CHAPTER 5

SELECTION OF WILT RESISTANT TOBACCO FROM TISSUE CULTURE

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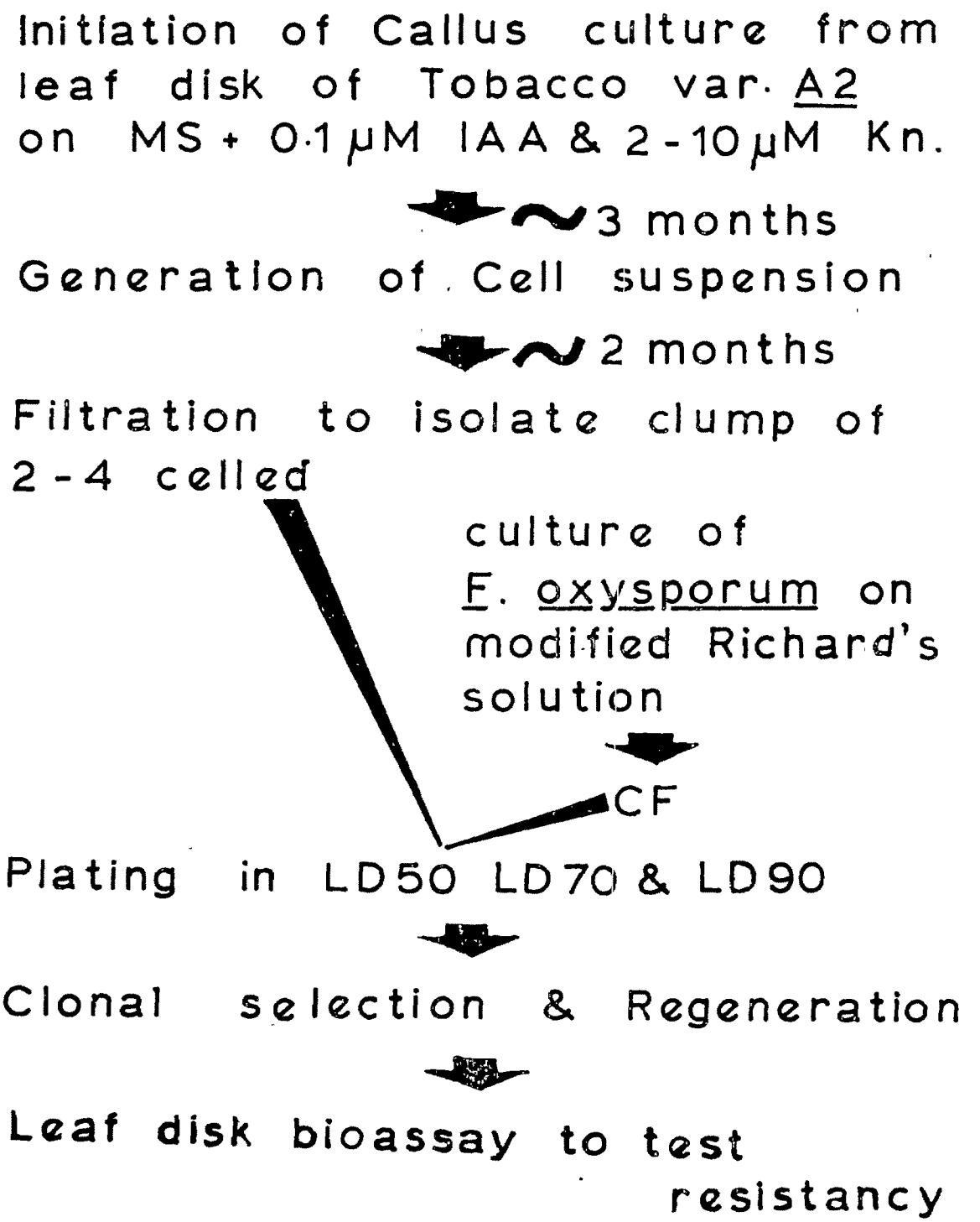
EXPERIMENTAL PROTOCOL 3



obtained from the inoculated leaf disks was subcultured every 2 weeks in the same medium for 3-4 times. The friable callus was transferred to liquid media of the same chemical composition minus agar.

5-B.2 Preparation of the CF of F. oxysporum :

The CF obtained from the 10 days old Fusarium culture, was filter sterilized (as mentioned in previous chapters) and was used as the selection agent. It was mixed with sterile MS medium* in various volume to volume proportions.

5-B.3 Determination of sensitivity of the tobacco cultures to the fungal CF :

Before performing a selection experiment it is necessary to determine the sensitivity of the host cells to the toxin under standard selection conditions. This is more appropriately done by establishing a dose response curve between the degree of toxicity of CF on cultured tobacco tissues vs. concentration of CF.

The fungal CF was mixed with sterile, molten (at 45 to 50°C) 1.2% agar based (final agar conc. after mixing with cells is 0.6%). MS medium* with different concentrations of CF viz.,

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* MS medium was supplemented with 0.1 μM IAA, and 2 μM Kn and 2% sucrose at final concentrations.

10, 20, ...60% v/v. The concentrations of the MS medium was adjusted such that after mixing with CF, full strength concentration of MS medium was achieved.

