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Resistant tobacco plants from protoplast-derived calluses selected for their resistance to *Pseudomonas* and *Alternaria* toxins

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Summary. Protoplast-derived calluses of tobacco (*Nicotiana tabacum* cv. 'Samsun') were selected for their resistance to toxins from *Pseudomonas syringae* pv. *tabaci*, which causes wildfire disease, and from *Alternaria alternata* pathotype tobacco, which causes brown spot. A number of plants were regenerated from each of the toxin-selected protoplast-derived calluses. A large percentage of the plants obtained from the second selection cycle calluses were resistant to infection by these pathogens. Resistance to wildfire disease, however, seems to be unrelated to resistance to brown spot disease. Variations in the morphological characteristics of the regenerated plants were found. Results of an assay of the R_1 generation indicate that the resistance shown by R_0 plants against both disease is heritable.

Key words: *Pseudomonas syringae* – *Alternaria alternata* – In vitro selection – Disease resistance – Heritable selected variation

Introduction

Several techniques have been used to breed crop plants that are disease resistant. One is the introduction of resistance from wild species, although the genetic source is not always available. Another means is the artificial induction of mutations that show resistance.

This technique has been studied intensively for many years (Simons 1979), but the frequency of the resistant mutants so far produced has fallen far short of early expectations. The most recent technique in use is the selection of resistant variants from artificially cultured cells or tissues. Since Takebe et al. (1971) reported the ability of a single tobacco leaf protoplast to regenerate an intact plant, much attention has been placed on the screening of novel variants of agronomic importance.

Large scale screening of cell populations by conventional in vitro procedures has been successful in producing resistant plants against some amino acid derivatives (Carlson 1973; Marton and Maliga 1975; Widholm 1977), antibiotics (Umiel 1979; Maliga et al. 1975; Maliga 1978), pesticides (Palacco and Palacco 1977; Chaleff and Parsons 1978) and some pathotoxins (Gengenbach et al. 1977; Mattern et al. 1978; Brettel and Ingram 1979; Thomas et al. 1979; Siegemund 1981; Sacristan 1982).

We have selected leaf protoplast-derived calluses from tobacco (*Nicotiana tabacum* cv. 'Samsun') for their resistance to toxins from the pathogens of wildfire and brown spot diseases, and have regenerated intact plants from these calluses. The morphological characteristics and disease resistance of the regenerated plants and their progenies also have been investigated. Our results show that resistance to both pathogens is heritable.

Materials and methods

Protoplast isolation and culture

Fully expanded leaves of tobacco plants (*Nicotiana tabacum* L. cv. 'Samsun') that had been grown in a greenhouse under natural daylight at 25 ± 5 °C were used for protoplast isolation. The leaf surface was initially sterilized with 70% ethanol for 15 s and then with 0.2% mercuric chloride for 15 s. After rinsing the leaf three times with sterile water, its lower epidermis was removed and pieces of the leaf were floated epidermisless side downwards on a protoplast isolation solution composed of 9.1% mannitol, 1% cellulase and 0.05% Macerozyme (Yakult Pharmaceutical Industry Co. Ltd. Nishinomiya, Japan). The pH of the enzyme solution was adjusted to 5.8 with 0.2 N KOH prior to sterilization through a Millex-GS filter (Millipore Corporation Bedford, Mass. USA). All subsequent operations were carried out aseptically in a laminar flow cabinet. Leaf materials were kept in the enzyme solution at 30 °C for 3 h without agitation until a mesophyll protoplast suspension formed. This suspension was filtrated through four layers of cotton cheese cloth. Its filtrate was placed in centrifuge tubes and spun at 100 × g for 2 min. After

removal of the supernatant, the pellet was washed four times with sterilized 0.5 M mannitol solution containing 0.1 mM $CaCl_2$, then suspended in a small amount of the same solution to give the desired protoplast concentration, approximately 5×10^7 cells/ml.

Protoplasts were cultured at a concentration of about 1×10^4 cells/ml (ca. a 500-fold dilution of the original suspension) in plastic petri dishes (9 cm in diameter) containing 5 ml of Nagata and Takebe's medium (1971) supplemented with 3 mg/l β-naphthyl acetic acid (NAA), and 1 mg/l benzylaminopurine (BAP), and 0.8% agar. These dishes were sealed with Parafilm M and incubated first in the dark for 7 days then in diffuse light of 700 lux at 26 °C for 5 weeks.

