CHAPTER 5
;ENETICAL ANALYSIS OF INHERITANCE OF VILT DISEASE RESISTANCE CHARACTER IN THE REGENERATED PLANTS

CHAPTER 5 - GENETICAL ANALYSIS OF INHERITANCE OF WILT DISEASE RESISTANCE CHARACTER IN THE REGENERATED PLANTS.

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INTRODUCTION

Fusarium wilt disease is reported in diverse crops such as banana (Vakili, 1965), Peas (Linford, 1928), tomato (Wellman, 1941, Foster, 1946), tobacco (Valleau, 1952), Cotton (Kappelman, 1975, Smith and Snyder, 1975), Chickpea (Kanniyan et al, 1984), Sugarbeat (Mc Donald et al, 1976), flax (Wilhelm, 1981) and numerous other crops.

Among all the fungi, Fusarium and Verticillum are the most common vascular pathogens (Mace et al, 1981). Production of the highly prized banana variety 'Gros Michel' was abandoned in many parts of western hemisphere because of the 'panama disease' caused by Fusarium oxysporum f.sp. cubense (E.F.Sm) Synd & Hans (Green, 1981). The genus Fusarium is a persistent 'soil inhabitant' and once established, persists for several years, rendering the soil unfit for profitable crop production.

Fusarium is toxonomically complex genus. Synder and Hansen (1940) grouped all forms into one species F. oxysporum comprising of several formae specialis named on the basis of their specialized hosts. Thus F. oxysporum f.sp. vasinfectum (Atk) Synd. & Hans infects

cotton, F. oxysporum f.sp. lycopersici (sacc) Synd. & Hans infects tomato, F. oxysporum f.sp. cubens (E.F. Sm.) Synd. & Hans infects banana; F. oxysporum f.sp. lini (Bolley) Synd & Hans. infects flaxs and F. oxysporum f.sp. nicotianae (J. Johnson) Synd & Hans. infects tobacco.

CONVENTIONAL BREEDING TO DEVELOP FUSARIUM RESISTANCE IN CROP PLANTS:

Despite the difficulty in isolating host genes that would confirm absolute resistance to Fusarium spp., breeders have produced several tolerant varieties to this pathogen by conventional breeding techniques. The first wilt resistant flax, 'N.D. 52', 'N.D. 73' and. 'N.D. 114' were selected by Bolley in 1907 (Cf. Wilhelm, 1981) from common varieties by screening in highly infested field with Fusarium in North Dakota, U.S.A.

Resistance to F. oxysporum f.sp. albedinis (Killian & Maire) Malecon has been identified in a number of date varieties (Pereu-Leroy, 1958). In 1982, Nene and Kannaiyan screened 11,000 entries of Cajanus cajan to F. udum and identified 33 resistant lines. However, only one line ICP 8863 was found to be resistant in both

green house and laboratory screening tests.

MUTATION BREEDING FOR INDUCED RESISTANCE TO FUSARIUM spp:

Success in obtaining wilt resistant mutants through induced mutation by ionizing irradiation was reported by Broertjes (1969) and Horner and Melouk (1976) as mentioned by Whilhelm (1981). 11,000 rhizome pieces of Scotch spermint (Mentha cardiaca Gerard ex Baker) were irradiated with 8,000 to 12,000 radiation dosage units of cobalt^{60} gamma rays. Shoots growing from these rhizomes were excised and immersed in the suspension of Verticillium spp.. Out of an initial 1,500 plants that were found resistant in soil, several showed high degree of durable resistance. But the same technique failed in soybean to select for Phyphthora wilt (Chamberlain and Bernard, 1968). Genetic variability in N. tabaccum is very limited and varieties having beneficial characters like disease or pest resistance are very low. Further, the occurrence of spontaneous mutations for such characters of economic value in tobacco is very low probably due to the amphiphloid nature of the cultivated species.

SOMATIC CELL GENETICAL METHOD FOR DEVELOPING WILT RESISTANT PLANTS:

In vitro selection method for developing resistant plants to fungal disease, its merits and demerits with specific examples has been aptly described in the introductory part of this thesis. In general the approach is successful where the pathogenicity can be mimicked by using the specific fungal toxin often called the 'host specific toxin'.

A successful in vitro selection programme does not end with the obtaining the resistant cell lines to a particular fungal toxin. Further screening of subsequent progenies for the inheritance of the resistant character is very much required to prove the mutational nature of the selected trait (Maliga, 1978). Genetically stable resistance to Fiji disease was first reported in the in vitro regenerated sugarcane. (Krishnamurthy and Tlaskal, 1974). In a true sense, the inheritance was not studied in the meiotic progenies, only mitotic progenies were studied. Resistance to Fusarium wilt in alfalfa (Hartman et al., 1984a), Potato (Behnke, 1980a, Schuchmann, 1985), Tomato (Sahin and Spivey, 1986) and celery (Heath Pegliuso et al., 1989) have been developed employing the approach of somatic

cell genetics. However analysis of wilt resistant phenotype has been carried out only in case of tomato and celery. In both these cases progeny plants were infected by the Fusarium to prove their resistance to the pathogen. Tomato plants resistant to the fungal pathogen, Fusarium oxysporum f. sp. lycopersici, race 2 were obtained using in vitro selection against fusaric acid, a non-specific toxin, as well as non-challenged cells (Shahin and Spivey, 1986). Protoplasts were isolated from cotyledonary tissue of tomato cv. 'UC-82', which is susceptible to Fusarium race 2. Protoplasts were challenged with the toxin, and the resistant calli were further subjected to the toxin. Plants regenerated from toxin-resistant calli were screened for resistance to the pathogen by using the Fusarium slurry inoculation technique. Seeds were collected from the surviving individuals, germinated and re-screened for resistance to the pathogen. Data obtained from this test showed a ratio of three resistant to one susceptible among R1 progenies. Further analysis of the R2 progenies confirmed that the fusarium resistant plants were either homozygous or heterozygous dominant for the gene conferring the resistance. Similar results were recorded for tomato plants regenerated from cells that

received no selection pressure. The nature of this single dominant gene-type of resistance is under investigation.

