BARD

FINAL REPORT

PROJECT NO. US-991-85

**Somaclonal Variation in Celery and Selection
Toward Resistance to *Fusarium Oxysporum*
F. SP. *APII* and *Septoria Apiicola***

L. Rappaport, E. Pressman, Y. Ben-Yephet

38

630.72

BAR/RAP

2nd copy

1991

35137

000000186674

BARD FINAL REPORT

BARD project No. US-991-85

TITLE:

Somaclonal variation in celery and selection toward resistance to *Fusarium oxysporum* f. sp. apii and *Septoria apiicola*.

INVESTIGATORS:

Principal: Lawrence Rappaport
Cooperating: Yepheth Ben Yepheth, Etan Pressman

PRINCIPAL INSTITUTION:

Department of Vegetable Crops/Plant Growth Laboratory,
University of California, Davis, CA 95616; Volcani
Agricultural Center, Bet Dagan, Israel 50250

PROJECT STARTING DATE: November, 1986

Lawrence Rappaport
Principal Investigator

R.W. Breidenbach
Coordinator

NOTED
TIME
9 1 1986

630.72
BAR/RAP : 634.75 : 632.44 : 631.53

2nd copy

b.

TABLE OF CONTENTS

c.	ABSTRACT	<u>Page No.</u> 4
d.	OBJECTIVES OF THE RESEARCH	4
e.	BODY OF THE REPORT	5
	I. Introduction	5
	II. Methods	7
	1. Tissue Culture	7
	a. Tissue Culture of 'Tall-Utah 5270R'	7
	b. Tissue Culture of Israeli Celery Varieties	7
	2. Pathogens and Inoculation Procedures	7
	a. <i>Fusarium oxysporum</i> f. sp. <i>apii</i>	7
	b. <i>Septoria apiicola</i>	7
	c. <i>Alternaria alternata</i>	8
	3. Disease Evaluation	8
	a. <i>Fusarium oxysporum</i> f. sp. <i>apii</i>	8
	b. <i>Septoria apiicola</i>	9
	c. <i>Alternaria alternata</i>	9
	4. Selection for Resistance	9
	a. <i>Fusarium oxysporum</i> f. sp. <i>apii</i>	9
	b. <i>Septoria apiicola</i>	9
	5. Bioassays	9
	6. Toxin from <i>Alternaria alternata</i>	10
	a. Purification	10
	i. Gel filtration chromatography	10
	ii. Reverse phase chromatography	10
	b. Identification	11
	7. Toxin from <i>Septoria apiicola</i>	11
	Purification	12
	i. Gel Filtration	12
	ii. Reverse Phase HPLC	12
	8. Ammonia Determinations	12

III. Results	13
1. Selection of Germplasm Resistant to <i>Fusarium oxysporum</i> f. sp. <i>apii</i>	13
a. 'UC-T3 Somaclone'	13
b. Genetics of Resistance	13
2. Selection for Resistance to <i>Septoria apiicola</i>	15
a. Media for co-culture	15
b. Production of a Toxic Principle by <i>Septoria apiicola</i>	15
c. Selection of Resistant Mutants	17
i. Co-culture of Cells with <i>Septoria apiicola</i>	17
ii. S ₁ Progeny from Callus Culture	18
iii. S ₁ Progeny from Selfed Plants	19
3. Horticultural Evaluation of Progeny Arrays of T-3 20	
4. A Possible Mode of Action for <i>Septoria apiicola</i> in Symptom Expression	22
5. The Role of Alternatin as a Pathotoxin	22
f. DESCRIPTION OF COOPERATION	23
g. MAIN ACHIEVEMENTS OF THE RESEARCH	23
IV. Conclusions	24
h. LIST OF PUBLICATIONS	25

c. ABSTRACT

Work on selection of regenerated celery plants against the fungus *Fusarium oxysporum* f. sp. *apii* was continued with the support of the BARD grant. First generation (S₁) progeny, second generation (S₂) progeny and backcross progeny of a celery (*Apium graveolens* L. var Dulce) somaclonal variant, UC-T3, obtained from a FOA₂-susceptible cultivar, 'Tall-Utah 527OR' (527OR), were evaluated for resistance to the fungus *Fusarium oxysporum* f. sp. *apii*, race 2. Selections T-2 and R-R₁ were also shown to have resistance. Chi-square analysis of progeny scored for resistance to the fungus showed that the new variation was heritable and appeared to be conditioned by the action of two dominant genes. UC-T3 was released as a germplasm line named 'UC T-3 Somaclone' which was also evaluated for its horticultural characteristics in comparison with the compatible parent, 527OR, and in crosses with 527OR, 527OHK and celeriac, *Apium graveolens* var *rapaceum*, a close relative of celery. There was a weak genotype x location interaction in the field tests. We believe that continued selfing of UC-T3 to generate homozygous individuals and then backcrossing to 527OR would result in celery having the horticultural qualities of 527OR plus enhanced resistance to FOA₂.

Similar approaches were used to select for resistance to *Septoria apiicola*, the causal organism of late blight in celery. Using the cultivar 'Tendercrisp', the Israeli scientists developed appropriate tissue culture techniques and then co-cultured celery cells and the fungus with the aim of selecting somaclonal cell survivors which they could regenerate from the tissue cultures.

In a search for a putative toxin from the fungus *Septoria apiicola* at Davis, the fungus was isolated from the surface of celery leaves. The fungus was cultured in Fries medium for 4 weeks and a nonpolar compound was separated from the stale culture broth which, when concentrated was toxic in several assays. Midway through the purification procedure it was determined that the culture had been contaminated and that the organism under study was not *Septoria apiicola* but *Alternaria alternata*. Since the metabolite seemed to be highly toxic and toxicity was related to concentration of the crude extract, it was decided to continue purification. Separation was achieved by column chromatography (sephadex LH-20, AG 1 x 8) and reverse phase C₁₈ HPLC. The structure of the compound was deduced by UV, IR, EIMS, CIMS, and NMR. The new toxin was given the name Alternatin.

The search for a toxin from *Septoria apiicola* yielded a water soluble compound which showed activity on the corn leaf prick assay. The nature of the compound is unknown. *Septoria apicola*-infected leaf disks were shown to increase the pH of the medium by more than 1 unit. The increase was associated with an increase in NH₄⁺N at a concentration sufficiently high to induce symptoms of the disease.

d. OBJECTIVES OF THE RESEARCH

This proposal had two objectives: 1) to complete research on the selection and characterization of regenerated celery plants resistant to the fungus *Fusarium oxysporum* f. sp. *apii* (FOA₂) in order to obtain disease resistant lines, and 2) to select for resistance to *Septoria apiicola*, the fungus causing late blight of celery, using two approaches: i) selection of

plated cells from suspension cultures against a putative toxin produced by the fungus, and ii) selection of resistant regenerated plants by treating the leaves with fungal spores, recovering and selfing the survivors, and selecting resistant progeny against the fungus.

e. BODY OF THE REPORT

I. Introduction

The most important disease of celery in California is fusarium wilt, sometimes called fusarium yellows, caused by the fungus *Fusarium oxysporum* f. sp. *apii*. In some years up to 30% of the crop has been lost to this disease in the United States. The disease is not known to occur in Israel. At the time this research was begun, there were no effective, economic methods for control of fusarium wilt. The fungus survives in the soil for many years. It can be killed by fumigation, but the method is expensive and has to be repeated. The only reasonable solution was to find genetic resistance. Since conventional breeding methods had not resulted in discovery of resistance, a novel approach was adopted to seek resistant germplasm. The underlying and essential feature of this program is the occurrence of transmissible genetic variation in plants regenerated from tissue and cell suspension cultures. Such somaclonal variation was found at a high level (0.05%) in regenerated plants of 'Tall Utah 5270R' (Rappaport, 1981-84; Heath-Pagliuso et al., 1988; Heath-Pagliuso and Rappaport, 1988; Rappaport et al., 1988; Heath-Pagliuso et al., 1990).

The first approach (i, above) requires the use of a toxin, a cell regeneration system and, finally, a selection system against the fungus. The second approach (ii, above) requires an efficient plant selection system. Our hypothesis was that, as with *Fusarium* resistant mutants, once selected, the progeny would carry the resistance gene(s).