An actively growing cell suspension culture (log phase) of tobacco (12 days old culture) was removed from the shaker and filtered through successively smaller sizes of sterile stainless steel meshes to obtain free cells and small cell clumps made up of 2-4 cells and they were plated in MS-CF combinations as mentioned in section 4-D.1.

After 12 days of incubation, numerous 10-15 celled colonies were observed. Their number was counted under an inverted microscope to calculate the plating efficiency (section 4-D.1). Besides the determination of number of colonies, the diameter of individual colony was measured.

5-B.4 Leaf disk bioassay :

Regenerated plants were bioassayed for the disease resistance using a simple leaf disk assay. Leaf disks of 0.9 cm diameter were cut from the surface sterilized leaf and inoculated on the callus induction medium. The medium was supplemented with LD50 v/v concentration of CF*. The change in their fresh weight was determined after incubation for 10 days.

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* This concentration inhibits 50% of growth of leaf disk compared to control.

5-C.2 Morphogenesis from callus and cell suspension culture:

Differentiation of shoots from callus was achieved on solid MS medium supplemented with 1 µM IAA and 0.1 to 1 µM Kn and that of roots with 10 µM IAA and 1 µM Kn. The appearance of shoot required 12 days and of roots 10 days after the transfer of callus on morphogenetic media.

5-C.3 Sensitivity of tobacco cells to the CF of F. oxysporum:

Survival of tobacco cells on agar plate in increasing concentration of the CF was determined (Fig.5-2). When number of colonies per plate was counted, it was found that the CF concentration above 20% v/v, of MS medium reduced the number of colonies. None of the plated cells could divide when the CF concentration was increased to 50%. Richard's medium by itself when mixed with equal volume of MS medium had no inhibitory effect on plating efficiency.

The MCD was found inversely proportional to the PE (Fig.5-2).

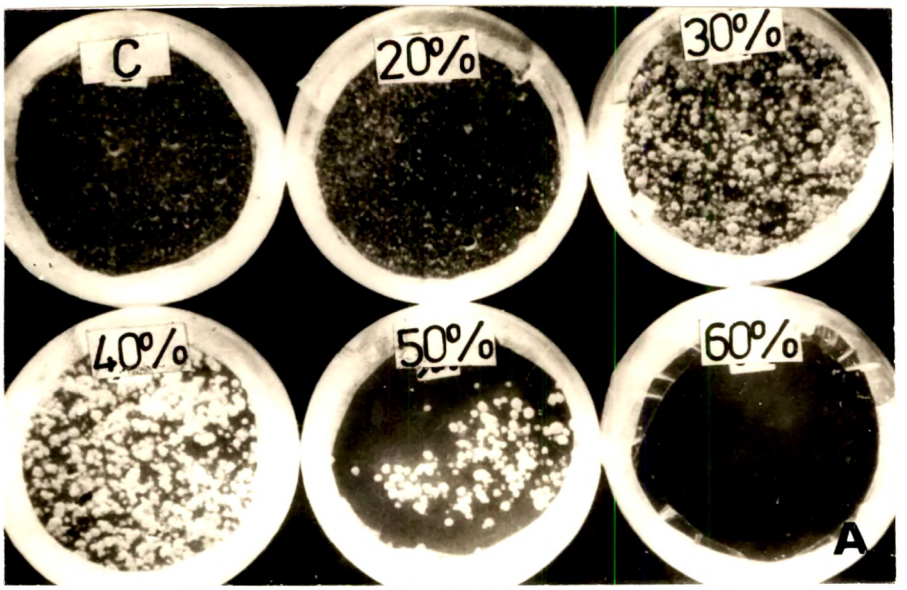
5-C.4 Selection of resistant cell lines :

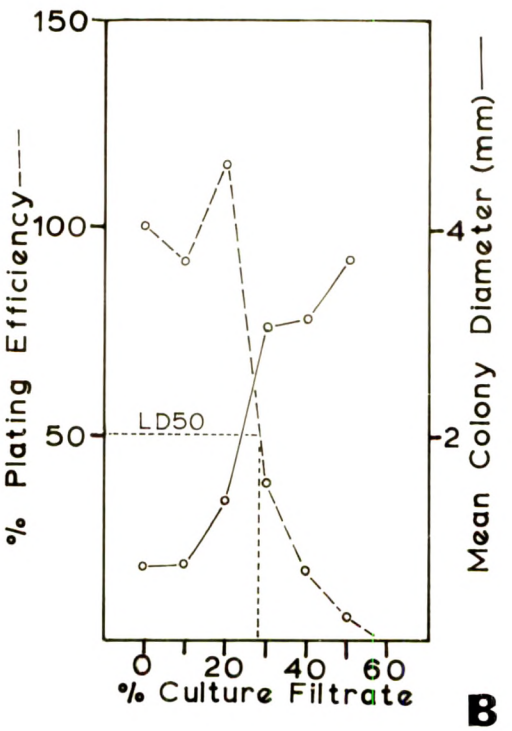
The technique of recurrent selection (Brettell et al., 1980) involves plating of host cells from its suspension culture on medium containing sublethal concentration (Handa et al.,1982) of fungal toxin or the crude CF in case where the toxin is not known, and selecting out the colonies that appear from the remainder of the cell units killed by the toxin. Lethal dose 90 (LD90) was registered at 47% CF

Fig.5-2 Dose sensitivity of CF of *F. oxysporum* on the cell plating efficiency of tobacco.