Selection of resistant calluses and plant regeneration

Cell colonies (0.3–0.8 mm) that appeared on the Nagata and Takebe medium after 4–6 weeks of incubation were transferred to Linsmaier and Skoog (1965) basal medium containing 2 mg/l NAA, 0.2 mg/l BAP and 1–1.5% agar (LS-1 medium) for callus differentiation. The transferred calluses were maintained at 26 °C under continuous illumination of 800 lux. After 2 weeks of incubation, each callus was cut into pieces of ca. 2.5 mm.

Ten pieces were placed in separate petri dishes (9 cm in diameter) containing 15 ml LS-1 medium, to which 1% *P. syringae* toxin or 0.7% *A. alternata* toxin (both in a v/v ratio) was added aseptically as the selective agent. At this concentration both toxins produced modest growth inhibition. The dishes were sealed with Parafilm M and kept under dim light of 400 lux at 26 °C for 3–6 weeks.

The LS-2 agar medium (Linsmaier and Skoog basal medium supplemented with 0.1 mg NAA and 5.0 mg BAP/l) was used for plant regeneration. Calluses that survived on the selective medium were transferred to this medium and incubated in a growth chamber adjusted to 26 °C and 50% relative humidity under a 16 h/day of 13,000 lux. Three to six weeks later the shoots that had developed were transferred to LS-3 medium (Linsmaier and Skoog basal medium containing 0.1 mg NAA and 0.5 mg BAP/l) for further shoot development and root initiation.

Pathogens and toxin preparation

Pseudomonas syringae pv. *tabaci* (Wolf and Foster) Young, Dye and Wilkie (Isolation No. 7426) was obtained from Dr. K. Ono of the Okayama Tobacco Experiment Station, Japan. The bacterium was kept on YDC agar slants (1% yeast extract, 2% sucrose and 2% $CaCO_3$) at 20 °C and subcultured every 2 weeks. The identity of each culture was confirmed before use from its growth on King's and TCA medium (Harrigan and McCance 1966) and by its virulence against tobacco plants.

To prepare the toxin, we grew bacterial cells in Wooley's liquid medium (Wooley et al. 1952) at 24 °C for 5 days with agitation. Cells were centrifuged and the supernatant passed through Seitz-filters.

The filtrate was concentrated 30 fold at 35 °C in vacuo. This crude filtrate then was passed through a Sephadex G-25 column and eluted with distilled water. The fractions toxic to tobacco leaves were collected, pooled and reconcentrated to the original content in vacuo. The toxin samples used were filtered through a Millex-GS filter before their use in selecting resistant calluses.

Alternaria alternata pathotype tobacco was supplied by Prof. S. Nishimura of Nagoya University, Nagoya, Japan. This fungus was maintained on PSA (potato sucrose agar) medium at 28 °C and was subcultured at 2-week intervals.

To prepare its toxin, we cultured the fungus in Richard's medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 50 g sucrose and 1 g yeast extract/l) and kept the culture stationary for 3 weeks at 28 °C.

Mycelial mats from 5 l of the culture fluid were removed by passing the fluid through Whatman No. 2 filter paper. The toxic filtrate was absorbed with 0.5% activated charcoal for 30 min, then the charcoal was washed twice with distilled water and was collected on Whatman No. 2 filter paper by filtration. The toxin was extracted from this charcoal with 70% acetone which then was removed from the toxin extract by evaporation at 40 °C. The water solution remaining was adjusted to pH 4.0 with 1 N HCl. This acidified solution was treated with ethylacetate to remove any admixtures in the water.

The crude toxin was partially purified eluting it with 1 N NH_4OH from a Dowex 50×8 column. Ammonia was removed by evaporation, then the volume of the crude toxin was reduced to 50 ml (a 100-fold concentration of the content of the original solution). The toxin samples used first were filtered through a Millex-GS filter to sterilize them.

Testing plant resistance to pathogens

Test of regenerated plants. Regenerated plants and their offspring were tested with both pathogens for their responses to infection.

P. syringae pv. *tabaci:* Leaves were infected by pricking them with a needle dipped in bacterial suspension (10^8 cells/ml). In susceptible plants, chlorosis appeared on the leaves within 2–3 days, and typical symptoms of wildfire disease were present in inoculated plants 14–21 days after treatment.