Cell lines of Medicago sativa have been selected for resistance to the toxin(s) produced by Fusarium oxysporum f.sp. medicaginis. (Hartman et al, 1984b). Selected cell lines showed resistance in vitro on media containing F. oxysporum culture filtrate. Plants regenerated from resistant cell cultures showed resistance to the pathogen in two greenhouse studies. In addition, cell lines reinitated from regenerated plants showed resistance on the media containing culture filtrate. Initial resistant selections from long-term culture showed elevated ploidy levels (octoploid and hexaploid). Subsequent selections of resistant cell lines from a shorter selection cycle yielded resistant plants with the normal tetraploid chromosome number.

Screening of progeny plants in the field is not only time consuming but also quite arbitrary as it is not possible to obtain uniform infectivity. Therefore simple but rapid screening methods are prerequisite for the study of segregation pattern of disease resistant trait. Such methods should be highly sensitive, reliable and also should be able to detect the slight

variations that occur during Cellular selection (G. Wenzel et al, 1990). Preliminary screening of tissue culture regenerants is normally done with rather small number of plants in green house. Work with field-grown plants in large numbers can only use less sophisticated tests and does not differentiate slight changes. Such slight improvements are however of importance although the breeder commonly make use of them after further combination in breeding programmes. (G. Wenzel et al., 1990).

In our case the progeny plants were screened for disease resistance using a novel and quantitative leaf disk assay method deviced in the present investigation. Here the resistancy was judged by the % loss in chlorophyll content in the excised leaf disks incubated in LD 50 concentration of the culture filtrate of the pathogen. Further this method was found not only rapid but also reproducible because the plants that were found to be resistant in the leaf disk bio-assay were also found to be resistant to direct fungal infection method.

THE Ro PLANTS :

Selvapandiyan (1987) regenerated 76 tobacco plants from the cell lines that were resistant to LD 50 (= 28% CF)

concentration of the culture filtrate of F. oxysporum f.sp. nicotianae using cell selection technique. They were named as Ro generation. The frequency of obtaining resistant plants from the originally plated cell was recorded as 0.38%.

Screening of the Ro progeny plants with leaf disk bio-assay as well as by direct fungal infection revealed an array of resistance varying from highly resistant, moderately resistant and sucsecptible. Highly resistant plants grew very slow further they wer sterile. I therefore selected 8 moderately resistant Ro regenerants for subsequent genetical analysis of wilt resistance because of their high fertility. Ro plants thus obtained from the resistant cell lines to Fusarium wilt were selfed to obtain R1 plants. Individual R1 plants were further selfed to obtain R2 plants (See the flow diagram Fig. 6). Genetical analysis of R1 and R2 progenies for their inheritance pattern to wilt resistance were investigated and described in the present chapter.

FIG.6. Experimental protocol :

Cell line selection and subsequent Genetic analysis of tobacco plants resistant to F. Oxysporum

Plating of 2 x 10^4 tabacco cells of suspension culture in two kinds of media

(a) R. medium = selection pressure of LD 50% concentration of sterile culture filtrate of Fusarium oxysporum f.sp. nicotianae

(b) W. medium = Standard culture medium without selection pressure.

|

(a) Appearance of resistance clones at 0.38% frequency in R medium

(b) Appearance of wild type plants at 99% frequency from unselected plates in W medium.

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Regeneration of plants = R0 generation

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Selfing of R0 plants to obtain seeds OF R1

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R1 generation,screening plants for resistance to

Fusarium oxysporum.

|

Selfing individual R1 progeny to obtain R2 seeds.

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R2 progenies. Screening plants for resistance to

Fusarium oxysporum.

MATERIALS AND METHODS

GROWING Ro PLANTS FOR SEED PRODUCTION:

Seeds of Ro progeny were sown in the 6" earthen pots in the green house. Soil was throughly drenched with bordeaux mixture before sowing to avoid damping off, which is common disease in tobacco nursery. Seedlings of 1-2 month old were transplanted to field with 90 X 75 cms of spacing. Farm yard manure was added to the field at the rate of 56 kg/ha before transplantation. Field was irrigated three times at an interval of 30 days. Occasionally TMV infection was noticed, such plants were removed from the field and thus were never processed for genetical analysis upon flowering, the young flower buds (= unopened) were covered with paper bags (Fig. 7) to ensure self-pollination. Seeds were harvested from individual plants from dry capsules and stored in cool and dry place with proper labeling of progeny plant code number.

PRODUCTION OF R1 AND R2 PLANTS:

Seeds obtained after selfing Ro plants were used to obtain R1 generation plants. R1 plants were grown in the field and were selfed in the same manner as described

FIG.7.— Growing of R1 progeny plants in the field. Young flower buds were covered with paper bags to ensure self pollination.

above. R1 generation plants were inturn selfed to obtain R2 generation plants.

GENETIC ANALYSIS OF R1 AND R2 PROGENIES FOR FUSARIUM WILT RESISTANCE:

Atleast 20-30 plants from each of R1 and R2 progenies were analysed for their Fusarium resistance reaction using the leaf disk bio-assay as well as by the direct pathogen infection assay. Data obtained from these were used to grade phenotypes of regenerants in reference to the parent A2 variety as shown in (Fig.8). Discrepancies between observed and expected values of progenies were stastically tested by Chi-square test (Redei, 1982).