A) Growth of colonies in different CF conc.

B) Correlation between the numbers of colonies and their size.


C
20%
30%
40%
50%
60%
A


150
100
50
LD50
% Plating Efficiency
Mean Colony Diameter (mm)
4
2
0
20
40
60
% Culture Filtrate
B

(at 10% PE), LD70 as 34% (30% PE) and LD50 as 28% (at 50% PE). At these CF levels free cells and small cell clumps were plated. Resistant colonies appeared in these two lethal levels were individually isolated after 20 days of incubation and subcultured in fresh medium containing the same levels of selection pressures.

The colonies which appeared at LD90 failed to grow further when transferred to fresh medium with same (LD90) level of CF. They became gradually brown and dead. Some of them transferred to even toxin-free medium could not survive. The colonies that had appeared at LD70 and LD50 concentration of CF proliferated in subsequent transfers to respective selective media.

5-C.5 Regeneration of plants from the cultures selected as toxin resistants :

The callus cultures selected and grown continuously for 50 days at LD50 and LD70 were transferred to shoot inducing medium (MS+1 μM IAA + 0.1 to 1 μM Kn + 2% sucrose + 0.8% agar), in presence of the same level of CF. 20 to 30% of the colonies survived at LD50 and majority of LD70 (upto 80%), however, could not survive in this medium. The Rest of the callus masses in both the cases turned green and gave rise to shoot primordia. The primordia of LD50 developed after 20 days of incubation tiny shoots with few small leaves emerged (Fig.5-3). On the other hand no further growth occurred of the shoot primordia in the presence of LD70 level of CF. Some of these

Fig.5-3 In vitro morphogenesis of tobacco in presence of selection pressure.

A) Shoots differentiation on diploid callus.

B) roots differentiation on shoot.

C) regenerants transplanted to vermiculite.


A
B
C

primordia became necrotic at the proximal end and further growth could not be achieved even when they were transferred to non-selective medium.

Callus masses (picked up from the control) on transfer to shoot induction medium formed green nodules at first and healthy shoots after 20 days of incubation. Their growth was found faster by 20 days than LD50 shoots. Moreover, all LD50 shoots later on showed vitrification. Their leaves were longer than those of control shoots, but the deep green lamina was narrower. These leaves appeared succulent, brittle and slightly transparent. This problem was, however, totally overcome by increasing the agar concentration from 0.8% to 1% as mentioned by Vieitez et al. (1985). Healthy shoots, 2 to 3 cm in control and LD50, after 30 days of incubation in shoot inducing medium, were separated and transferred onto root inducing medium (1/2 MS + 10 µM IAA + 1 µM Kn + 1% sucrose). Rooting medium for LD50 plants also contained LD50 CF (the selection medium). Roots appeared within 10 days of incubation, penetrated the medium, and branched out rootlets.

5-C.6 Transplantation to the soil :

Tiny plantlets of 6 to 10 cm were transplanted to pots. Care was taken to remove agar from the roots in order not to damage the roots. About 25% plantlets survived after transplantation into greenhouse. This was raised to 75% when they

were transplanted to autoclaved vermiculite and soil mixture (1:1). Their survival rate was enhanced to almost 100% when older leaves from the plants were removed while transplanting. Similar success was achieved when the intact leaves were smeared with either 50% glycerine or paraffin wax* to avoid excessive desiccation.

The potted plants were covered with larger glass beakers to maintain humidity. They were watered every day with tap water and once a week with 1/3rd strength MS inorganic salts. The plants were gradually hardened by exposing them to the culture room atmosphere by removing the beaker periodically. After a week they were transferred to the greenhouse and repotted in the garden soil mixture. Some plants were lost in greenhouse culture due to pest infection. The pest was later identified as Spodoptera littoralis Boisd. Endocel (35 EC Endosulfan Broad. spectrum insecticide - 1000 ppm conc.) was sprayed on the plants to save the rest.

5-C.7 Haploid culture :

Anthers inoculated on NS medium (Nitsch, 1969) were squashed in acetocarmine to see the androgenetic development of the microspores. As in Datura (Sunderland et al., 1974; Reinert et al., 1975), two similar nuclei were formed (Fig. 5-4a, b), as a result of direct division of the microspore

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* Wax was dissolved in diethyl ether and applied at once.