Plants that had chlorotic lesions less than 0.4 cm in diameter 4 days after inoculation, or those that showed no symptom when harvested were considered resistant. Plants that formed chlorotic lesions 0.5 to 1.0 cm in diameter or those that had only a few spots on aged leaves were classified intermediate. Plants that had chlorotic lesions ca. 1 to 2 cm in diameter with dead areas in their centers 4 days after inoculation were considered susceptible (Fig. 1). In highly sensitive plants, the wildfire lesions spread rapidly to nearly all the tissues and leaves within 2 weeks and caused severe infection.

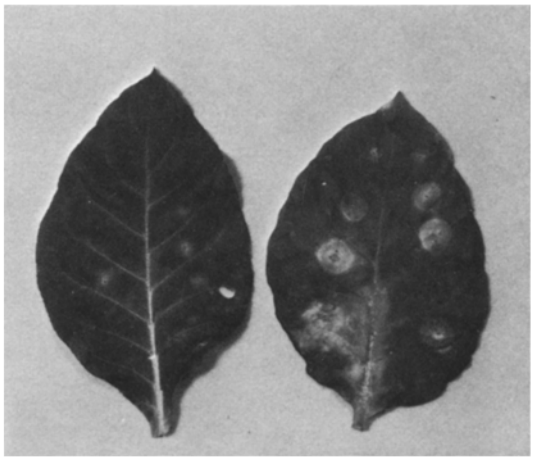

Fig. 1. Evidence of resistance to *P. syringae* pv. *tabaci* on leaves of regenerated plants 4 days after inoculation. Four to six drops of the bacterial suspension (10^8 cells/ml) were placed on each side of a leaf. Typical chlorotic lesions developed severely on leaves of susceptible plants (*right*)

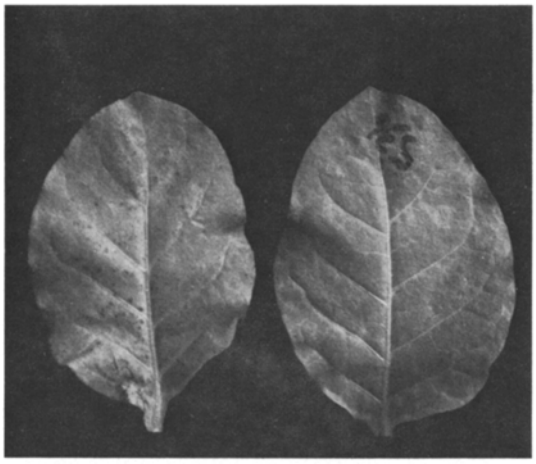

Fig. 2. Evidence of resistance to *A. alternata* pathotype tobacco on excised leaves of regenerated plants. Leaves were sprayed with 1×10^6 spores/ml then kept in 100% relative humidity at 10 000 lux for 3 days. The leaf of the susceptible plant is covered with expanded necrotic flecks (*left*)

A. alternata pathotype tobacco: Leaves were detached and tested by spraying them uniformly with a spore suspension (10^6 spores/ml) and maintaining them at 28 °C under 10,000 lux for 3–5 days. Susceptibility to the fungus was shown by the number of small necrotic flecks present on the inoculated leaves. Plants were divided into 3 classess of resistance according to the number of necrotic flecks that had appeared by the 5th day after inoculation in a square centimeter of each inoculated leaf (Fig. 2): resistant (R); 0–10 necrotic flecks/cm²-intermediate (I); 11–50 necrotic flecks/cm², and susceptible (S), had more than 50 flecks/cm².

Test of the progeny of regenerated resistant plants. Seeds were produced by open pollination of regenerated plants that had been selected for their resistance. Seedlings raised from these seeds were tested for their disease resistance.

In the test for resistance to *P. syringae* pv. *tabaci*, progenies of resistant plants from callus 348R were heavily inoculated by a pressurized spray of 10^8 cells/ml to the lower surfaces of individual leaves. Development of the disease was checked weekly for 4 months after inoculation. Plants that showed any of the chlorotic lesions caused by wildfire disease were considered susceptible. Plants that did not have visible symptoms were assumed to be resistant. In the progeny test on resistance to *A. alternata* pathotype tobacco, the offspring of resistant plants regenerated from 348R callus were inoculated and checked as described above.