The hypothesis was that the fungus produces a toxin which, when provided to the celery tissue culture, could be used to select for surviving resistant colonies in tissue culture. This method was first demonstrated for plant cells Carlson (1973) who sought resistance to wild fire disease in tobacco. The approach has two technical requirements: discovery and characterization of a toxin and development of a tissue culture system from which plants resistant to *Fusarium oxysporum* f. sp. *apii* (FOA₂) could be selected.

The search for a toxin ultimately led to the isolation and characterization of fusaric acid (Rappaport, 1982), a well known compound produced by many *Fusarium* species, whose chemistry and metabolism were well established. After a number of unsuccessful attempts to select cells and newly regenerated plants for resistance to fusarium wilt in tissue culture (Heath-Pagliuso et al., 1988; Rappaport et al., 1988) and for a variety of other reasons (Rappaport et al., 1988; Heath-Pagliuso et al., 1988), we concluded that fusaric acid is not the primary cause of the disease, although it likely contributes to its expression.

We next decided to select for resistance by growing regenerated plants in soil infested with the fungus. This approach proved successful, (Rappaport and Heath-Pagliuso, 1988; Heath-Pagliuso et al., 1988) and led to the

development of a germplasm line, 'UC Somaclone T-3' (Heath-Pagliuso et al., 1988).

At the time this research was begun, resistance to *Fusarium oxysporum* f. sp. *apii* in celery had not been obtained by conventional breeding methods. Because celery is relatively easy to culture and regenerate in cell culture (Chen, 1976; Williams and Collin, 1976; Al-Abta and Collin, 1978; Dunstan et al., 1982; Rappaport et al., 1988; Nadel et al., 1989) and selection procedures using regenerated plants to obtain resistance to FOA₂ and other diseases seemed promising (Rappaport, 1982; Pullman and Rappaport, 1983; Heath-Pagliuso et al., 1988; Wright and Lacy, 1988), we decided to try selecting for resistance to *Fusarium oxysporum* f. sp. *apii* by growing regenerated plants in soil infested with the fungus. Success with this approach was documented in a variety of reports to the celery growers who supported the early research of Rappaport (1977) and Rappaport and Heath-Pagliuso (1983-88). Wright and Lacy (1988) showed that regenerated plantlets from two celery lines ranged from highly susceptible to highly resistant for three fungal pathogens and one bacterial pathogen.

Cell culture can be useful in increasing genetic diversity in plant populations (Larkin et al., 1981; Scowcroft et al., 1983; Evans and Sharp, 1983). Much of the variation is genetic and heritable (Earle et al., 1978; Evans and Sharp, 1983; Larkin et al., 1984; Orton, 1984; Scowcroft et al., 1983; Zong-xiu et al., 1983), affecting single and polygenic characters (Evans and Sharp, 1983; Evans et al., 1984; Larkin et al., 1984; Shepard, 1983). This variation may result from alterations in such processes as chromosome translocation, inversion, deletions, or gene amplification (Karp and Bright, 1985; Lee and Phillips, 1987, 1988). The longer the culture is maintained, the more mutations are likely to accumulate (Larkin and Scowcroft, 1981). Hence, any regenerant may contain one or more new mutations in its genome, the desired mutation and possibly undesirable mutations. Thus, plants regenerated from cell culture must be examined for extraneous mutations which may affect important horticultural traits.

A celery tissue culture system was developed (Fujii, 1982; Heath-Pagliuso et al., 1988; Rappaport, 1980; Rappaport et al., 1988) and plants were regenerated and grown in the field and greenhouse. Surprisingly, we found a large number of aberrant plants displaying astonishing variation in leaf morphology, petiole height, and color. At the same time, Scowcroft and Larkin (1982) published their research on "somaclonal variation" describing transmissible genetic variation that arose spontaneously and at a high rate in plants regenerated from sugar cane tissue cultures. In our studies, somaclonal variation was found at a high level (0.05%) in regenerated plants of 'Tall Utah 5270R' (Heath-Pagliuso et al., 1988; Rappaport et al., 1988; Heath-Pagliuso et al., 1990).

The successful selection of *Fusarium* resistant lines encouraged the cooperative study of late blight in celery, a very important disease both in the US and Israel. Conceptually, the underlying feature of the research on *S. apiicola* was the same as for *Fusarium oxysporum* f. sp. *apii*: the isolation and characterization of a toxin and the development of cell and plant selection systems to find resistant germplasm. Actually, we first attempted to utilize a cell culture system to select for resistance to *Septoria apiicola* in the UC

Davis laboratory (Rappaport, 1979), but was abandoned because of the greater need for research on fusarium wilt.

The proposed research on *Septoria* resistance in the US lab was aimed at characterizing a toxin from the fungus that could be used in selecting resistant colonies and at regenerating genetically resistant plants. The work in Israel was aimed at developing a cell culture system for regenerating plants, characterizing the fungal pathogen *Septoria apiicola*, in selection of resistant plants against the fungus, and at obtaining seed resistant to the disease from the selfed plants. There were also efforts to find a toxin in fungal cultures.

II. Methods

1. Tissue Culture

a. Tissue Culture of 'Tall-Utah 5270R' and Selected Lines

Methods for tissue and suspension culture of celery cultivars was reported by Rappaport (1977; 1978; 1979), (Rappaport et al., 1987) and Heath Pagliuso et al. (1988). The most recent version of the technique was reported in Heath-Pagliuso et al. (p.447, 1988).

b. Tissue culture of Israeli Celery Cultivars and Lines

Petioles of 'Tendercrisp' were used as a tissue source for callus initiation. Several media, based on Murashige Skoog (modified) medium (1962) were tested and were identified by letter symbols. Cell suspension cultures were prepared from callus and grown in R medium. Multicellular clumps and proembryonic masses were observed in the suspension cultures. Embryogenesis was found to be optimal with E liquid medium and G medium was useful for plant regeneration from calli and cell suspensions.

2. Pathogens and Inoculation Procedures

a. *Fusarium oxysporum* f. sp. *apii*

Barley straw inoculum. Ten ml. of FOA₂ microconidial suspension in Charudattan media (Charudattan and DeVay, 1972) containing 10⁶ spores/ml were used to prepare a stable inoculum following the method of Schneider (1984). Five or 10 g of inoculum were used per 1 kg of soil.

b. *Septoria apiicola*

In the UC laboratory *Septoria apiicola*, obtained from infected leaves of greenhouse-grown celery, was maintained on solid PDA medium. For large scale production, several small colonies of the fungus were homogenized in a glass tissue grinder and several drops of the spore suspension were inoculated into Fernbach flasks which contained 500 ml modified Fries liquid medium (NH₄NO₃, 1.0 g; KH₂PO₄, 1.0 g; MgSO₄; 7H₂O, 0.5 g; CaCl₂, 0.13 g; NaCl, 0.1 g; sucrose, 30 g; yeast extract, 1.0 g; water, 1 liter).

In Israel, attention was directed both to development of a suitable medium and to growth and toxin production by the wild type fungus, obtained

from infected leaves, and a UV-induced hypovirulent mutation, 5A. The wild type, 4A, induced symptoms of late blight 10-14 days after inoculation. This isolate grows well on PD, asparagine, asparagine with 0.1% yeast extract, or asparagine plus 1% PD. The 5A mutant grows well on PD, but very slowly on asparagine medium; the mutant grows as rapidly as the wild type on asparagine in 0.1% potato dextrose agar. When plants were inoculated with pycnidiospores of 5A, disease symptoms appeared 30-40 days after inoculation. Symptoms produced by 5A were mild as compared with those resulting from infection with 4H, the wild type fungus.

For inoculation of celery plants, 3 1 of pycnidiospore suspensions of *Septoria apiicola* were applied to the leaves and the inoculated plants were covered with polyethylene bags and kept at an ambient temperature of $23 \pm 2^\circ$. Surprisingly, disease expression was observed on plants 10 to 40 days after treatment, but not in plants ranging from 45 to 90 days of age. The pathogenicity of 16 isolates of *Septoria apiicola* obtained from two regions in Israel was found to be similar.

c. *Alternaria alternata*

In the UC Davis 1987 annual report, we indicated that a highly toxic fraction was detected in purified extracts of the culture filtrate from an *S. apiicola* culture. As the purification of the toxic compound advanced we checked the cultures only to find that the fungus under study was not *S. apiicola*, but another fungus. It was identified by Dr. Edward Butler, Department of Plant Pathology, UC Davis, as *Alternaria alternata* (Fries) Keissler (*A. tenuis*, Auct. and *A. longpipes*), a saprophytic fungus which also occurs on the leaves of celery, the source of the inoculum. Apparently, as *A. alternata* grows much more rapidly than *S. apiicola*, it overtook the latter and we isolated *A. alternata*, a saprophyte, instead. Therefore, we had in fact isolated a toxin produced by *Alternaria alternata*. Stock cultures of the organism were maintained on medium similar to that for *Septoria apiicola*.