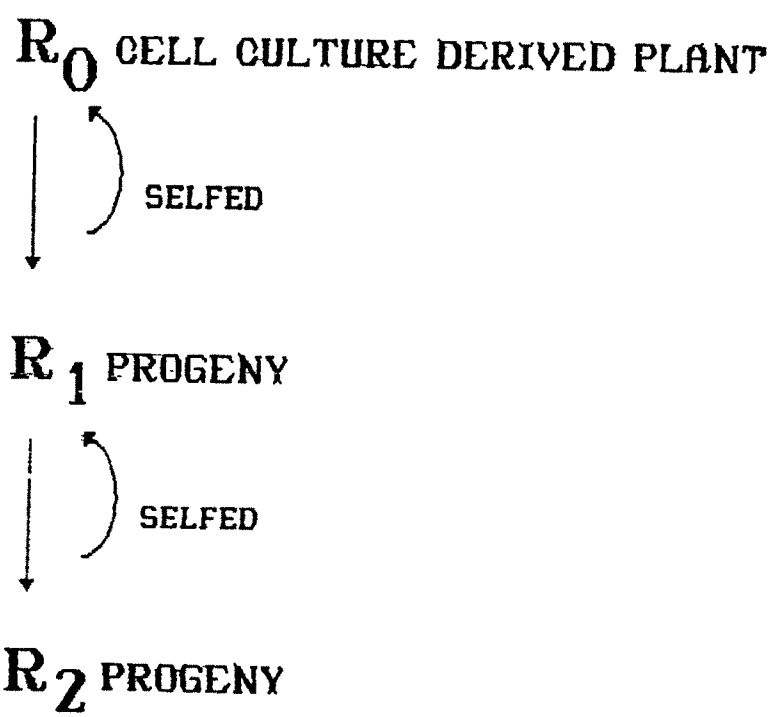


FIG- .8

SCREENING METHODS FOR FUSARIUM WILT RESISTANCE

1. LEAF DISK BIOASSAY (Selvapandiyan, 1987):

Incubation of leaf disk in LD 50 concentration of fungal culture filtrate (28% v/v) at 25°C for 24 h.

↓

Assay of chlorophyll using Arnon's procedure (1949)

↓

Rating of plants based on values of chlorophyll degradation as % of control.

5-15% loss of chlorophyll = Highly resistant (RR)

15-35% loss of chlorophyll = Moderately resistant (Rr)

> 50% loss of chlorophyll = Susceptible (rr)

2. LIQUID CULTURE METHOD (ROBERTS AND KRAFT, 1971)

Incubation of 3-4 month old seedling in LD 50 dose of culture filtrate for 10 days at 25 ± 2°C and 80% RH in glasshouse.

↓

Rating of plants based on Wilt Index:

0-20% wilt index = Resistant (RR)

20-40% wilt index = Moderately resistant (Rr)

> 50% wilt index = Susceptible (rr)

3. SICK SOIL METHOD (KAMAT, 1953) Growing of tobacco plants in soil containing Fusarium mycelia (10%)

Rating of plants based on wilt index

5-15% wilt index = Highly resistant (RR)

25-35% wilt index = Moderately resistant (Rr)

> 50% wilt index = Susceptible (rr)

χ^2 test or Goodness of Fit

$$\chi^2 = \Sigma \left(\frac{(\text{observed} - \text{expected})^2}{\text{expected}} \right)$$

When Σ stands for sum of the values.

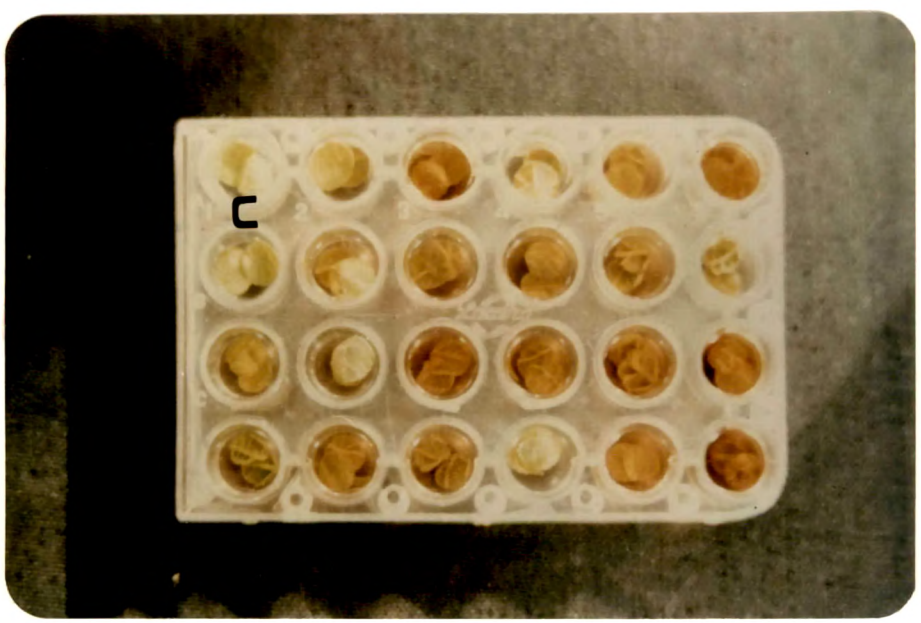
χ^2 = summed up value.

This calculated χ^2 value based on the χ^2 distribution with the appropriate degree of freedom.

$$Df = n - 1$$

Here Df was taken as 2 since we have observed three types of phenotypes namely Highly resistant, moderately resistant and susceptible.

FIG 9. A typical analysis of RØ regenerants for their disease resistance using the leaf disk bio-assay.