Chromosome counts

Chromosome numbers of regenerated plants and their progenies were determined by the acetocarmine squash method. It was used on root tips that had been treated with 2 mM 8-hydroxyquinoline and fixed with acetic alcohol (1 : 3 ratio).

Table 1. Reaction of protoplast-derived calluses to toxin preparations form *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco

Toxin	No. calluses tested	Surviving calluses[a]			
		1st transfer		2nd transfer	
		No.	%	No.	%
P. syringae pv. *tabaci*	700	74	10.6	6	0.86
A. alternata pathotype tobacco	700	36	5.1	5	0.71

[a] The percent of survival is the number of calluses surviving/the number of calluses tested

Table 2. Morphological classification of plants regenerated from calluses that had been selected against the toxins of *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco

Class	Distinguishing characteristics	No. plants
1	Normal	379
2	Flowers pale colored; slightly reduced in size and sterile; otherwise normal	9
3	Normal height with slightly thick, dark green leaves	29
4	Normal height; thick broad leaves with round tips; some plants sterile	100
5	Taller than normal; long stem internodes; long, narrow leaves with sharply pointed tips; pale-colored, sterile flowers	59
6	Taller than normal; long stem internodes; small, narrow leaves	14
7	Taller than normal; long stem internodes; slightly small, light green narrow leaves; no petiole	10
8	Normal height; discoloration of leaves; pale, sterile flowers	1
9	Slightly reduced height; delayed maturity; slightly small, light green, thick leaves, somewhat broad at the base with sharply pointed tips	10
10	Subnormal height; delayed maturity; slightly broad light green, thick leaves with somewhat curled tips and less pronounced petioles	40
11	Dwarf; thin, small, narrow leaves with sharply pointed tips; sterile	2
12	Crinkled dwarf; small, dark green, thick leaves with round tips; no flowering	45
13	Crinkled dwarf; small, leaves long, narrow, thick and curled leaves; no flowering	2

Results

In vitro selection of protoplast-derived calluses for toxin resistance and regeneration of plants

A good yield of protoplasts was obtained with the present enzyme system (Fig. 3 a). More than 65% continued cell division and developed into small calluses within 3 weeks (Fig. 3 b, c). From the large number of cell colonies formed on the culture plates (Fig. 3 d), 700 were chosen at random and transferred to LS-1 medium for callus growth.

