disease resistance would be anticipated.

Higher frequencies of disease resistance were recorded following glasshouse compared with *in vitro* inoculations in the screening of the 270 somaclones. Initially, conditions within the glasshouse led to plants becoming woody within seven days of being placed there and

hence more resistant to fire blight (Van der Zwet and Keil, 1979). Modifying the glasshouse to ensure conditions of high humidity led to plants remaining green and fleshy, resulting in higher frequencies of susceptible reactions. This reflects the effect of factors which influence the growth of the plant on the subsequent development of fire blight symptoms (Aldwinckle and Beer, 1979). Such factors need to be taken into account in any selection programme.

Replicate plants of any individual somaclone varied in resistance to fire blight following both glasshouse and *in vitro* incoculations. Differences in growth rates between replicate plants could explain the differences in symptom severity observed. This reinforces the need to screen individual somaclones many times and at different stages during development. Plants also need to be grown in the field to determine whether traits such as fruit yield and quality, and resistance to insects and other pathogens have been altered. Further screening for fire blight resistance is also required to determine whether plants retain their increased resistance when infection takes place via blossoms, the usual site of entry by the bacterium (Van der Zwet and Keil, 1979).

Pretreating leaf discs with *E. amylovora* prior to regeneration had no significant effect on the proportion of regenerated somaclones showing increased fire blight resistance. Although infiltrated bacteria remained viable, shown by plate dilutions of infiltrated leaves on selective media, *E. amylovora* failed to multiply within leaf discs placed on regeneration medium C81. Increased resistance to fire blight therefore appeared to be due to inhibition of bacterial growth within the plant rather than by failure to induce disease symptoms. Bacterial numbers in resistant somaclones did not reach threshold levels required for symptom development.

When *E. amylovora* strain T was cultured on plant tissue culture regeneration medium (C81), growth was poor. Previous work (Haberlach *et al.*, 1978, Holliday and Klarman, 1979) showed complex growth media and high cytokinin concentration affected the growth and pathogenicity of pathogens in *in vitro* studies. Whilst there is the possibility that residual cytokinin in regenerated tissues may initially affect pathogen virulence the relative ease with which these compounds are metabolized by plant cells would discount their importance when either *in vitro* rooted plants or greenhouse plants were challenged. The earliest ultrastructural changes in cells of inoculated apple stems take place approximately 48 h after inoculation with *E. amylovora* (Huang and Goodman, 1976). By this time, the growth of *E. amylovora* in infiltrated leaf discs may be restricted, resulting in no effective selection of *E. amylovora* resistant cells for regeneration.

As somaclonal variants possess a common genetic background, they are likely to be near isogenic lines. Such material may be of value in studying fundamental aspects of host–pathogen interaction aimed at understanding mechanisms of resistance (Evans and Sharp, 1986). For diseases such as fire blight, where many factors are thought to be involved in resistance, somaclonal variants may be particularly useful.

From these results several recommendations for any future study of somaclonal variation in apple can be advanced. Pre-treating leaves with *E. amylovora* had no benefits as regards regenerating somaclones with increased fire blight resistance in the cv. Greensleeves. A high frequency of resistant somaclones was obtained using no leaf pre-treatment. Testing somaclones *in vitro* allowed early selection for fire blight resistance thus removing the need to produce large numbers of different somaclones for evaluation in the glasshouse. Parallel application of the testing procedures for *in vitro* and glasshouse-grown plants developed in our laboratory (Donovan, 1991) could be utilized to maximise the efficiency of selection of resistant somaclones.

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