C = CONTROL

VARIOUS SCREENING METHODS USED FOR FUSARIUM WILT RESISTANCE IN TOBACCO Ro, R1 AND R2 PLANTS:

LEAF DISK BIO-ASSAY:

Preliminary screening of Ro, R1 and R2 plants was done using leaf-disk bio-assay as described in detail in the earlier chapter (FIG.9). Concentration of culture filtrate which caused 50% reduction in the chlorophyll content as compared 'O' h leaf disks of parental variety A2 was judged as LD 50 dose. Data of chlorophyll content was used to grade the phenotypes of regenerants.

LIQUID CULTURE METHOD:

Three months old 15-20 cm tall, field grown tobacco plants bearing 5-6 leaves of R1 and R2 generations were gently removed from pots in such a way to cause minimum damage to the roots. Further roots were surface sterilized using 0.1% $HgCl_2$ for 2-3 minutes and thoroughly washed with sterile distilled water for 3 to 4 times. These plants were positioned in 250 ml Erlenmeyer flasks in such a way that their roots remained fully immersed in LD50 CF (28% v/v i.e. the dose of CF in which more than 50% plants of A2 variety were killed). The detailed procedure for the

maintenance of fungus and the preparation of fungus culture filterate has been described in detail in the earlier chapter. Control plants were subjected to sterile Richard's medium (uninoculated). The neck of the flasks were closed by sterile cotton bungs which provided support to the arial shoots as well as prevented entry of contamination into the flasks.

The experimental set was incubated in the greenhouse at 25 ± 2° C and 60% humidity. The inhibitory effect of the pathogen on intact plants was expressed quantitatively as the wilt index (% wilt score). It was calculated according to Ebells (1967) after 10 days of incubation in the CF. Each plant was given a rating of 0-5 according to percent older leaves wilted as follows:

0 = No wilt symptom. All leaves fully turgid.

1 = Fewer than 25% leaves wilted.

2 = 25 - 50% leaves wilted.

3 = 50 - 75% leaves wilted.

4 = 75 - 100% leaves wilted but the apical shoot tip remained green bearing turgid leaf primordia.

5 = dead plant.

Sum of wilt score of individual plants in each treatment was done to calculate wilt index (% wilt score for each treatment as follows :

$$= \frac{\text{sum of wilt scores of all plant in the treatment.}}{\text{Number of plants per treatment.}} \times 100$$

RESULTS

It may be necessary here to briefly describe the method used by Selvapandiyan (1987) to regenerate Fusarium resistant tobacco plants from in vitro cell cultures. Initially filtered cells of suspension culture was plated in the culture medium supplemented with LD50, LD70 and LD90 concentration of filter sterilized culture filtrate of F. oxysporum f.sp. nicotianae. LD50, LD70 and LD90 respectively refer to the 50%, 70% and 90% reduction in number of colonies of tobacco cells on agar plate when the MS culture medium was supplemented with 28%, 34% and 49% v/v of the culture filtrate of pathogen. These doses were used to select resistant cell clones using a population of 2 X 10^4 live cells. Clones selected at LD 90 failed to grow further upon subculture even when the selection pressure was removed from the medium. About 20-30% of clones selected at LD 50 and LD 70 survived subcultures and formed large calli. After their continuous growth for 50 days in the selective media, they were transferred to the shoot induction medium. Surviving calli formed shoots within a period of a month. Shoots derived from LD 50 grown calli were healthy and showed continuous growth, but those of LD 70 grown calli were highly vitrified and did

not grow further even after media were modified to overcome vitrification as suggested by Ziv et al (1983). Eventually 5-10 cm long shoots of only LD 50 treated callus were transferred to rooting media and subsequently grown in pots. In all 76 such regenerants (Ro) were produced as LD 50 resistant plants from plated cells. The entire procedure is shown in the flow sheet diagram in(Fig. 10).

Total 76 regenerants were obtained in LD50 medium from the original 2 x 10^4 live cells by Dr. Selvapandiyan (1987). When these plants were analysed for wilt resistance using leaf disk bioassay, they showed an array of resistance to the fungus as shown in Fig ( ). Out of 76 plants 8 plants were selected which showed around 2 fold increase in fungal resistance when subjected further by the liquid culture method of screening. All these 8 Ro plants were given code no. RoC1 - RoC8. R represents the generation status and C represents the clone number. Thus Ro are the plants asexually produced directly from cell culture, which is equivalent to "P" generation of conventional genetic term.

FIG.10. Experimental protocol

Cell line selection of tobacco resistant to F. Oxysporum.

EXPERIMENTAL PROTOCOL

Cell line selection of tobacco resistant to *F. oxysporum*

Initiation of callus culture from leaf disk of Tobacco var. A2 on MS + 0.1 µM IAA & 2 - 10 µm Kn.

3 months

Generation of cell suspension

2 months

Filtration to isolate clump of 2-4 celled

Culture of *F. oxysporum* on modified Richard's Solution

CF

Plating in LD50, LD70 & LD90

Clonal selection & regeneration

Leaf disk bioassay to test resistancy

Genetic analysis of R1 progeny - Each of the moderately resistant plants (RoC1 - RoC8) were selfed to obtain the seeds which were grown to produce R1 generation plants equivalent to F1 plant of conventional genetic term. About 15 - 20 number of progeny plants from each of the clone were screened for Fusarium resistance. The data obtained was used to segregate the R1 population. Out of 8 moderately resistant regenerants, 6 showed the segregation pattern of 1 (highly resistant) : 2 (moderately resistant) : 1 (susceptible) ratio. One showed segregation pattern of 3 (moderately resistant) : 1 (susceptible) and another one did not fit significantly into either 1:2:1 or 1:3 pattern of inheritance as of other Ro clones (Table.16) .