3. Disease Evaluation

a. *Fusarium oxysporum* f. sp. *apii*.

Fusarium wilt infection was scored on the level of infection of the roots (designated mean root scores or MRS). After 10 weeks in FOA₂ infested soil, plants were sliced longitudinally and the roots and crown tissues were evaluated for evidence of the disease. Depending upon the extent of root and crown rot present, the plants were rated on a scale of 0-7 in greenhouse tests and 1-5 in field tests. The numbers indicate a range from no root or crown discoloration to completely decayed and dead. Only plants receiving a 0, 1 or 2 rating were saved for seed production by rinsing the roots in a solution containing Benlate and transplanting them to FOA₂ free soil. Survivors were self pollinated. Plants that received a rating of 2 on a scale of 0-7 rarely survived to maturity; therefore, only 0-1 ratings were considered resistant. Particularly successful lines were also returned to tissue culture for reselection.

b. *Septoria apiicola*

Plants were examined for typical brown, initially circular, ultimately fused, lesions on the leaves. Disease symptoms were apparent after approximately 10 days. In Israel, disease incidence was expressed as the percentage of lesions formed on inoculated plants and the number of lesions on the sprayed plants. Plants of the commercial cultivar 'Tendercrisp' was used as the control and were kept under the same conditions and treated in the same way as the regenerated plants. After they were scored for disease, the infected leaflets were removed and new leaves were permitted to develop for subsequent analysis.

c. *Alternaria alternata*

Spores of *Alternaria alternata* were applied to healthy plants at high density. Under no circumstances did the leaves show any evidence of toxicity.

4. Selection for resistance

a. *Fusarium oxysporum* f. sp. *apii*

The methods employed for selection of regenerated plants against *Fusarium oxysporum* f. sp. *apii* were reported (Heath-Pagliuso et al., 1988; Heath-Pagliuso et al., 1989; Heath-Pagliuso and Rappaport, 1990 and Rappaport et al., 1988) and are summarized in the Introduction.

b. *Septoria apiicola*

In Israel, plants regenerated from callus were treated with the fungal spores and were evaluated for disease expression throughout their life cycle. Plants which showed 0 to 20% disease incidence were inoculated repeatedly, as described above. In each of the four inoculation cycles plants with the lowest susceptibility were reinoculated and disease incidence was scored. These treatments were applied to select S₁ and S₂ generations of plants.

5. Bioassays

In the work at UC Davis, various bioassays were tried for assay of a putative *Septoria apiicola* toxin and for the *Alternaria alternata* toxin: leaf puncture assay, root growth inhibition, electrolyte leakage and bacterial growth inhibition assay. Of these, the leaf puncture assay was the simplest to perform and to consistently detect the presence of toxic compounds; the corn leaf assay proved to be most reproducible and adaptable for testing large numbers of samples.

Corn seedlings were grown singly in vermiculite in paper cups, and watered daily with one-tenth strength commercial liquid fertilizer (14-14-14). For bioassay, the container was placed on its side so that a leaf blade could be extended on a horizontal surface. The leaf was punctured with a hypodermic needle and then a 10 μ l test sample was applied in water to the puncture wound. The sample was allowed to dry and the treated leaf was checked for necrosis after 24 hours. When the sample was in an organic solvent, a 10 μ l drop of water was pipetted onto the puncture wound, followed by a 1 μ l aliquot of column fraction. This effectively diluted the organic solvent in the

samples to less than 10%. Toxin activity was measured by extent of necrosis in the vicinity of the wound. Leaf necrosis developed within two days. The bioassay is only semi-quantitative. Control tests showed that 10% methanol or isopropanol, usual solvents, did not induce necrosis or other symptoms on leaves. Up to 60 assays could be performed on a corn leaf blade. When the column eluant contained 10 mM acetic acid, 10 mM phosphate buffer, pH 7.0, was used as a diluent instead of water.

In Israel, the putative toxin from *Septoria apiicola* was obtained by growing fungal isolates in petri dishes on K1 or K9 Medium and, after four weeks growth, filtering off the medium from the mycellium. The crude fractions were tested on celery leaves using a special leaf infiltration device. The sample is injected into the leaf mesophyll and diffuses from the point of injection. Toxic effects include early development of a yellow halo around the site of injection followed by necrosis.

6. Toxin from *Alternaria alternata*

a. Purification

At UC Davis, purification of a toxin from cultures of *Alternaria alternata* was initiated by filtering the spent culture broth (200 ml) through four layers of cheese cloth and filter paper. The fungal mass was crushed with a glass rod in a small amount of sterile water. More water was added and the inoculum suspension was then distributed to the culture flasks. The inoculum was thus a suspension mixture of spores and mycelial fragments. The cultures were incubated without shaking at ambient light and temperature for four weeks.

Bioassays were performed at each step to ascertain the location of the activity and the level of purification. Droplets of the broth induced small necrotic spots on the leaves of corn and other test plants. The spots normally did not exceed 5 mm in diameter even after the toxin was concentrated.

After four weeks of incubation the mycellium was removed by filtration through filter paper. The culture filtrates were adjusted to pH 6 with NaOH and then concentrated 40-fold in a rotary evaporator. Two volumes of methanol were added and the resulting precipitate was removed by filtration through filter paper. The methanol was removed by evaporation. The sample was concentrated to one-half of the original volume; the precipitation procedure was repeated once more.

The aqueous phase was diluted to 400 ml and then partitioned three times with ethyl acetate. The water phase was next extracted three times with butanol. The butanol was removed from these extracts as well as from the original water phase by evaporation. The ethyl acetate fraction showed very weak activity (see Assay, below) while the butanol phase showed strong biological activity.

i. Gel Filtration Chromatography

The butanol fraction was concentrated and then applied in 30 ml 60% isopropanol to a LH-20 Sephadex column (2.5 x 100 cm). The column was

eluted with 60% isopropanol and 5 ml fractions were collected. Activity, detected by the leaf-prick bioassay in one broad activity peak, was found in fractions containing a dark brown pigment. These fractions were pooled, evaporated to dryness and then redissolved in 50% methanol.

ii. Reverse Phase HPLC

A Rainin HPLC system, equipped with an Alltech Econosil C₁₈ reverse phase semi-preparative column, 10 x 250 mm, was used throughout the project to further separate the active principle. The system was operated isocratically with three different mobile phases: 45% methanol, 60% methanol and 10 mM acetic acid in 50% acetonitrile. The runs were monitored with a Kratos UV detector at 300 nm wavelength. One ml fractions were collected and the major biological activity was detected in fractions 48-50 ml on the corn leaf prick bioassay. The purified compound was a colorless oil, insoluble in water, but soluble in methanol, acetone, isopropanol and acetonitrile.

b. Identification

The structure of the toxic compound was elucidated by UV, IR, EI-MS, CI-MS, ¹H-NMR, ¹³C NMR and 2D NMR techniques such as ¹H (¹H, ¹H-COSY), ¹³C-¹H (¹³C, ¹H-COSY) shift correlations and ¹H-¹H long-range couplings shift, ¹³C-¹H long-range couplings shift (¹³C, ¹H-COLOC). The compound was given the name Alternatin. A manuscript describing the isolation and characterization of Alternatin is in preparation. Table 1 shows mass spectral data for Alternatin.

Table 1. Mass spectral data for Alternatin. CI-MS *m/z* (rel. int): 395 [M+1-28]⁺ (34%) ([M]⁺ 422, C₂₀H₃₀O₆N₄), 393 [M+1-30]⁺ (11%), 383 [M-39]⁺, 238 [M/2+27]⁺ (15%), 212 [M/2+1]⁺ (3%), 198 [M/2+1-14]⁺ (100%), 196 [M/2-15]⁺ (26%), 186 (15%); EI-MS *m/z* (rel int): 198 (31%), 195 (34%), 185 (34%), 183 (66%), 168 (47%), 157 (69%), 154 (76%), 141 (100%), 85 (39%), 69 (25%), 57 (80%), 43 (93%), CI-MS for methylated 1 *m/z* (rel. int): 479 [M+1]⁺ (2%) (C₂₄H₃₈O₆N₄), 451 [M+1-28]⁺ (21%), 254 [M/2 + 15]⁺ (18%), 240 [M/2+1]⁺ (16%), 226 [M/2+1-14]⁺ (100%), 211 (26%). UV $\epsilon_{\max}$ (nm): 280, 238. IR $\max$ cm⁻¹: 3100-3400 (NH and OH), 1550-1700 (CO and CN), 2860, 2920, 2950 (CH).