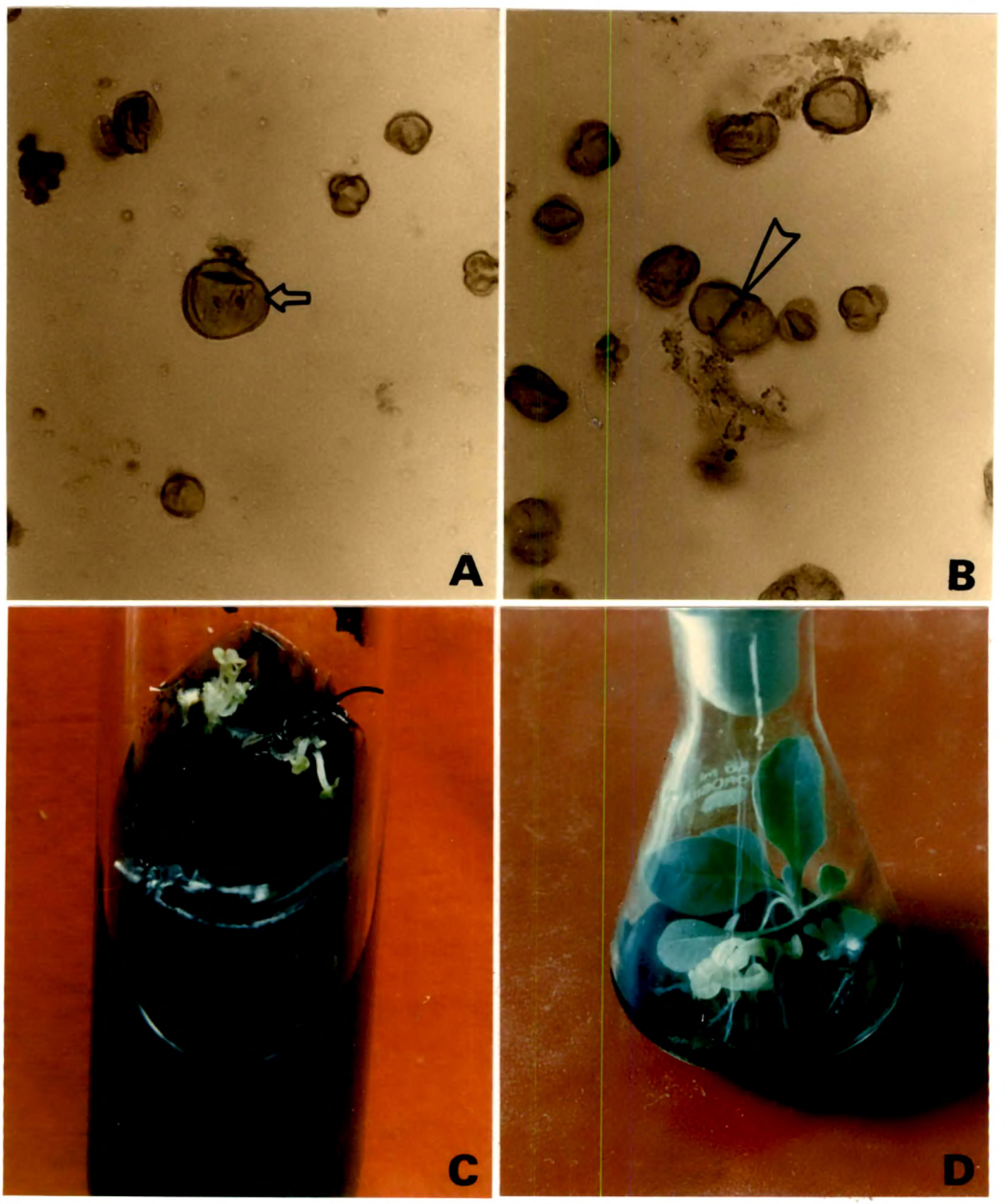
Fig. 5-4 In vitro culture of excised anthers of tobacco, undergoing androgenesis after 4 weeks :

A) microspore showing first mitosis with two similar-looking nuclei (arrow) obtained from 1-week-old anthers;

B) a bicelled embryonal microspore formed as a result of segmentation (arrow);

c) pollen plantlets emerging from cultured anthers.

D) well grown haploids.