Total 23 plants of R1 C1 clone were analysed for their disease resistance (Table 18.1). Out of them, plant no. 9, 18, 20, 21, 22, 23 showed the least chlorophyll degradation compared to control and it was in the range of about 5-15% mg/g/ fresh wt. % to control in the leaf disk bioassay. Here control represents the leaf disk of tobacco plants produced from unselected cells. Further when subjected by the liquid culture method, all these plants did not show any wilt symptom even after 10-15

days of incubation in CF. Therefore all these 6 plants were considered to be highly resistant plants. 5 plants (no. 1, 15, 16, 17, 19) showed the highest (i.e. more than 50%) chlorophyll degradation to control and the wilt index for these plants was high i.e. 70-100%. They are therefore rated as susceptible plants. Remaining plants (Plant No. 2,3,4,5,6,7,8,10,11,12,13 and 14) displayed the chlorophyll degradation in the range of 25% - 35% of control and the corresponding average wilt index of all these plants were 30% and thus were considered as moderately resistant (Table 18.1).

The plants that were found highly resistant, moderately resistant and susceptible in the leaf disk bio-assay were also found to fall into same categories when whole plants were infected with the pathogen in the sick soil method.

Only 4 plants of clone no 4 of R1 generation were tested, of which two were moderately resistant, one was highly resistant and the remaining one was susceptible, indicating 1:2:1 ratio of inheritance of disease resistance (Table.16).

Similarly R1 clone no. 2,3,5,6 showed the segregation pattern of 1 (highly resistant) : 2 (moderately

resistant) : 1 (susceptible ratio). Their x^2 values and the probabilities were in close agreement with their inheritance pattern of 1:2:1 (Table). R1 clone no. 7 showed the segregation pattern of 15 (highly resistant), 0 (moderately resistant) and 6 (susceptible) out of the 21 plant tested. Its x^2 value (0.158) and probability (90-100%) were in close agreement with its 3:1 ratio of inheritance. R1 clone no. 8 did not show any known pattern of inheritance. In this clone out of 22 plants tested 11 plants were found to be highly resistant, 7 were moderately resistant and 4 were found to be susceptible. Thus it did not show any known pattern of Inheritance. The x^2 value of this clone was very high 7.3 and the probability was 5% suggesting its disagreement with both 1:2:1 and 3:1 pattern of inheritance (Table. 16).

Wilt symptoms appeared within 10 days in the whole plant infection of progeny plants with CF of Fungus when compared to the direct fungal mycelial infection i.e. 30 days.

In the homozygous susceptible plants root portions were heavily infected with sever browning and rottening.

Disease symptoms appeared at a slower rate in the

heterozygous plants than in the homozygous susceptible plants. Thus the heterozygous plants were not totally immune to the pathogen infection. It could only stop further colonization of the pathogen whereas homozygous resistant plants did not show any visible wilt symptoms further, they formed new adventiteous roots. (Fig.11).

Genetical analysis of R2 generation - Inheritance pattern of each three different types (Highly resistant, moderately resistant and susceptible) of R1 plants was studied in R2 generation after selfing them. Four moderately resistant, two highly resistant and two susceptible R1 plants were selfed to obtain R2 progeny.

Pattern of R2 segregation confirmed that the progenies of highly resistant plants (=R1) and susceptible plants behaved true to their parents whereas progenies of moderately resistant plants were again segregated into three types of highly resistant, moderately resistant and susceptible with 1:2:1 ratio (Table.17). Thus all the 10 R2 progeny plants of highly resistant R1 plants (C1 plant No. 9 and 18) were found to be highly resistant.

And similarly all 10 R2 progeny plants of susceptible R1 (C1 plant no. 15 and 16) were turned out to be susceptible, suggesting their homozygous recessiveness

FIG.II. Infection of R2 generation susceptible(rr), moderately resistant (Rr) and resistant (RR) tobacco plant by Fusarium oxysporum using the liquid culture procedure. Note the development of new adventitious roots from the resistant plant.

C rr Rr RR

C rr Rr RR

for susceptibility to wilt.

Whereas moderately resistant R1 plants (C1 plant No. 2, 3, 4 and 5) again segregated into 1:2:1 ratio of inheritance. Their X^2 values were in the range of 0.2 - 1.32 (Table 17) and their corresponding probability values were in the range of 80-90%, indicating their close fit to the theoretical ratio of 1:2:1.

TABLE 16

SEGREGATION OF MODERATELY RESISTANT RO PLANTS IN R1 GENERATION. PLANTS OF RO WERE SELFED TO OBTAIN R1 PROGENY. THE R1 PROGENIES WERE SCREENED FOR THEIR DISEASE REACTION USING THE LEAF DISK BIOASSAY AND FUNGAL INFECTION.

Clone No.	No. of Plants Screened	Observed ratio among R1			Expected Ratio	X^{2}	P
		Highly Resistant	Moderately Resistant	Sucseptible			
Unselected Control	5	0	0	5	-		
Clone No. 1	23	6	12	5	1:2:1	0.13043	90-100
Clone No. 2	13	3	8	2	1:2:1	0.8461	50-70%
Clone No. 3	14	5	7	2	1:2:1	1.2856	50-70%
Clone No. 4	4	1	2	1	1:2:1	0.00	100%
Clone No. 5	21	6	11	4	1:2:1	1.125	50-70%
Clone No. 6	29	6	19	4	1:2:1	2.861	25%
Clone No. 7	21	15	0	6	3:1	0.0158	90-100
Clone No. 8	22	11	7	4	1:2:1	7.30	5%

TABLE 17

SEGREGATION PATTERN OF R2 GENERATION. INDIVIDUAL R1 PLANTS OF CLONE NO. 1 WERE SELFED TO OBTAIN R2 PROGENY. THE R2 PROGENIES WERE SCREENED FOR THEIR DISEASE REACTION USING THE LEAF DISK BIOASSAY AND FUNGAL INFECTION.