7. Toxin from *Septoria apiicola*

Although the toxin we had isolated was found to be a metabolite of *Alternaria alternata*, we continued the search for a toxin from *Septoria apiicola*. Lesions containing pycnidiospores were dissected from celery leaves, air dried and stored at 4°. A few flakes of this material were added to 50 ml sterile deionized water in a 250 Erlenmyer flask. After 20 minutes of shaking, a drop of the liquid was checked for filiform spores under the microscope, serial dilutions were made and all the dilutions were plated out on PDA plates. *Septoria apiicola* colonies appeared as pin-heads at 10 days. Single spore isolates were obtained from these colonies. Stock cultures were maintained on PDA. Agar plugs with the fungus were excised from the petri dishes and were added to 500 ml modified Fries medium in Fernbach flasks; the cultures were harvested after one month; the pH of the culture filtrates ranged from 2.5-2.8.

a. Purification

After four weeks of incubation, the mycelial mass were removed by filtration through filter paper. The pH was adjusted to 6.0 with NaOH and the filtrate was concentrated 40-fold in a rotary evaporator. Two volumes of methanol were added and the precipitate was removed by filtration through filter paper, after which the sample was concentrated to one-half the original volume. This procedure was repeated twice more.

i. Gel Filtration Chromatography

The first chromatographic step was elution of the sample, dissolved in 50% isopropanol, through a Sephadex LH-20 column. The 1 ml fractions were tested on the corn leaf prick assay, and those showing toxicity were pooled, evaporated to dryness and redissolved in 50% methanol.

ii. C₁₈ Reverse Phase HPLC

The sample was next subjected to reverse phase HPLC using an Alltech Econosil C₁₈ column eluted successively with 50%, 35%, 20% and 2.5% methanol plus 10 mM acetic acid, and 10mM acetic acid. All of the activity invariably was eluted in the void volume, even when 0.10 mM acetic acid or water was used as the developing solvent.

8. Ammonia determinations

The possibility that the toxicity produced by *Septoria apiicola* and by purified fractions from the fungus was due to the effects of ammonia was measured using the bleach-phenate method. Aliquots of fractions taken directly from the column were applied to leaf disks floating in 5 ml deionized water.

The effect of leaf disk infection with *Septoria apiicola* on ammonia concentration was measured in the culture medium. Celery plants were raised in the greenhouse in 6-in pots until they were 20 cm tall. The plants were infected with *Septoria apiicola* by rubbing the spore suspension on both sides of the leaf. The plants were enclosed in plastic bags and placed in a growth chamber at 18°C and 70% relative humidity. After three days, the plastic bags were removed and the infected areas were excised with a razor blade. Comparable uninfected areas from healthy plants were used for controls. The samples were ground separately in 5 ml deionized water with a mortar and pestle. After the debris was removed by centrifugation, the pH of the supernatant was determined and a 3 ml aliquot of each sample was taken for ammonia determination. Each sample was first decolorized by adding 2 ml activated charcoal suspension (2% w/v). After the mixture was shaken for 15 min, the activated charcoal was pelleted by centrifugation and 4 ml of the supernatant was taken for ammonia determination.

III. RESULTS

1. Selection of Germplasm Resistant to *Fusarium oxysporum* f. sp. *apii*

Much of the research on methods of selection for resistance to *Fusarium oxysporum* f. sp. *apii* was accomplished before the BARD grant was awarded. However, with the aid of the grant, we have been able to test the progeny, study the genetic basis for resistance, evaluate the resistant lines horticulturally, and cross this germplasm line with standard varieties to test heritability of the resistance while improving horticultural characteristics.

Selection of regenerated '5270R' celery plants against the fungus has been successful in providing new resistance to the fungus. Two lines, T-3 and R-R₁, selected from about 1030 plants, survived exposure to the fungus and the selfed progeny maintained stable resistance over two seed generations (Heath-Pagliuso et al. 1988). T-3, derived from a recultured line which showed some resistance, had superior resistance and was the line of greatest interest for investigation. T-3 has proved superior in resistance to most other cultivars tested in some 20 field tests in five locations in California (Heath-Pagliuso et al., 1988; (Heath-Pagliuso and Rappaport, 1990), Orton (private communication) and Michigan (Lacy, private communication).

a. 'UC-T3 Somaclone'

Seeds of a line named 'UC-T3 Somaclone' were made available to celery breeders since August, 1988 and it was also published as a germplasm release (Heath-Pagliuso et al., 1989).

b. Genetics of Resistance

Previous results from this laboratory showed that the S₁ progeny obtained by selfing a somaclonal variant, 'UC-T3 Somaclone' (Heath-Pagliuso et al., 1989), had increased resistance to *Fusarium oxysporum* f. sp. *apii*, race 2 (FOA2) (Heath-Pagliuso et al., 1988). In order to determine the stability and the nature of the resistance, additional studies were carried out with the S₁ and S₂ progeny. In addition, the disease resistance of progeny resulting from crosses of UC-T3 S₁ individuals to 'Tall-Utah 5270R' (wild-type) and the tolerant variety 'Tall-Utah 5270HK' were investigated. The possible genotype of 'UC-T3 Somaclone' and the value of this new germplasm in a breeding program are also discussed. The salient observations made in this research, as reported by Heath-Pagliuso et al. (1988), were:

* The S₁ progeny, obtained by self-pollinating UC-T3, best fit a two locus model with two dominant genes. Similar results were reported for celeriac, a close relative of celery, which indicated that resistance was governed by a single dominant gene with modifiers (Orton et al., 1984). A more recent study on the F₂ progeny of crosses between highly resistant celeriac (112) and the moderately tolerant 5270HK, and the highly susceptible 5270R showed that at least two loci are involved (Quiros, 1987).

* In some 16 tests in the greenhouse and field with soil infested with the fungus, all progeny of 'UC-T3 Somaclone' were superior to the parent, 5270R and showed as good or superior resistance than 5270HK, a tolerant variety.

* There was a weak genotype x location interaction in the field tests.

* In tests in non-infested fields the progeny of backcrosses generally exhibit the most horticulturally acceptable morphological characteristics (Heath-Pagliuso and Rappaport, submitted; Rappaport and Heath-Pagliuso, 1987). In our opinion that continued selfing of UC-T3 to generate homozygous individuals and then backcrossing to 527OR would result in celery having the qualities of 527OR plus enhanced resistance to FOA₂.

In a more recent study, first generation (S₁) progeny, second generation progeny (S₂) and backcross (BC) progeny of UC T-3 were investigated to determine the level of resistance to *Fusarium oxysporum* f. sp. *apii* (Heath-Pagliuso and Rappaport, 1990). Confirmation that resistance was not due to a single gene was obtained using chi-square analysis. All families proved to be superior to 527OR, indicating that the disease resistance obtained by plant selection against the fungus was heritable.

The salient features of the paper by (Heath-Pagliuso and Rappaport, 1990) are as follows:

* Tests with the selfed (S₁) progeny in the greenhouse and with 12 families indicated that resistance to FOA₂ is attributable to at least two genes.

* In tests of backcrosses of UC-T3 S₁ individuals to 527OR a wide range of mean root scores (MRS) were obtained, depending upon the parents used in the crosses. However, the overall MRS of the progeny of all crosses were significantly lower (the plants were healthier) than those for 527OR and, comparable to, or in one case significantly lower than, the tolerant cultivar, 527OHK.

* Even though the progeny of UC-T3 appeared to be segregating for two loci, the average resistance among the S₁ families or BC₁ progeny arrays was always significantly greater than that found in 527OR.

* Excellent levels of resistance were obtained for many of the S₁ families (S₂ progeny) and among the progeny of crosses between UC-T3 S₁ individuals and the moderately tolerant variety, 527OHK.