A
B
C
D

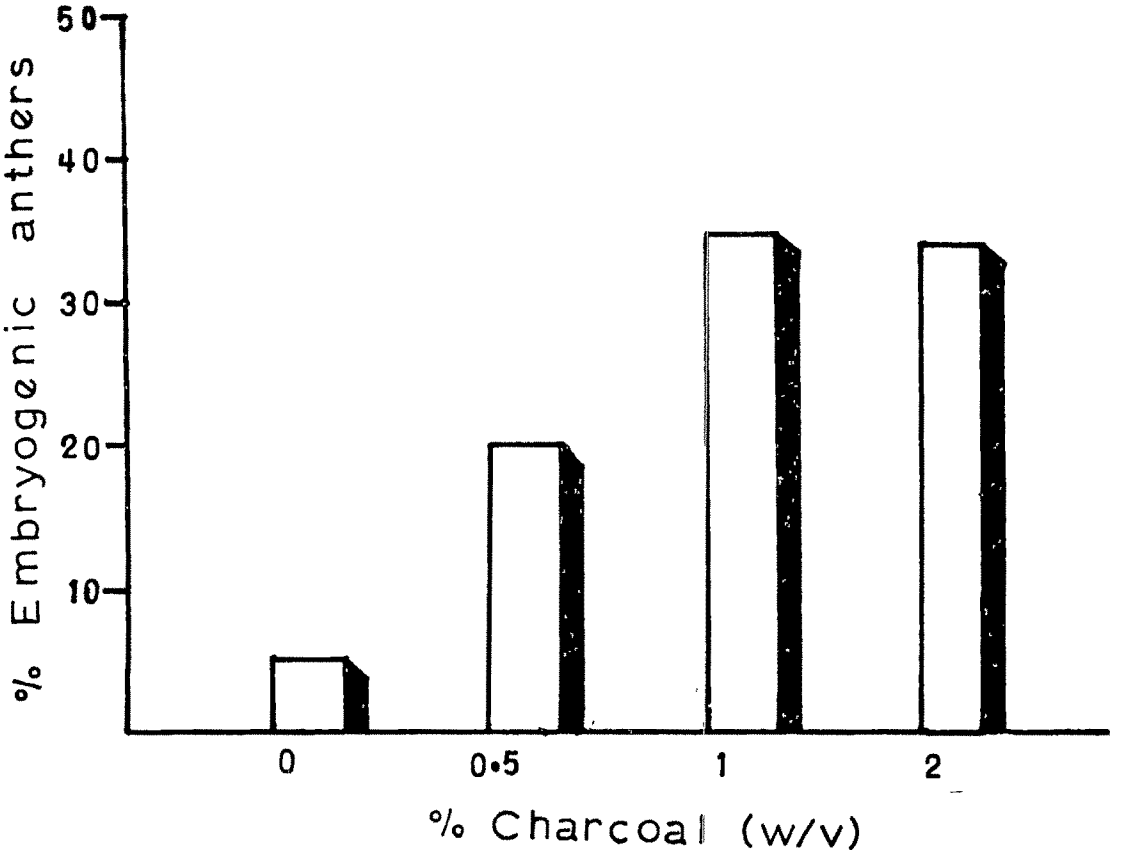
nucleus. Subsequent divisions and growth stages were observed till young torpedo shaped embryos emerged by repturing the anther wall (Fig. 5-4C). Well grown young haploids containing small roots were then transferred to 1/2 MS medium supplemented with 0.1 μM NAA. Plants of 5 to 10 cm in height were transplanted to vermiculite soil mixture after removing the older leaves.

5-C.8 Response to activated charcoal by anther culture :

To enhance the per cent of productive anther, anthers were inoculated on NS medium containing different concentrations i.e., 0.5, 1 and 2% of activated charcoal along with 15% of coconut milk. The medium without any charcoal served as control. After 30 days of incubation in dark, the frequency of anthers showing embryogenic response (per cent productive anthers) was calculated. The productive anthers observed on 0 (control), 0.5, 1 and 2% of activated charcoal media were 5, 10, 34 and 33% respectively (Fig.5-5).

5-C.9 Testing the regenerated plants for their stability of disease resistance :

It is important that the plants regenerated ~~from~~ in vitro should be critically examined for stability and inheritance of the disease resistance character. The leaf disk bioassay as described in materials and methods was, therefore, preferred to make preliminary screening of the regenerants due to its


50
40
30
20
10
% Embryogenic anthers
0
0·5
1
2
% Charcoal (w/v)

simplicity and rapidity, over whole plant screening due to their destructive nature. The plants which appeared resistant in the leaf disk assay were then screened for their resistance to the pathogen by direct infection with the pathogen.

In all, 72 LD50* regenerated plants were screened. The change in fresh weight of leaf, disks as per cent of control was determined. Leaf disks measuring 0.9 cm in diameter from young leaves were inoculated on callus inducing medium incorporating 30% CF (nearer to LD50 = 28%). The control medium contained no CF. Leaf disks were removed after 10 days incubation and the fresh weights were determined. The data presented in Table 5-1 and Fig.5-6 are expressed as % of control of leaf disks weighed. The results showed large variations in resistance among the regenerants. Maximum resistancy was recorded in the plant 37 as 86.9% (if the fresh weight change to per cent control considered as such as per cent resistancy). Four plants were in the range 75 and 85%. Majority of the plants had fallen in the range between 30 and 65% (39 plants). Twenty two plants were placed below 30% resistancy. Plant No.21 was found to be the most susceptible one with -3.8% of the control.

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* LD50 regenerants are those plants which come from cells that grew in plate containing CF that inhibited growth and cell division of 50% of plated cells.

Table 5-1 : Evaluation of LD50 regenerants in the leaf-disk bioassay

Plant No	Fresh weight (g)	Fresh weight % control	Plant No	Fresh weight (g)	Fresh weight % control
0	0.153 ± 0.007				
1	0.096 ± 0.006	56	21	0.018 ± 0.022	- 3.8
2	0.026 ± 0.021	2.3	22	0.037 ± 0.011	33.8
3	0.1 ± 0.003	59	23	0.085 ± 0.015	47.7
4	0.051 ± 0.01	21.5	24	0.063 ± 0.006	30.77
5	0.046 ± 0.046	17.69	25	0.103 ± 0.011	61.5
6	0.075 ± 0.011	39.9	26	0.111 ± 0.023	67.7
7	0.034 ± 0.047	8.5	27	0.11 ± 0.02	66.9
8	0.072 ± 0.046	37.7	28	0.042 ± 0.036	14.6
9	0.09 ± 0.011	51.5	29	0.078 ± 0.013	42.3
10	0.047 ± 0.041	18.5	30	0.064 ± 0.02	31.5
11	0.063 ± 0.027	30.8	31	0.026 ± 0.008	2.3
12	0.074 ± 0.024	20.8	32	0.04 ± 0.005	13
13	0.074 ± 0.024	39.2	33	0.048 ± 0.005	19.2
14	0.046 ± 0.008	17.7	34	0.087 ± 0.04	49.2
15	0.076 ± 0.013	40.8	35	0.106 ± 0.006	63.8
16	0.096 ± 0.007	56.1	36	0.025 ± 0.008	1.5
17	0.074 ± 0.016	39.2	37	0.136 ± 0.006	86.9
18	0.03 ± 0.014	5.4	38	0.032 ± 0.013	6.9
19	0.074 ± 0.015	39.2	39	0.046 ± 0.005	17.7
20	0.067 ± 0.35	10.77	40	0.006 ± 0.006	33.1