Plant Code	No. of Plants Screened	Observed ratio among R2			Expected Ratio	X^2	P
		Highly Resistant	Moderately Resistant	Sucseptible			
Unselected Control	5	0	0	5	–	–	–
C_1. Plant 2	10	2	5	3	1:2:1	0.2	90%
C_2. Plant 3	10	2	5	3	1:2:1	0.2	90%
C_4. Plant 4	12	2	8	2	1:2:1	1.32	50-70%
C_{11}. Plant 5	10	2	5	3	1:2:1	0.2	90%
C_{15}. Plant 15	10	All are homozygous recessive					
C_{16}. Plant 16	10	All are homozygous recessive					
C_9. Plant 9	10	All are homozygous dominant					
C_{18}. Plant 18	10	All are homozygous dominant					

* Each plant has a control in which leaf disk were incubated in sterile Richards medium.
* Control plants for liquid culture method were incubated in sterile water.
* Chlorophyll content at 0 h as well as after 72 h of incubation were calculated based on which the % chlorophyll degradation of each plant in treatment to control were calculated.
* The values represented here are mean of at least 3 replicates.
* The data clearly shows the segregation of plants of each clone into moderately resistant, highly resistant and susceptible with respect to both the chlorophyll degradation as well as the wilt index in the same degree and thus were rated into these groups based on the values as shown in the Figure.8 of Material and Methods.
* R1 = Generation Status

  C1 = Clone No.

TABLE 18.1

R1 CLONE NO. 1

C1 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	0.98	0
1	73.60	100
2	35.25	40
3	28.12	25.5
4	35.21	40
5	35.13	40
6	34.96	40
7	31.48	30
8	35.19	40
9	12.30	10
10	28.85	25
11	25.16	20
12	34.19	32
13	25.75	20
14	35.12	40
15	68.14	80
16	86.83	100
17	75.03	100
18	12.40	10
19	72.44	100
20	6.61	0
21	5.70	0
22	8.05	0
23	6.67	0

TABLE 18.2

R1 CLONE NO. 2

C2 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	0.98	0
1	73.46	100
2	80.91	100
3	35.12	38
4	26.12	20
5	12,30	10
6	33.90	35
7	31.38	30
8	29.91	28
9	33.96	35.5
10	26.46	20
11	5.16	0
12	33.18	35
13	10.16	10

TABLE 18.3

R1 CLONE NO. 3

C3 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	1.10	
49	6.46	100
50	70.11	100
51	70.11	100
52	15.16	10
53	35.18	40
54	14.92	10
55	35.18	42
56	30.12	28
57	26.48	20
58	35.58	40
59	14.08	10
60	15.26	10
61	26.42	25
62	28.76	25

TABLE 18.4

R1 CLONE NO. 4

C4 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	0.98	0
1	35.28	40
2	35.32	40
3	75.88	100
4	5.08	0

TABLE 18.5

R1 CLONE NO. 5

C5 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	0.98	0
1	26.42	20
2	35.32	10.5
3	15.30	10
4	35.11	38
5	33.28	30
6	26.38	25
7	66.67	80
8	28.12	25
9	14.92	10
10	35.36	40
11	33.31	30
12	72.59	100
13	35.1p	38
14	35.20	38.5
15	15.11	10
16	35.11	38
17	11.99	8
18	15.27	10
19	13.07	10
20	72.90	100

TABLE 18.6

R1 CLONE NO. 6

C6 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
Control	1.10	0
93	33.12	32
94	77.48	100
95	28.92	25
96	33.12	32
97	70.68	100
98	70.68	100
99	27.91	25
100	35.12	42
101	15.28	10
102	14.11	10
103	35.38	40
104	14.98	10
105	14.00	10
107	34.26	32
108	28.92	20
109	34.82	32
110	33.12	30
111	33.22	30
112	15.31	10
113	32.89	28

Contd...

TABLE 18.6 (Contd...)

R1 CLONE NO. 6

C6 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
114	35.89	40
115	35.41	40
116	35.01	40
117	70.48	100
118	14.09	10
119	33.98	30
120	33.16	30
121	35.32	38

TABLE 18.7

R1 CLONE NO. 7

C7 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
1	11.99	10
2	13.05	10
3	14.96	10.5
4	72.56	100
5	15.30	10.5
6	15.30	10.5
7	72.90	100
8	13.08	10
9	66.67	70
10	13.05	10
11	13.62	10
12	13.67	10

TABLE 18.8

R1 CLONE NO. 8

C8 Plant Code No.	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
134	30.16	28
135	32.89	30
136	10.76	0
137	15.18	10.5
138	15.28	10.5
139	8.60	5
140	15.26	10
141	62.17	60
143	40.20	37.5
144	60.16	60

R1 = Generation Status

C_n = Clone No.

TABLE 18.9

Plant Code	Chlorophyll loss % to Control mg/g/ fresh weight	Wilt Index
A2 variety	56.50	100
Unselected Control 1	66.00	100
Control 2	70.00	100
Control 3	72.00	100

DISCUSSION

The genus Nicotiana to which tobacco (tabaccum and rustica) belongs, has provided excellent material for the past half a century for fundamental genetic studies. These studies have contributed to an understanding of certain major problems such as quantitative inheritance, heterosis, self and cross incompatibility, interspecific hybridization and gene transfer and monosomic analysis.