* It was concluded that somaclonal variation for FOA₂ resistance is heritable and the new germplasm could be incorporated into a breeding line. The progeny of UC-T3 have been well characterized for disease resistance (Heath-Pagliuso et al, 1988; Rappaport and Heath-Pagliuso, 1986 and 1987) and for horticultural traits (Rappaport and Heath-Pagliuso, 1987). In support of a proposal in the earlier publication (Heath-Pagliuso et al, 1988) the data support the view that 'UC-T3 Somaclone' (Heath-Pagliuso et al., 1989) is a valuable source of FOA₂-resistant germplasm.

2. Selection for Resistance to *Septoria apiicola*:

In Israel, culture of the native *Septoria apiicola* was studied i) to better understand its life cycle, ii) to develop a medium and suitable conditions for co-culture with celery cells as a means of selecting for resistance, and iii) to develop suitable conditions for production of a putative toxin.

a. Media for Co-culture

To test the hypothesis that co-culture is a useful way to select for resistance, a circle of celery cells about 2.5 cm wide around, was pipetted around the periphery of 9 cm petri dishes. *Septoria apiicola* pycnidia were inoculated in the center of the plate, not touching the fungus colony. However, efforts to co-culture *S. apiicola* and celery cells in suspension culture were thwarted initially by alterations in pH of the medium attributable either to the plant cells or the different fungal strains. Table 2 shows the effect of pH on survival of celery cells in co-culture.

Table 2. The effect of pH on survival of celery cells co-cultured with *Septoria apiicola*.

pH	Cell Response
3.0-4.0	most cells died
5.0-6.0	vigorous growth
7.0	weak growth
8.0	most cells died

To gain further information about this phenomenon, celery cells were added to K1 liquid medium after it was adjusted to different pH levels with 1N HCl. In media with an initial low pH of 3 or with a high pH of 7 or 8.0, some 50% of the cells died. Interestingly, the presence of celery cells served to buffer the medium. Thus cultures whose initial pH was adjusted in a range above 3 and below 7.0 were maintained at pH 6.0 ± 0.3 pH units and at the intermediate pH levels, the cells grew vigorously. Therefore, in subsequent studies, the pH of the medium was adjusted to maintain rapid growth and with minimum fluctuation.

b. Production of a Toxic Principle by *Septoria apiicola*

Evidence that *S. apiicola* produces toxic compounds(s) was provided in an experiment in which the two fungal isolates, 4H and 5A, were grown in K1 and 9 liquid media (Table 3) for 4 weeks. The mycelia were filtered off and aliquots of the filtrates were injected into the celery leaves after the pH was adjusted to 3.8. Table 3 shows that the filtrate from both isolates had toxic effects.

Table 3. Induction of chlorotic symptoms on celery leaves by culture filtrates of two *Septoria apiicola* isolates (4H and 5A) grown on two media (K1 and 9). The culture filtrates and controls were adjusted to pH 3.7-3.9 and then infiltrated into celery leaves.

Medium pH				
Medium	Isolate	Collected	Infiltrated	Symptoms
K1	4H	3.9	3.9	Chlorosis
K1	5A	3.9	3.9	Chlorosis
K1	--	5.7	3.9	No chlorosis
9	4H	3.7	3.7	Chlorosis
9	5A	3.8	3.8	Chlorosis
9	--	5.0	3.7	No chlorosis

Clearly, the filtrate from both isolates had toxic effects. Culture filtrates of wild type and mutant cultures which had been grown on asparagine or potato dextrose media were extracted three times with butanol, the butanol was evaporated and, without further purification, the extract was dissolved in water in a dilution series ranging from 1 ml filtrate to 30 to 50 ml of water. The fractions were assayed by the leaf infiltration method. Ten days later, intense chlorotic symptoms were observed on leaves injected with extract from the wild type isolate, whereas symptoms were variable and slight in leaflets injected with the extract prepared from the mutant. Table 4 shows the effect of butanol extracts of the culture filtrates of the two isolates that had been grown on either potato dextrose medium or asparagine with 0.1% potato dextrose medium.

Table 4. Chlorosis induced in celery leaflets by infiltrated butanol extracts of culture filtrates from wild type (4H) and mutant (5A) isolates of *Septoria apiicola*. The isolates were grown on PD or asparagine media (As). The data are the number of plants exhibiting symptoms out of the total number injected in 7 different experiments.

Chlorotic Symptoms									
Exp. No.	1	2	3	4	5	6	7		
Medium	PD	As PD	As PD	As PD	As	As	PD	PD	
Isolate									
4H	6/6	6/6 6/6	12/12 10/12	12/12	16/16	27/27	27/27		
5A	0/6	4/6 5/6	3/12 2/12	8/12	0/16	6/27	2/27		
Control	0/6	3/6 1/6	0/12 0/12	0/12	0/16	0/27	2/27		

Several plates of callus from plant 11b were co-cultured on solid medium with the two isolates of the fungus, 4H and 5A. The isolates were inoculated at the center of the plates to which celery cells had previously been pipetted in a ring around the fungal colony. Control plates were not inoculated with the fungal isolates. Six plants survived without evidence of infection, despite the heavy selection pressure. Plants 11b and 11c were particularly resistant and were induced to flower and selfed. The results of selection of plants 11B and wild type against isolates 4H and 5A are presented in Table 6.

Table 6. Co-culture of cells on solidified agar medium of plant 11b with isolates 4H and 5A. Isolates were placed in the center of the dish which had been plated with celery cell suspension.

Source Plant	Isolate	No. of Plates	Plates with Survivors
11B	4H	7	1
11B	5A	4	2
11B	-	5	5
Control (wild type)	4H	6	0
Control (wild type)	5A	5	0
Control	no fungus	5	5

ii. S₁ Progeny from Callus Culture

One hundred forty plants were regenerated from callus obtained from plants E, 11a, 11c and 'Tendercrisp'. These were inoculated and disease expression evaluated. Plants showing less than 20% disease compatibility were again inoculated in four cycles. Plants derived from plant E showed susceptibility equal to that of the wild type, whereas those from plants 11a and 11c were less susceptible. However, not all 11a and 11c plants which exhibited symptoms after the first inoculation behaved in the same way after the second inoculation (Table 7).

iii. S₁ Progeny from selfed plants

Plants 11b, 11c, 4, 8a and 'Tendercrisp' were induced to flower and seed was collected. To determine whether there was transmissible resistance, plants from this seed were inoculated in the usual manner. After 21 days all the wild type plants showed a very high level of infection (Table 8).

Of the selections, there was a broad distribution of disease incidence. Only a few plants in the 11b and 11c populations showed a relatively low level of infection, between 0 and 20%.

In a further test with the more tolerant S₁ plants, one 8a, three wild type, three 11b, and four 11c plants were retested. Leaves were inoculated with 300 or 3000 spores per inoculation

Table 7. Susceptibility of plants regenerated from callus from wild type and plants E, 11a and 11c.

Inoculation No:		Percent distribution of plants in each disease category ²															
Source of plants	No. of plants tested	1				2				3				4			
		Disease category				Disease category				Disease category				Disease category			
		0-20	21-40	41-60	61-80	81-100	0-20	21-40	41-60	61-80	81-100	0-20	21-40	41-60	61-80	81-100	0-20
E	24	50	29	15	-	0.5	22	23	9	9	18	41	5	40	60	2	50
Wild type	7	57	-	14	14	14	5	40	60				4	75	25	2	100
11a	64	58	27	9	6	-	19	26	32	21	5	16	4	25	-	1	100
Wild type	15	33	40	20	0.7	-	10	30	30	10	10	20	7	14	28	2	100
11c	48	60	21	13	2	-	19	47	21	16	5	10	8	62		4	25
Wild type	7	43	29	14	-	-	7	14	14	28	28	28	3	67		4	100

²Plants were inoculated with microdroplets with 300 and 3000 viable spores per microdroplet and 30 microdroplets from each were placed on each plant. Disease incidence was recorded 21 days after inoculation.

Table 8 . Number of second generation celery plants in each disease category regenerating from wild type and plants 4, 8a, 11b and 11c.

Source of plants	disease category				
	0-20	21-40	41-60	61-80	81-100
4		1	2	7	8
8a		3	1	9	5
11b	1	1	1	7	9
11c	1	2	1	8	6
Wild type				2	8

Each plant was inoculated with 60 microdroplets, 30 with 300 viable spores and 30 with 3000 spore per each droplet. Disease incidence was recorded 21 days after inoculation.