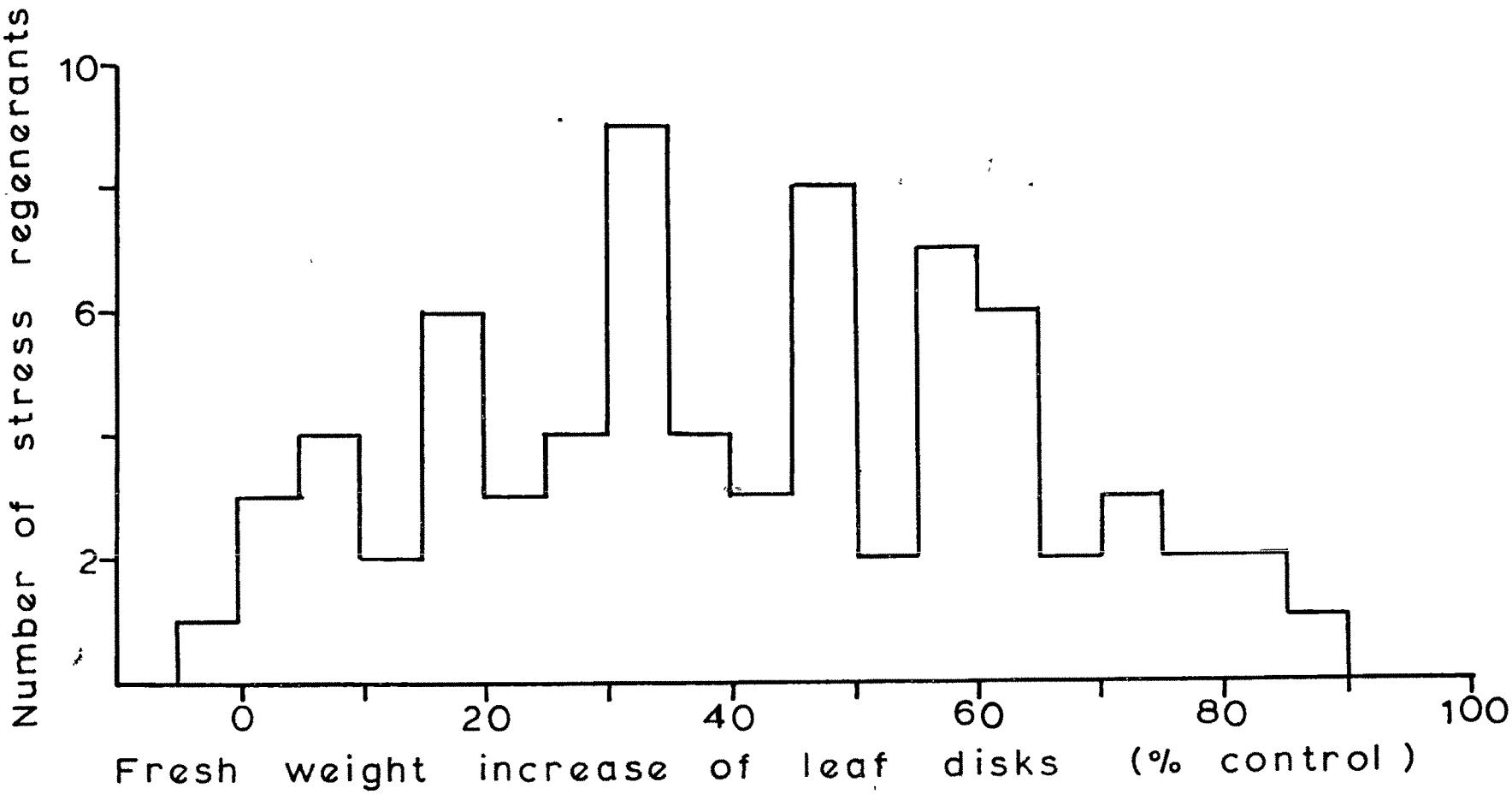
Table 5-1 (Contd.)