However, the polyploid nature of both the cultivated species, viz. N. tabaccum and N. rustica, has restricted the scope of critical genetic studies on them. It has been more difficult in the case of economic characters which are polygenic. On account of these limitations, investigations were concentrated on simply inherited characters mostly in N. tabaccum and some in N. rustica.

The genetic control of disease resistance has been established in certain cases. For example, Mosaic resistance was controlled by two recessive genes (Clayton et al., 1938). Fusarium wilt resistance was controlled by a partially dominant gene (Johnson and Fulton, 1952). Resistancy to Powdery mildew was controlled by two recessive genes (Wan, 1962). Whereas

resistance to Bacterial mildew was controlled by multiple genes.

Fusarium Spp are pathogenic on large number of economically important plants causing the wilt disease (Mace et al, 1981). Resistance to this Pathogen in alfalfa (Hartman et al, 1984) Potato (Behnke, 1980a , Schuchmann, 1985) Tomato (Shahin and Spivey, 1986) and Celery (Heath Pagliuso et al, 1989) has been developed employing the approach of somatic cell genetics.

Many of plant Pathogens produce phytotoxic substances which have varying degree of role to play in disease development. Some phyto toxins e.g. Methionine sulfoximine from Pseudomonas tabacii (Carlson, 1973) produce only part of the disease symptom. Selection for resistance to these kinds of toxins will probably give at best only partial resistance to disease.

The selection of tissue cultures with resistance to a host-specific toxin (T-Toxin) has been used to obtain maize plants resistant to Southern corn leaf Blight (Gengenbach et al., 1975). Oat plants (Avena sativa) insensitive to the pathotoxin victorin which is produced by Helminthosporium victoriae, were obtained by in vitro selection (Rines and Luke, 1985). Since then a number

of investigators (Ling et al., 1985; Wenzel, 1985) have used the pathotoxins in form of crude and/or purified toxins in selecting disease resistant mutants.

Previous workers have used either the crude culture filtrate of Fusarium oxysporum as in alfalfa (Hartman et al, 1984a) and Potato (Behnke, 1980a) or the general non-specific toxin fusaric acid of this pathogen as in tomato (Shahin and Spivey, 1986) for the in vitro selection of resistant culture.

In our studies, treatment of fusaric acid to susceptible variety A2 of tobacco plant showed neither the differential varietal toxicity nor could it induced other disease symptoms like yellowing of leaves and blackening and softening of root cortical tissue. However the crude culture filterate of the pathogen could induce many of the disease symptom as a result, we preferred the crude culture filtrate as the selection agent for the in vitro selection. The range of culture filtrate concentration (LD 50 = 28% v/v of the culture medium) used in our selection experiment is identical to that used by Behnke (1980a) in potato and Hartman et al (1984a) in alfalfa. We believe that the use of culture filtrate for the selection experiment is appropriate in the absence of any host specific toxin as in the case of

Fusarium Oxysporum. We had to compromise with the culture filtrate as it could induce many of the disease symptoms akin to the fungus.

Thus the better we understand the mode of action of the toxins used in the screening process, the more effectively we can design experiments for in vitro selection.

There are two methods to select cell line resistant to the toxin. One method uses low concentration (= sub lethal) of CF in the first round of selection. Gradually stepwise increase of culture filterate is done in each successive round of selection. This method proved successful in selecting maize plant resistant to the T toxin of Helminthosporium maydis race T (Gengenbach et al, 1977). However, a problem with the step-up procedure is that it is long-term and may result in accumulation of undesirable genetic and chromosomal change, in cluding elevated ploidy levels which then complicates inheritance studies of resistant plants.

Exposure to the high concentration of culture filterate in the medium and subsequent intense but short term selection resulted in stable resistant cell lines to Fusarium in alfalfa (Hartman et al., 1984a). Harbor and

Green demonstrated a similar benefit from short term selection in maize.

The recovery of disease resistant mutants without selection pressure was not a surprise, since there are frequent recovery of disease resistant regenerants from unselected cultures (Brettel et al 1980; Larkin and Scowcroft 1983; Umbeck and gengenbach 1983; Ling et al 1986). For example Evans and Sharp (1986) recovered 13 plants with single gene mutations from a population of 230 regenerated tomato plants without employing any selection pressure.

Shahin and Spivey (1986) regenerated Fusarium-resistant tomato plant from mesophyll protoplasts from both non-selective and selective medium. This is in contrast to the findings of Sarcristan (1982,1985) on Brassica/phoma selection system using toxic culture filtrate. In this case not all the regenerants from selected cultures showed increased resistance, but the proportion of resistant or tolerant plants was upto six time higher among regenerants in the selected cultures than among the plants from unselected control. This further indicates that the frequency of a defined and desirable mutation frequency can be enhanced by the usage of host

specific toxin of the pathogen as was reported in our selection system.

In contrast to both these findings in our selection system the unselected regenerants of both R0 and their R1 progenies obtained after selfing were susceptible to the pathogen like the parental plant A2. Hence in our case the resistancy in the selected resistant regenerant to Fusarium wilt is clearly due to the selection pressure.

We wish to give here two possible reasons for our successful production of stable resistant mutant of tobacco to Fusarium wilt coupled with the absence of variations among the unselected regenerants. They are (1) We used cell suspension cultures for our selection experiment to achieve uniform exposure to the CF of the pathogen in contrast to the callus used by many earlier researchers and (2) We have adopted a short-term and single step method of selection in which relatively milder selection pressure of LD-50 dose of culture filtrate was used.