Table 9. Susceptibility of second generation celery plants to Septoria apicola

Disease incidence (%) ^y													Number of lesions/leaf ^z					
Inoculation	1	2	3	4	5	6							1	2	3			
Source of Plants	Spores number per inoculation sites											Average						
	300	3000	300	3000	300	3000	300	3000	300	3000	300	3000	300	3000	Average			
Wild type ₁	76	96	67	83	98	98	47	43	47	100	-	73	67	82	65	117	65	82
Wild type ₂	100	92	13	10	64	80	51	20	33	87	-	27	52	53	58	76	16	50
Wild type ₃	52	92	17	40	73	96	52	51	-	-	-	-	49	69	60	-	28	44
11b ₄	28	68	50	47	67	90	-	-	-	-	-	75	48	68	4	99	52	52
11b ₅	8	24	47	40	64	76	-	-	-	-	-	-	40	47	-	-	-	-
11b ₆	24	100	43	60	69	78	-	-	-	-	-	-	45	79	-	-	-	-
11c ₁	28	76	73	60	-	-	18	16	43	60	-	37	41	50	32	66	33	44
11c ₂	28	40	40	57	58	76	24	7	73	100	-	72	45	59	34	106	25	55
11c ₃	0	32	53	47	53	73	57	31	70	80	-	55	47	53	27	80	29	45
11c ₄	22	44	30	17	44	53	64	22	33	-	-	35	39	34	31	65	30	42
8a ₆	32	96	-	-	-	-	7	2	-	60	-	83	18	60	36	149	-	93

^YEach plant was inoculated with 60 microdroplets, 30 with 300 and 30 with 3000 viable spores per each droplet.

^ZEach plant with 5 leaf₁ was sprayed with the 15 ml of spore suspension, 10⁵ spores/ml.

site, up to six inoculation sites per plant, and disease incidence (%) was evaluated. The number of lesions per leaf was obtained by spraying each plant with 15 ml of spore suspension, 10^5 spores per ml. (Table 9).

Both Tendercrisp, the wild type plants and selections responded variably to the inoculation and spray treatments. Tendercrisp was highly susceptible and selections 11c, especially progeny of plant 11c₄ showed significant resistance. The other selections also showed more tolerance than wild type but were more susceptible than plants of 11c. It should be noted that the amount of inoculation used in these studies far exceeds that which would occur in the field. Nor would the environmental conditions in the field likely be as favorable as those in the humidified greenhouse.

While the process of selfing and selection should be repeated with plant 11c₄, the results are highly indicative that plants of this line have significantly greater resistance than the parent line.

3. Horticultural Evaluation of Progeny Arrays of UC-T3.

An evaluation of several horticultural traits was conducted on the S₁ and S₂ progeny of 'UC-T3 Somaclone' in order to assess the potential of UC-T3 progeny for use in a breeding program. UC-T3 was self-pollinated to produce S₁ progeny. Selections from the S₁ population with resistance to FOA₂ were self-pollinated to produce S₂ families or were backcrossed to 527OR. All horticultural tests were conducted in apparently fusarium wilt-free fields with celery harvested near market maturity. Controls were plants of the susceptible cultivar 'Tall-Utah 527OR'. Other lines and cultivars were included in all field tests which were run in commercial fields.

The following characteristics were assessed: (1) Number of side shoots - direct count of extraneous shoots per plant; (2) Petiole length - an average obtained by measurement from the base of the crown to the blade junction of four or five of the largest petioles; (3) Plant height - direct measurement of the distance from the base of the crown to the tip of the longest leaf; (4) Conformation - a visual estimate of uniformity and compactness of the petioles on the stalk. Rating scale ranged from 1 (bowed petioles) to 9 (petioles straight and compact); (5) Color - a visual comparison with 527OR as a standard, using a scale of 1 (white) to 9 (dark green); (6) Petiole smoothness - a visual estimate of the smoothness of the convex surface of the petiole. The scale ranged from 1 (deeply ribbed) to 9 (no ribbing); (7) Weight - the weight of 5 to 10 plants severed at the base of the crown at near market maturity and trimmed to a height of 36 cm, the standard commercial height, after the side shoots and roots were removed; (8) Pithiness - rated on a scale of 1 (all plants in a family with severe pith) to 9 (no pith in any plant) on petioles remaining after the top of the plant was cut off 3 inches above the crown; (9) Susceptibility to blackheart of leafminer - a visual estimate of the extent of damage. The scale ranged from 1 (all plants of a family with severe blackheart or leafminer) to 9 (no disease present on any of the plants); (10) Bolting - number of plants in a family with visible seed stalks; (11) Taste - sensory ranking of petioles from 1-5, where: 1 = strong bitter flavor, tough; 2 = mild bitter flavor, tough; 3 = bland flavor, tough; 4 = sweet mild flavor, tender; 5 = nutty flavor, tender.

UC-T3 progeny produced significantly more petioles than 527OR in two fields and exhibited less pithiness in the Watsonville trial. In two of the three trials, S₁ progeny of UC-T3 had significantly shorter petioles than those of 527OR (Table 1). If reduced plant height was a residual effect of tissue culture, the progeny of the next generation should have been uniform in height. The heights observed among the S₂ family means were broadly distributed and generally shorter than 527OR (Table 2), indicating that shorter petiole length is probably not a residual effect of cell culture, but is a heritable characteristic. Because petiole length is important for marketability, it is noteworthy that the means for petiole length of some families equaled those of 527OR (Table 2).

In Watsonville, the members of one family (T3-575) measured 14 cm and were dwarfed by plants of normal height (Table 2). This family had not been included in the two Oxnard trials so it was evaluated at two other locations, Oxnard and Chular. In both locations progeny of T3-575 were approximately one half the size of 527OR (12.2cm vs 22.5 cm and 18.6cm vs 30.4 cm).

Table 3 shows that height was restored in the backcross progeny. Even progeny of the cross involving the dwarf, T3-575, were similar in height to the progenitor line (Table 3).

In addition to variation in petiole length among S₂ families, there were significant differences in the number of side shoots, conformation, petiole smoothness and weight, but not at all locations (Table 2). With the exception of number of side shoots, the overall mean of the 527OR individuals tested fell within the range of family means. Interestingly, there were large differences in weight among S₂ families. Resistance to leafminer and blackheart were assessed in the Watsonville test where variability for resistance to leafminer (range 6-9) and to blackheart (7-9) was detected (Table 2).

Taste was evaluated in this field and was variable, the scores ranging from 2-5 (Table 2). Among the S₂ families flavors ranged from bitter to sweet or nutty. Phenotypic correlations were calculated between taste and other traits from the Watsonville data. The strongest correlations were seen for taste and height ($r=0.729$). Strong correlations between resistance to *Septoria apiicola* L. and flavor-producing compounds in celery have been reported (Donovan et al., 1986). Therefore, the significant negative correlations between taste and resistance to leafminer ($r=0.584$) and blackheart ($r=-0.560$) were of interest. It would appear that in a breeding program aimed at increasing pathogen resistance, it would be of additional value to monitor components of taste.

The S₂ progeny were uniform and virtually identical to 527OR for bolting, pithiness, and color (Table 2).

In conclusion, the S₁ progeny of the somaclonal variant UC-T3 closely resembled the phenotype of 527OR, with the exception of petiole length. The origin of the variability observed among the S₂ families for height, conformation, number of side shoots and resistance to leafminer and blackheart is unknown, but is very likely related to inbreeding depression. A definitive statement about the origin of this variability can not be made without comparing selfed families of 527OR. Nevertheless, it appears that 'UC-T3

Somaclone', the fusarium resistant germplasm developed from UC-T3 (Heath-Pagliuso et al., 1989), could be very useful in celery breeding programs.

4. A Possible Mode of Action of *Septoria apiicola* in Symptom Expression

The unusual solubility of the toxic principle in water suggested that it was a small molecule--a peptide, amino acid, sugar or soluble ion. The culture medium was found to contain a high concentration of NH_4^+N , sufficient to cause necrotic symptoms similar to those we observed in the corn leaf prick assay. The relationship between toxicity and ammonia concentration are shown in Table 10.

Table 10. Correlation of toxin activity (corn leaf prick bioassay) and ammonia concentration (NH_4^+N , g/ml) in C18 reverse phase HPLC fractions eluted with acetic acid. Relative toxicity was determined by visual observation.