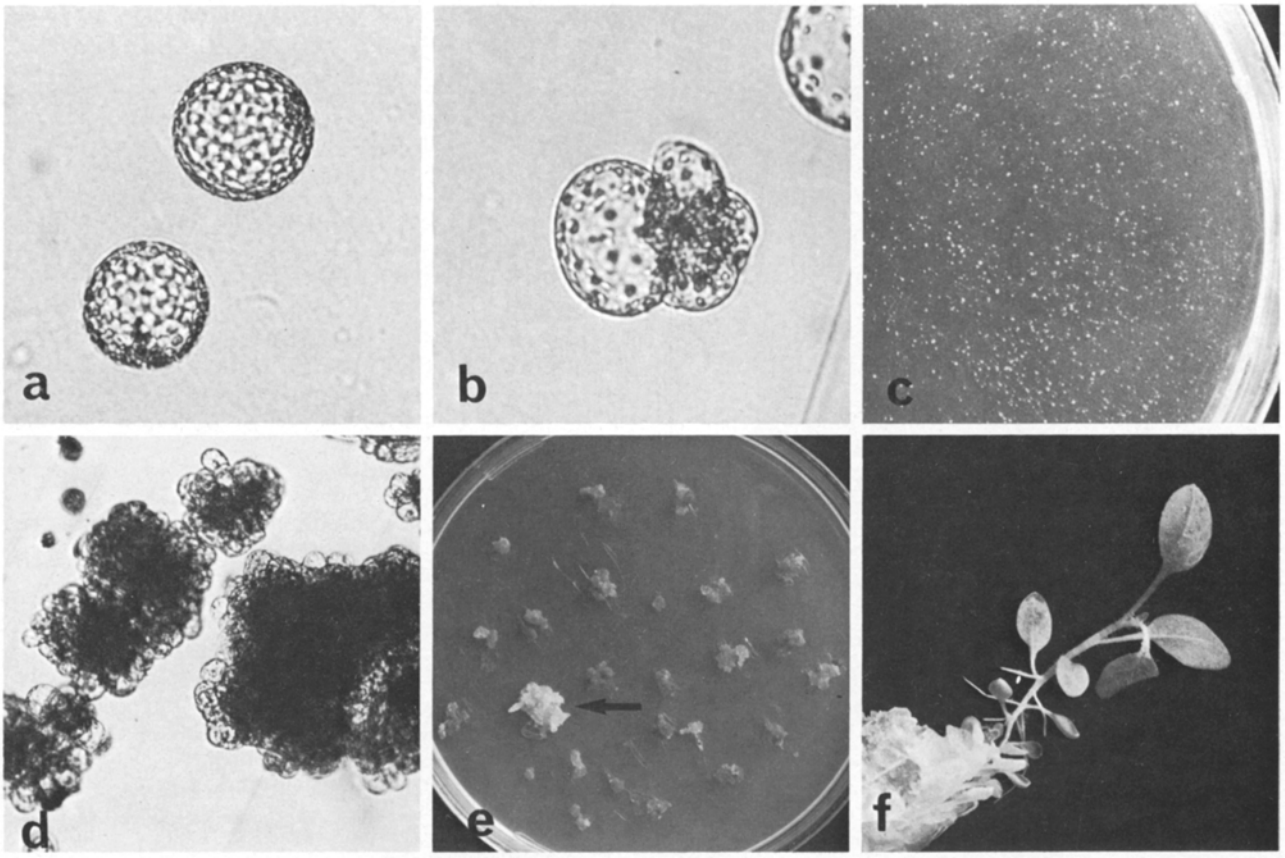

Fig. 3. **a** Freshly isolated protoplasts (× 350); **b** First division after 3–5 days in culture (× 350); **c** Cell colonies that formed on the agar medium within 3 weeks; **d** Callus cluster from the cultured protoplasts; **e** Calluses after 4 weeks of culture on toxin medium. Only one colony grew on this plate (the *arrow* indicates the growing callus); **f** Plantlet regenerated from the callus that survived in **e**

The lowest toxin concentration that inhibited callus growth was 0.05% (v/v) for the *P. syringae* pv. *tabaci* toxin and 0.025% (v/v) for the *A. alternata* pathotype tobacco toxin. Much higher concentrations, 1.0% for the former and 0.7% for the latter, were used to select resistant calluses. Most calluses transferred to toxin-containing LS-1 medium became brown and died within 4 weeks of transfer (Fig. 3 e).

Results of our selection of resistant calluses through two cycles of selection against these toxins are given in Table 1. Calluses that survived after the second transfer to toxin-containing medium were allowed to regenerate plants. The number of plants regenerated ranged from 4 to 30 per callus (Fig. 3 f).

Morphological variations in regenerated plants

Plants regenerated from resistant calluses varied in many of their morphological characteristics; plant height, leaf shape and flower characteristics. We classified plants visually into 13 morphological types (Table 2). Many of the morphological anomalies were accompanied by slow growth and reduced vigor and by malformed leaves, flowers and stems and/or poor root development. Such plants were particularly numerous among offspring regenerated from calluses that survived the second selection cycle. However, about 70% of the plants obtained were normal or almost normal in their gross morphology. Regenerated plants were allowed to grow to maturity in a greenhouse. Seeds were collected from all fertile plants for use in the progeny test.

Disease resistance of regenerated plants

Results of the testing response of the regenerated plants to both pathogens are given in Table 3. Plants regenerated from calluses untreated with a pathogenic toxin were all as susceptible, that is highly susceptible, as the original stock of cv. 'Samsun'. Calluses that survived one selection cycle produced large numbers of plants, but 80–90% of these were susceptible, or highly susceptible to the pathogen whose toxin had been used as the selective agent. No resistant plant was obtained from these calluses.