41	0.09 ± 0.005	51.5	57	0.038 ± 0.006	15.9
42	0.086 ± 0.005	48.5	58	0.051 ± 0.009	28.7
43	0.121 ± 0.01	75.4	59	0.07 ± 0.005	61.7
44	0.068 ± 0.053	34.6	60	0.087 ± 0.009	61.7
45	0.129 ± 0.048	81.5	61	0.069 ± 0.008	44.9
46.	0.118 ± 0.007	73.1	62	0.467 ± 0.018	8.4
47.	0.1 ± 0.02	59.2	63	0.055 ± 0.009	31.8
48	0.075 ± 0.015	39.9	64	0.029 ± 0.011	7.5
49	0.098 ± 0.008	57.7	65	0.113 ± 0.017	85
50	0.121 ± 0.023	75.4	66	0.083 ± 0.01	57.9
0	0.128 ± 0.009		67	0.096 ± 0.006	70.7
51	0.054 ± 0.006	30.8	68	0.097 ± 0.005	71
52	0.071 ± 0.027	46.7	69	0.074 ± 0.017	49.5
53	0.052 ± 0.015	29	70	0.088 ± 0.015	62.6
54	0.09 ± 0.011	64.5	71	0.089 ± 0.007	63.3
55	0.072 ± 0.02	47.7	72	0.057 ± 0.012	33.6
56	0.08 ± 0.009	15.9			

NB i) The mean fresh weight of inoculated leaf disks for plant 1 to 50 = 0.023 g.

ii) The mean fresh weight of inoculated leaf disks for 51 to 72 = 021 g.

iii) Fresh weight % control include at least five fresh weight of leaf disk of control unselected regenerants as well as leaf disk from seed derived plants.


Number of stress regenerants
10
6
2
0
20
40
60
80
100
Fresh weight increase of leaf disks (% control)

5-C.10 Screening the haploids for CF resistance :

Totally 40 haploids were screened for disease resistance by the leaf-disk-assay as conducted in the preceding experiment to test the LD50 regenerants. Change in the fresh weight of leaf disks as per cent of the control was calculated (Table 5-2 and Fig.5-7). Different levels of resistance to CF were observed among the haploids ranging from -17.5 to 58.7%. Two plants 21st and 33rd showed 56 and 58.7% resistance respectively. Majority of them (25 plants) have fallen between 10 and 40%, of which 7 showed 25 to 30%, resistance. Five were considered to be the most susceptible as they were below 0%.

5-C.11 Confirmatory study :

To confirm resistant character determined in the leaf-disk-assay, 3 plants showing the susceptible nature, (Plant No.2, 32 & 38; see Table 5-1) and 3 showing resistant phenotype (Plant No.37, 43 & 65) were inoculated with fungus (inoculum density = 4%) using the liquid culture technique described in chapter 3. Wilt index was calculated and recorded in Table 5-3 along with the data of leaf-diak-assy. On comparison, the wilt index was found to coincide significantly with the results of the leaf-disk -assay. The plants recorded as susceptible in the leaf-disk-assay were attacked on fungal inoculation, showing severity of disease symptoms. On the other hand, resistant plants 43 and 65 showed slight wilting of one basal leaf in the beginning. Even some portions of root regions of all 3 turned brown initially. But there was no sloughing

Table 5-2 : Evaluation of haploids in the leaf-disk bioassay for disease resistance

Plant No.	Fresh weight (g)	Fresh weight % control	Plant No.	Fresh weight (g)	Fresh weight % control
0	0.083 ± 0.014				
1	0.035 ± 0.013	23.8	21	0.055 ± 0.005	56
2	0.027 ± 0.015	10.4	22	0.042 ± 0.006	34.9
3	0.036 ± 0.01	25.87	23	0.047 ± 0.005	42.86
4	0.039 ± 0.02	30	24	0.05 ± 0.011	47.62
5	0.031 ± 0.024	18.4	25	0.035 ± 0.018	24.7
6	0.023 ± 0.002	5.7	26	0.009 ± 0.006	-17.46
7	0.032 ± 0.004	19.05	27	0.022 ± 0.003	3.2
8	0.02 ± 0.009	0	28	0.038 ± 0.008	45.4
9	0.027 ± 0.01	12.06	29	0.049 ± 0.004	28.57
10	0.031 ± 0.003	17.46	30	0.036 ± 0.002	24.7
11	0.054 ± 0.006	54.9	31	0.028 ± 0.005	13.18
12	0.039 ± 0.012	29.68	32	0.039 ± 0.001	29.5
13	0.039 ± 0.016	30.16	33	0.057 ± 0.006	58.7
14	0.014 ± 0.008	-9.5	34	0.05 ± 0.004	47.6
15	0.039 ± 0.001	30.16	35	0.035 ± 0.015	24.3
16	0.03 ± 0.016	15.87	36	0.042 ± 0.013	34.3
17	0.038 ± 0.004	28.57	37	0.021 ± 0.004	1.59
18	0.044 ± 0.008	38.1	38	0.021 ± 0.013	0.95
19	0.035 ± 0.007	23.8	39	0.019 ± 0.002	-1.59
20	0.038 ± 0.005	29.57	40	0.012 ± 0.008	-12.7

N.B. : The mean fresh weight of leaves inoculated = 0.02 g.