	Tube Number						
	<u>14</u>	<u>16</u>	<u>18</u>	<u>20</u>	<u>22</u>	<u>24</u>	<u>26</u>
Toxicity	0	++	+++	++	++	+	0
NH_4^+N (g/ml)	0	114	182	137	54	10	0

Therefore, we sought evidence for *in planta* production of NH_4^+ in infected celery leaves. The necrotic spots were excised and ground in water and the pH and NH_4^+N concentration were determined. The uninfected area was processed separately in the same way as a control. The results showed a high correlation between pH and NH_4^+ concentration in the healthy and diseased disks. Within a range, the uninfected leaf disks had a pH of 5.97 and an NH_4^+N concentration of 30 g/g fresh weight, whereas the necrotic spots had a pH of 7.5 and an NH_4^+N concentration of 110 g/g. Thus there was a good correlation between symptomology and concentration of NH_4^+N .

There is a body of literature which suggests that ammonia can be a causal agent in disease expression (Lovrekovich et al, 1970; Bashan, 1980). In both these studies, increased ammonia level and toxicity were correlated. The authors of both papers pointed out significant complexities in the interpretation of their data but expressed confidence that ammonia was causally related. This interesting observation requires further investigation, especially in view of the results of the *Septoria apiicola* experiments in Israel in which co-culture resulted in significant changes in pH of the medium.

5. The Role of Alternatin as a Pathotoxin

Alternaria alternata (Fries) Keissler (Simmons, 1967) is a diversified group of molds which comprises specific plant pathogens and ubiquitous, saprophytic members commonly found on decaying plant material. The saprophytes produce a variety of mycotoxins (Harvan and Pero, 1976; Luke and

Biggs, 1976; Stack et al., 1986; Stinson, 1985; Stack and Mazzola, 1989). At least seven pathovars are known, each of which produces a host selective toxin (Scheffer, 1983; Nishimura and Kohmoto, 1983). A non-host selective toxin (tentoxin) (Meyer et al., 1975) is also produced by a member of this species of fungus which causes chlorosis in seedlings.

The structure of Alternatin bears strong resemblance to the barbiturate amytal (Florey, 1973) and the known *Alternaria* metabolite tenuazoic acid (Stickings, 1959; Harvan et al., 1976). The latter was identified from *Alternaria alternata* (Fr.) Keissler which causes leaf blight of *Datura innoxia* Mill. It was identified by ultraviolet and infrared analyses.

At this time it is not possible to state whether Alternatin is involved directly in disease expression. The fungus was dusted liberally on celery leaves without any evidence of infection. Like other *Alternaria* it is a saprophyte and probably enters only through a wound or as a secondary infection. Until the necessary physiological and biochemical studies are performed (Gilchrist, 1983), we are inclined to consider Alternatin as a phytotoxin.

f. DESCRIPTION OF COOPERATION

The research on selection and genetic analysis of resistance to *Fusarium oxysporum* f. sp. *apii* was carried out at Davis by S. Heath-Pagliuso. The study of *Septoria apiicola* was a joint effort of the UC and Israeli workers. The discovery and characterization of Alternatin was by two postdoctorals, Oliver Kwok and Nianbai Fang, at UC Davis. Studies of pH changes, NH_4^+N production and toxicity induced by *Septoria apiicola* were performed by O.C.H. Kwok in the UC laboratory. Production of a hypovirulent strain and characterization of *Septoria apiicola* in Israel was performed by Yepheth Ben-Yepheth. Development of a cell culture system in Israel was the responsibility of Etan Pressman and he, together with Ben-Yepheth, developed the tissue culture screening system to select Israeli celery cultivars for resistance to *Septoria*.

Cooperation in the first year consisted mainly of exchange of ideas and methodologies. However, the toxin research was enhanced by the visit of Ben Yepheth to Davis. The US research on selection focused mainly on *Fusarium*.

In Israel, the research being performed by Dr. Ben Yepheth on the characterization of the fungus will enable us to effectively pursue selection of resistant plants. Dr. Pressman's research in developing a cell selection system will make it possible to seek celery lines resistant to *Septoria* both by the use of a putative selective toxin and by selection of regenerated plants.

g. MAIN ACHIEVEMENTS OF THE RESEARCH

The results with *Fusarium oxysporum* f. sp. *apii* are a milestone because, although selection of germplasm resistant to the disease was accomplished previously, this is the first example of a somaclonal variant that has been reported to show stable resistance over two generations. Moreover, the resistance is transmissible in crosses and backcrosses with celery cultivars.

RECEIVED
MAY 1986

Resistance has been characterized and is believed to result from the action of two co-dominant genes. We also have obtained significant horticultural data which indicate that the line will have commercial value with relatively little improvement. In sum, one aim of the proposed research has been achieved successfully.

The positive results obtained in Israel with *Septoria* are strongly indicative that resistance can be found using somaclonal variation obtained in tissue culture as a source of germplasm. More generally, it appears that this technique should have value for selection in other systems.

The isolation of Alternatin is a significant discovery. It is a new toxin from *Alternaria alternata* which has powerful toxic effects on plants. It will be of interest to investigate further its mode of action and to determine its role in infection.

IV. CONCLUSIONS

We believe that our results have substantiate the hypothesis that useful germplasm can be obtained using somaclonal variation as a source of new germplasm. It is not "trendy" in the sense that transformation biotechnology is trendy. Nevertheless, no virulence genes have been transformed into host plants. The appropriate comparison, therefore, is with conventional breeding. The effort with *Fusarium oxysporum* f. sp. *apii* paralleled the conventional celery breeding programs in the Vegetable Crops department which involved crossing of celery to resistant celeriac and backcrossing to obtain horticultural lines with the proper morphology. This has been successful but has taken a long time, about 10 years. The tissue culture work, which began at about the same time, required development of appropriate techniques and approaches to find the best way to select for resistance. This took much labor and much time. Nevertheless, resistant germplasm was reported about 5 years after the beginning of the research. The results obtained with *Septoria*, which appear to be very promising, required about 3 years. It will require another 2 years to attain the level of resistance and its inheritance. One feature that both programs had in common was that at the time the work was started there was no known resistance to these diseases and we were in an unknown sphere. We believe that even if better resistance is found, as with the celeriac crosses, the new germplasm will be of significant value as back up and should be integrated into future varieties to broaden their base of resistance.

The discovery of Alternatin adds yet another toxin to the number that are currently known. In isolating and characterizing it chemically we have achieved the first goal toward understanding its role in disease expression. It is worth pursuing and it is our intention to do so.

Septoria apiicola has the ability to effect very significant increases in leaf pH, presumably by raising the level of NH_4^+N which in turn produces toxicity. This is only a first step, but the data are highly suggestive and should be followed up.

Considering this has been a 3-year program it has proved remarkably successful and is deserving of further support.