The number of plants regenerated was greatly reduced by the passage of two selection cycles. On the plants regenerated, 10 (about 13%) were resistant to

Table 3. Number of regenerated plants showing different reactions to infection with *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco

Pathogen	No. selection cycle	No. calluses isolated	No. regenerated plants[a]				
			R	I	S	HS	Total
P. syringae	1	74	0 (0.0)	129 (18.4)	499 (71.2)	73 (10.4)	701 (100.0)
tabaci	2	6	10 (12.8)	49 (62.8)	18 (23.1)	1 (1.3)	78 (100.0)
A. alternata	1	36	0 (0.0)	74 (10.6)	619 (89.1)	2 (0.3)	695 (100.0)
pathotype tobacco	2	5	5 (21.7)	7 (30.4)	11 (47.8)	0 (0.0)	23 (100.0)

[a] *R* = resistant, *I* = intermediate, *S* = susceptible, *HS* = highly susceptible. In parentheses their proportion in percentage is given

P. syringae pv. *tabaci* and 5 (about 22%) to *A. alternata* pathotype tobacco. The percentage of plants susceptible to each pathogen was greatly reduced. These facts clearly show that the use of two cycles of selection to select resistant calluses is very effective for obtaining resistant plants from callus.

To determine whether plants regenerated from a single callus vary, we classified all regenerated plants by their morphological characteristics and susceptibility to disease; results are shown in Table 4. Variations in morphological characteristics and in reaction to disease do exist among plants regenerated from a single toxin-resistant callus.

Plants resistant to *P. syringae* pv. *tabaci* that had been regenerated from callus No. 348 (348R plants) all showed the normal morphology and vigorous growth typical of the parent 'Samsun' tobacco. Similarly, plants resistant to *A. alternata* pathotype tobacco that had been produced from callus No. 48 (48R plants) had normal morphology and showed vigorous growth. The offspring of these plants were given the progeny test.

Disease resistance and chromosome numbers of the progeny of regenerated plants

The progeny of 348R plants were tested for infection by *P. syringae* pv. *tabaci* and those of the 48R plants for infection by *A. alternata* pathotype tobacco to ascertain whether their resistance was heritable. Results are shown in Table 5. About 26% of the progeny of 348R were resistant to wildfire disease; about 7% of the control were resistant. Approximately 9.3% of the progeny of 48R plants were resistant to *Alternaria* leaf spot; none of the control plants showed resistance to this disease. Our results clearly indicate that the resistance shown by these regenerated plants was heritable, and that it can be transmitted to offspring through sexual reproduction.

The somatic chromosome numbers of several resistant offspring were counted. All had the normal chromosome complement (2n=48) of *N. tabacum* cv. 'Samsun' (Fig. 4).

Discussion

Variations in varying degrees have long been known to occur in cultured tissues originating from the same source and in plants regenerated from a single callus (Skirvin 1978). Protoplasts can be isolated from many plant species and, in several cases, many cultures have been obtained (Vasil 1976; Vasil et al. 1979). Intact plants also have been successfully regenerated from

Table 4. Variations in morphological characteristics and responses to two pathogens in plants regenerated from individual toxin-resistant calluses

Selective toxin from	Isolation no. of original callus	No. regenerated plants	Morphological type[a]	Disease reaction[b]			
				R	I	S	HS
P. syringae	87	4	4	–	2	2	–
tabaci	282	4	2	1	2	1	–
	315	37	12	–	37	–	–
	348	12	1, 4, 6	3	4	4	1
	355	21	9, 10	6	4	11	–
A. alternata	48	10	3	3	5	2	–
Pathotype	458	5	3, 4	2	1	2	–
Tobacco	496	5	12	–	–	5	–
	500	3	13	–	1	2	–

[a] Refer to Table 2 [b] *R* = resistant, *I* = intermediate, *S* = susceptible, *HS* = highly susceptible

Table 5. Response of R_1 progenies to infection by *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco

Pathogen	Parent[a]	No. progeny tested	Reaction to pathogen[b]			% resistant
			R	I	S	
P. syringae pv. *tabaci*	348R	1913	498	–	1415	26.0
	Control (cv. 'Samsun')	206	14		192	6.8
A. alternata Pathotype Tobacco	48R	461	43	81	347	9.3
	Control (cv. 'Samsun')	60	0	8	52	0.0

[a] 348R; plants regenerated from callus no. 348, being resistant to *P. syringae* pv. *tabaci*, 48R; plants regenerated from callus no. 48, being resistant to *A. alternata* pathotype tobacco
[b] *R* = resistant, *I* = intermediate, *S* = susceptible