Different levels of resistance occur by using different concentrations of phytotoxin. This will lead to the false interpretations of the results (Nielson et al.,

1979). To avoid this we have screened all the progenies in Ro, R1 and R2 by using the same LD50 (= 28% v/v) dose from from which the resistant cell lines were obtained. Further, the LD50 dose of culture filtrate which caused death of 50% cells in vitro was found to be similar to that of LD50 of CF (28% v/v) which caused more than 50% death of the plants due to wilt.

In his review, Wenzel (1985) discussed several reports where resistant-mutants were recovered even by using non-host specific toxins. However, none reported the mode of inheritance of the recovered resistance or the nature of defense mechanism (S) of the involved. Thanutong et al (1983) observed nuclear inheritance in tobacco selected protoplasts for resistance to Pseudomonas syringae and Alternaria alternata. However, their experiments have not revealed the mode of inheritance of resistance to these two pathogens.

Sacristan (1982) reported a screening procedure for Brassica napus to Phoma lingam using its toxic CF to make selections. Not all regenerants from selected cultures showed increased resistance, but the proportion of resistant or tolerant plants was upto 6 times higher among regenerants from selected cultures than among plants from controls. The first three progeny

generations were tested for their level of resistance. Unexpectedly, segregation was found in the progenies of plants classified as resistant although in all cases the proportion of resistant plants was highest for the progenies obtained from resistant material, indicating genetically defined resistance.

Resistance to Fusarium has been developed in alfalfa (Hartman et al, 1984a) Potato (Behnke, 1980a, Schuchmann, 1985) tomato Shahin and Spivey, 1986) and Celery (Heath Pagliuso et al, 1989) by employing the approach of somatic cell genetics. However genetical analysis of wilt resistant phenotype in the regenerated plants has been carried out only in case of tomato and celery. Wilt resistance phenotype in tomato was governed by a single dominantly expressed gene and in celery by two genes.

In case of tomato Shahin and Spivey (1986) recovered 4 resistant mutants out of 4 x 10^{5} number of protoplasts that were challenged with 20 um concentration of fusaric acid. Thus the % frequency of obtaining resistant mutants was 0.0001. These 4 resistant Ro plants were selfed. About 79,R1 progeny plants were screened for their disease resistance to

fusarium using Fusarium slurry technique, 3-4 weeks after inoculation. The resistant plants were allowed to grow for additional 3-4 weeks and then scored again to eliminate escape. Data obtained from this showed a ratio of three resistant to one susceptible among the R1 progenies. Statistical analysis of the data using Chi-square test showed the low x^2 (between 0.19 - 1.85) and high probability value (between 0.05 - 0.8) indicating the high validity of the 3:1 ratio of R1 progeny plants for disease resistance. One of R1 progeny mutant lines was selfed and the resulted R2 progeny were screened with fusarium slurry method. Further analysis of the R2 progeny confirmed that the Fusarium wilt resistance phenotype in tomato was governed by a single dominantly expressed gene.

Another deviation of our finding from the known published results is with respect to the inheritance pattern of disease resistance in the regenerated tobacco plants.

Out of eight moderately resistant regenerants (=Ro) six individuals showed the segregation pattern of 1 (highly resistant) : 2 (Moderately resistant) : 1 (Susceptible) ratio. One showed segregation pattern of (3 moderately resistant) : 1 (susceptible) and another one did not fit

significantly to any known pattern of inheritance. Inheritance of each three different types of R1 plants was studied in R2 generation after selfing them.

Pattern of R2 segregation provided additional evidence for Mendelian inheritance. Progenies of highly resistant plants (=R1) behaved as true to their parent (= all were highly resistant). Progenies of moderately resistant plants were again segregated into three different types of highly resistant, moderately resistant and susceptible with 1:2:1 ratio and progenies of susceptible (R1) were again true to parents. This means that tissue culture selection procedure isolated majority of plants (6 out of 8) which were moderately resistant and thus were heterozygous for their resistance genotype. This pattern of inheritance indicates partial dominant nature of resistant gene.

A novel and quantitative leaf-disk assay method adopted for screening of plants was found to be rapid and highly reproducible. Because the plants confirmed as wilt resistant by leaf-disk study were also found to be resistant in whole plant infection with CF of the pathogen.

The homozygous dominant plants = highly resistant plant

possessing resistant gene (RR) were distinguished from the heterozygous plants (moderately resistant Rr) in that the former had the 0 value of wilt index even after 10 days of incubation by the pathogen. The heterozygous plants had 30-40% wilt index under this condition. The susceptible plants as well as the unselected control regenerants developed 100% wilt index during this period. Loss of chlorophyll in the leaf disk bio-assay was greatest for the leaves of susceptible plants, intermediate for the leaves of heterozygous plant and lowest for the leaves of resistant plants.

Disease symptoms appeared at a slower rate in the heterozygous plants than in the homozygous susceptible plants. In the homozygous susceptible plants the root portions are severely infected with the pathogen showing the complete browning and rottening. The heterozygous plants were not totally immune to the pathogen. The reason for their immortality is that in contrast to the susceptible individuals, further spread of the pathogen was checked. In the homozygous resistant plants no infection was seen.

Generally a resistance gene that is effective against one wilt fungus also confirms resistance against certain

other pathogens. For example resistance of soyabeans to Cephalosporium is effective against Verticillium (Tachibana 1971). Cotton plant resistance to Verticillium is resistant to Fusarium (Bell and Mace, 1981). Incidentally we have also observed that seedlings of Fusarium resistant RI plant were also resistant to the damping off disease caused by Pythium, which is common occurrence at nursery stage of tobacco plants.

Plants that were found resistant in the both leaf-disk bio-assay and the liquid culture method, were handed over to Agriculture University, Anand. Further they proved to be resistant by the direct fungal attack in sick-plot method and were commercially accepted. Thus these three step screening procedure was found to be highly effective and did not allow any escapes.