h. LIST OF PUBLICATIONS * and References

- Al-Abta, S. and H.A. Collin 1978 Control of embryoid development in tissue cultures of celery. *Ann Bot* 42:773-782.
- Charudattan, R. and J.E. DeVay 1972 Common antigens among varieties of *Gossypium hirsutum* and isolates of *Fusarium* and *Verticillium* species. *Phytopathology* 62:230-234.
- Chen, C.H. 1976 Vegetative propagation of the celery plant by tissue culture. *Proc. South Dakota Acad. Sci.* 55:44-48.
- Daub, M. 1986 Tissue culture and the selection of resistance to pathogens. *Ann. Rev. Phytopathol.* 24:159-186.
- Donovan, C. M., H. Collin, and S. Issac 1986 Selection for resistance to late blight in celery. In: *Secondary metabolism in plant cell cultures*. Ed: P. Morris, A. Scrogg, A. Stafford, M. W. Fowler. Cambridge University Press, pp 244-249.
- Dunstan, D.I., K.C. Short, M.E. Merrick and H.A. Collin 1982 Origin and early growth of celery embryoids. *New Phytol* 91:121-128.
- Earle, E.D., V.E. Gracen, O.C. Yoder and K.P. Germmill 1978 Cytoplasm-specific effects of *Helminthosporium maydis* race T toxin on survival of corn mesophyll protoplasts. *Plant Physiol.* 61:420-424.
- Evans D.A. and W.R. Sharp 1983 Single gene mutations in tomato plants regenerated from tissue culture. *Science* 221:949-951.
- Evans, D.A., W.R. Sharp and H.P. Medina-Filho 1984 Somaclonal and gametoclonal variation. *Ann. J. Bot.* 71:759-774.
- Evans, D.A., W.R. Sharp and H.P. Medina-Filho 1984 Somaclonal and gametoclonal variation. *Ann. J. Bot.* 71:759-774.
- Florey, K. 1973 *Analytical Profiles of Drug Substances*. Vol 2, Academic Press, New York and London.
- Fuji, D.S. 1982 *In vitro* propagation of celery (*Apium graveolens* L.) Masters Thesis, University of California, Davis.
- Harvan, D.J. and R.W. Pero 1976 In: *Mycotoxins and Other Fungal Related Food Problems*. Ed. by J. V. Rodricks, *Advances in Chemistry Series* 149, American Chemical Society, Washington D.C., pp. 344-355.
- * Heath-Pagliuso, S and L. Rappaport 1990 Somaclonal variant UC-T3: the expression of fusarium wilt resistance in progeny arrays of celery, *Apium graveolens* L. *Theor. Appl. Gen.* 80:390-394.
- * Heath-Pagliuso, S, J. Pullman and L. Rappaport 1989 'UC-T3 Somaclone': celery germplasm resistant to *Fusarium oxysporum* f. sp. *apii*, race 2. *HortScience* 24:711-712.
- * Heath-Pagliuso, S., and L. Rappaport 1989 Horticultural evaluation of celery lines derived from cell suspension cultures. *Abstr. Mtgs. Amer. Soc. Hort. Sci.*, Mich. State Univ., E. Lansing, MI, Aug. 12-17.
- * Heath-Pagliuso, S., J. Pullman and L. Rappaport (In press) 'UC-T3 Somaclone': celery germplasm resistant to *Fusarium oxysporum* f. sp. *apii*, race 2. *ASHS*.
- * Heath-Pagliuso, S., J. Pullman and L. Rappaport 1988 Somaclonal variation in celery: Selection for resistance to *Fusarium oxysporum* f. sp. *apii*. *Theor. Appl. Gen.* 75:446-451.
- * Heath-Pagliuso, S., J. Pullman and L. Rappaport 1989 'UC-T3 Somaclone': Celery germplasm resistant to *Fusarium oxysporum* f. sp. *apii*, race 2. *HortScience* 24:711-712.
- * Heath-Pagliuso, S., O.C.H. Kwok, and L. Rappaport 1988 Induction of linear furanocoumarins in celery and celeriac. *Phytopathology* 78:1556.

- Karp, A. and S.W.J. Bright 1985 On the causes and origins of somaclonal variation. *Oxford Surveys of Plant Molecular and Cell Biology* 2:199-234.
- Kwok, O.C.H. and L. Rappaport 1988 Isolation of toxins from *Septoria apiicola*. *Phytopathology* 78:1557.
- Larkin, P.J. and W.R. Scowcroft 1981 Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60:197-214.
- Larkin, P.J., S.A. Ryan, R.I.S. Brettell and W.R. Scowcroft 1984 Heritable somaclonal variation in wheat. *Theor. Appl. Genet.* 67:443-455.
- Lee, M. and R.L. Phillips 1987 Genomic rearrangements in maize induced by tissue culture. *Genome* 29:122-128.
- Lee, M. and R.L. Phillips 1988 The chromosomal basis of somaclonal variation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39:413-437.
- Luke, H.H. and R.H. Biggs 1976 In: *Mycotoxins and Other Fungal Related Food Problems*. Ed. by J. V. Rodricks, *Advances in Chemistry Series* 149, American Chemical Society, Washington D.C., pp. 344-355.
- Luke, H.H. and R.H. Briggs, In: *Mycotoxins. Phytopathogenic Toxins from Fungi: An Overview*. Ed. by pp. 296-317.
- McNay, J.W., P.S. Chourey and D.R. Pring 1984 Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of maize. *Theor. Appl. Genet.* 67:433-437.
- Nadel, B.L., A. Altman and M. Ziv 1989 Regulation of somatic embryogenesis in celery cell suspensions. I. Promoting effects of mannitol on somatic embryo development. *Plant, Cell, Tissue and Organ Culture* 18:181-189.
- Orton, T.J. 1984 Genetic variation in somatic tissues: method or madness? *Adv. Plant Pathol.* 2:153-189.
- Orton, T.J., M.E. Durgan and S.H. Hulbert 1984b Studies on the inheritance of resistance to *Fusarium oxysporum* f. sp. *apii* in celery. *Plant Disease* 68:574-578.
- Pullman, G.S. and L. Rappaport 1983 Tissue culture induced variation in celery for *Fusarium* yellows. *Phytopathology* (Abstr) 73:818.
- Quiros, C.F. 1987 Breeding celery for disease resistance and improved quality. In: *Annual Report, California Celery Growers Association*, 531-D, North Alta Ave., Dinuba, California 93618.
- Rappaport, L. 1977-1978 Selecting for resistance to *Septoria* leaf spot in celery. In: *Annual Report, California Celery Growers Association*, 531-D, North Alta Ave., Dinuba, CA 93618.
- Rappaport, L. 1978-1979 Cloning celery for plant propagation, inducing variation and screening for disease resistance. In: *Annual Report, California Celery Growers Association*, 531-D, North Alta Ave., Dinuba, CA 93618.
- Rappaport, L. 1979-1980 Cloning celery for plant propagation, inducing variation and screening for disease resistance. In: *Annual Report, California Celery Growers Association*, 531-D, North Alta Ave., Dinuba, CA 93618.
- Rappaport, L. 1982 Cloning celery for plant propagation, inducing variation and screening for disease resistance. In: *Annual Report, California Celery Growers Association*, 531-D, North Alta Ave., Dinuba, CA 93618.
- Rappaport, L. 1982 Cloning celery for plant propagation. In: *Annual Report, California Celery Growers Association*, 531-D N. Alta Ave., Dinuba, California 93618.

- Rappaport, L. and S. Heath-Pagliuso 1986 Cloning celery for plant propagation. In: Annual Report, California Celery Growers Association, 531-D N. Alta Ave., Dinuba, California 93618.
- * Rappaport, L. and S. Heath-Pagliuso 1987 Cloning celery for plant propagation. In: Annual Report, California Celery Growers Association, 531-D N. Alta Ave., Dinuba, California 93618.
- * Rappaport, L., S. Heath-Pagliuso, G. Pullman, S. Matlin S and D. Fujii 1988 Somaclonal variation as a genetic source of resistance to *Fusarium* wilt of celery: a case history. In: Proceedings of the Symposium on the Application of Biotechnology in Agriculture. Zhejiang Agricultural University, Hangzhou, P.R.C. Shanghai Scientific: Technical Publishers.
- * Rappaport, L., S. Heath-Pagliuso, J. Pullman, S. Matlin and D. Fujii 1988 Somaclonal variation as a genetic source of resistance to *Fusarium* wilt of celery: A case history. In: Li Xu Hsien, ed., Application of Biotechnology in Agriculture, Shanghai Scientific & Technical Publishers, Shanghai, PRC, pp. 137-154.
- Schneider, R.W. 1984 Effects of nonpathogenic strains of *Fusarium oxysporum* on celery root infection by *F. oxysporum* f. sp. *apii* and a novel use of the Lineweaver-Burk double reciprocal plot technique. *Phytopathology* 74:646-653.
- Scowcroft, W.R., P.J. Larkin, and R.I.S. Brettell 1983 Genetic variation from tissue culture. In: Use of Tissue Culture and Protoplasts in Plant Pathology. J.P. Helgesen and B.J. Deverall, ed. New York: Academic Press pp. 139-162.
- Shepard, J.F. 1981 Protoplasts as sources of disease resistance in plants. *Ann. Rev. Phytopathol.* 19:145-166.
- Singer, S.R. and C.N. McDaniel 1984 Selection of amitrole tolerant tobacco calli and the expression of this tolerance in regenerated plants and progeny. *Theor. Appl. Genet.* 67:427-432.
- Steel, R.G.D. and J.H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill, pp 72-75 and 113-114.
- Williams, L. and H.A. Collin 1976 Growth and cytology of celery plants derived from tissue cultures. *Ann. Bot.* 40:333-338.
- Wright, J.C. and M.L. Lacy 1988 Increase of disease resistance in celery cultivars by regeneration of whole plants from cell suspension cultures. *Plant Disease* 72:256-259.
- Zong-xiu, S., Z. Cheng-Zhang, Z. Kangle, Q. Xiu-Fang and Z. Ya-Ping 1983 Somaclonal genetics of rice *Oryza sativa*. *Theor. Appl. Genet.* 67:67-73.