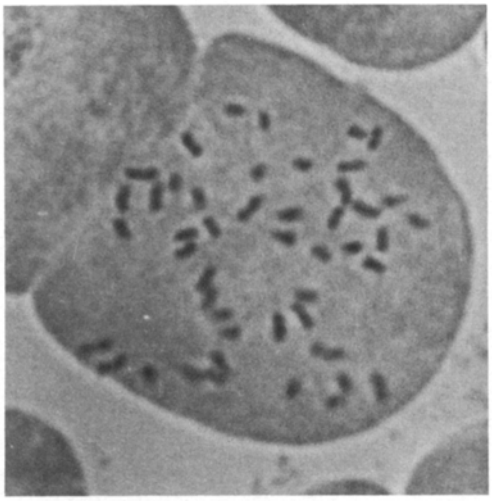

Fig. 4. Chromosomes from a root tip cell of a regenerated plant showing the normal chromosome complements (2n = 48) of tobacco plants

protoplasts of several important crops (Kartha et al. 1974; Xu et al. 1982), in which plants there have been many morphological variants.

In 1971, Takebe et al. reported morphological malformations and chromosomal aberrations among plants regenerated from tobacco mesophyll protoplasts. Similar regenerate variability has been reported for other plants, including potato and rape (Kao et al. 1970; Butenko and Kuchko 1979; Shepard et al. 1980).

Wenzel et al. (1979), however, assumed homogeneity of a protoclonal plant population derived from a diploid potato line.

In our study, morphologically as well as physiologically different tobacco plants were produced from leaf protoplast-derived calluses without mutagenic treatment (Table 2). Our results are comparable to those of Takebe et al. (1971). A two-cycle selection of calluses for resistance to *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco toxins gave resistant regenerates at frequencies as high as 13% for the former toxin and 22% for the latter. Carlson (1973) obtained tobacco mutants resistant to wildfire disease by selecting cell lines resistant to methionine sulfoximide, which produces the same chlorotic haloes on tobacco leaves as does the bacterial wildfire toxin (Braun 1955).

In sugarcane, 15–20% of the plants regenerated from calluses or suspension cell cultures were found resistant to both *Helminthosporium sacchari* and its toxin helminthosporide (Heinz 1973). Some clones of these regenerates also were resistant to mosaic virus (Nickell and Heinz 1973), Fiji disease and downy mildew (Heinz et al. 1977).

In vitro screening of calluses resistant to T-toxin produced maize plants with increased resistance to *Helminthosporium maydis* (Gengenbach and Green 1975; Gengenbach et al. 1977). In potato, Shepard and his co-workers (1980) reported that 1–2% of the plants raised from mesophyll protoplasts were strikingly more resistant to the early blight disease caused by *Alternaria solani*, or to the late blight disease caused by races of *Phytophthora infestans*, than the parent, 'Russett Burbank'. Behnke (1979, 1980a, b) selected potato cell lines that survived on medium containing of *P. infestans* or *Fusarium oxysporum* culture filtrate and obtained plants from these cell lines that showed weak symptoms. Recently, Sacristan (1982) produced rape plants resistant to the pathogen, *Phoma lingam*, from cell cultures selected and unselected by the pathogen's toxin.

All these cited results and our reported ones indicate that the variability in various characters, including disease resistance, found among regenerates, might be present beforehand in the cells from which the plants were derived. It is also possible that cell or callus cultures, or both, might cause genetic alterations in the cells of a callus or in regenerated plants.

In fact, variants exist in plants regenerated from callus of single cell origin (Table 4). In these cases, genetic differences most probably would be generated during cell division in vitro. Nevertheless, toxin-selected calluses do yield a high percentage of disease resistant plants.

Self-fertilization of regenerated plants proved resistance to both the pathogen and its toxin produced resistant plants that ranged from 9–26% f the offspring that retained the same degree of resistance to the pathogen. This is evidence that resistance to both *P. syringae* pv. *tabaci* and *A. alternata* pathotype tobacco is transmitted to the R_1 progeny through sexual reproduction.

The segregation data obtained for the R_1 generation, however, are not significant for determining the mode of inheritance of new traits because the parental resistant regenerates are suspected to be chimeric. Genetic analysis is needed to clarify this point.

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