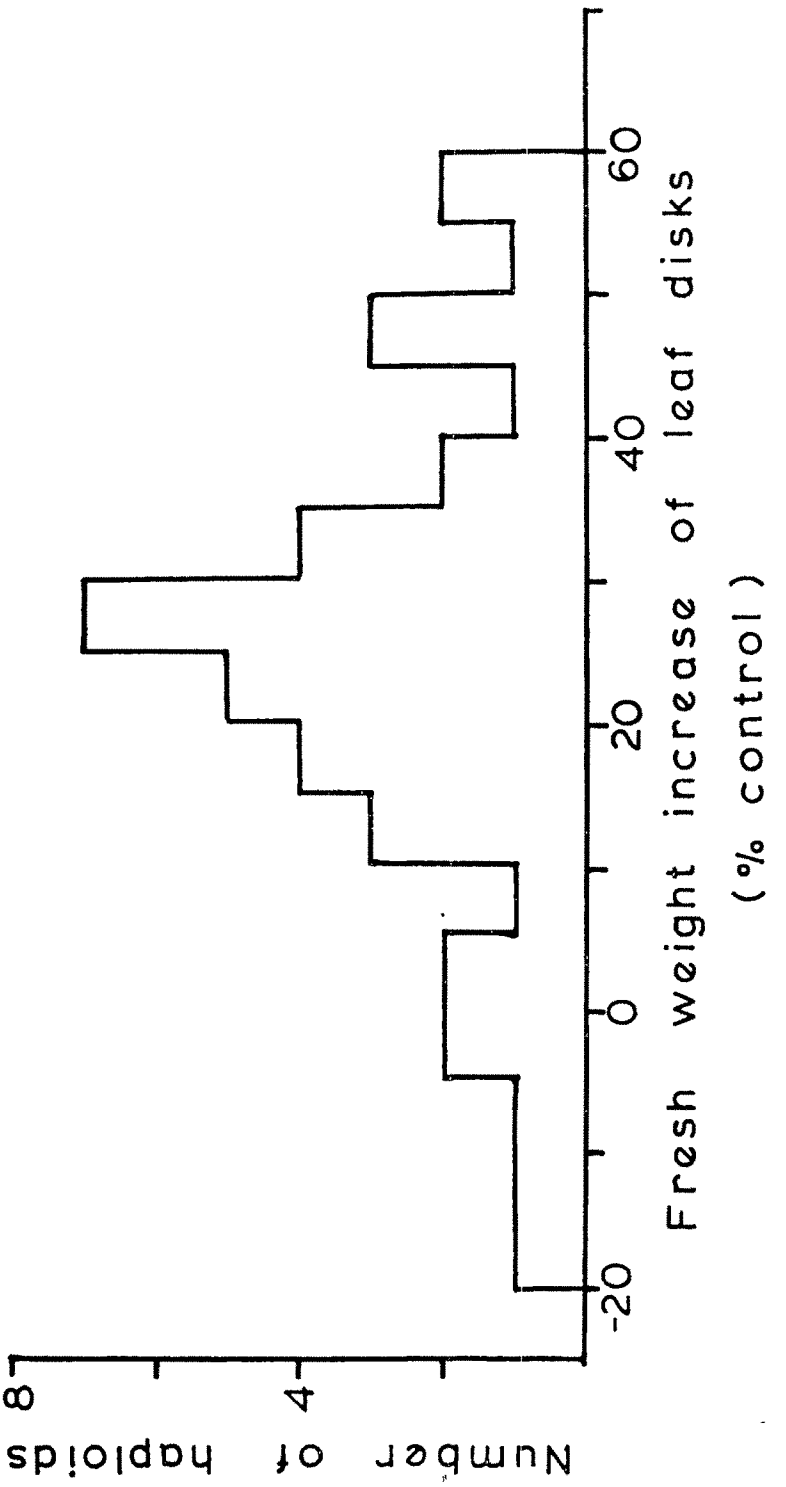

Number of haploids
8
4
-20
0
20
40
60
Fresh weight increase of leaf disks
(% control)

Table 5-3 : Correlation of leaf-disk-assay and wilt index on the LD50 selected tobacco.

Plant Code No.	Leaf-disk Fresh weight % control*	Wilt Index
2	2.3	100
32	13	100
37	86.9	0
38	6.9	100
43	75.4	20
65	85	20

* Mean of 5 replicates.

off of the cortical tissue in the roots and no rotting smell around these regions. Later on many healthy (new) roots appeared from the basal region of the stem (Fig.5-8) of all the resistant plants. These plants behaved like the control plants (kept in water) giving no disease symptoms.

5-C.12 Control study :

To examine the origin of resistant character obtained during in vitro selection, control regenerants were also evaluated for their disease resistance. This experiment will answer the question on whether the acquired resistant character in LD50 regenerant, is due to somaclonal variation or due to our selection protocol.

Four control regenerated plants and four LD50 regenerants (Plant No.26, 50, 57 & 68) were selected for the experiment. Dose sensitivity study of CF (as done on leaf disks in chapter 4) was conducted on the growth of leaf disks from all the plants in vitro. The fresh weight taken after 10 days of incubation is presented in Fig.5-9.

The control regenerated plants behaved like the paraental plants. Inhibition was noticed on the growth of the leaf disks when the concentration of the CF was raised beyond 20%. Growth was completely arrested at 50%, CF in all the cases. The disks became brown and dead in plants No.3 & 4. In all stress regenerants, slight inhibition of growth of disks was noticed beyond 30% CF with even at 50% CF registering increase in fresh

Fig.5-8 A) LD50 selected tobacco plants transplanted to pots containing garden soil mixture in greenhouse.

B) Infection of LD50 selected tobacco plant in lethal inoculum density of Fusarium (see new roots emerging out from the basal portion of the stem).

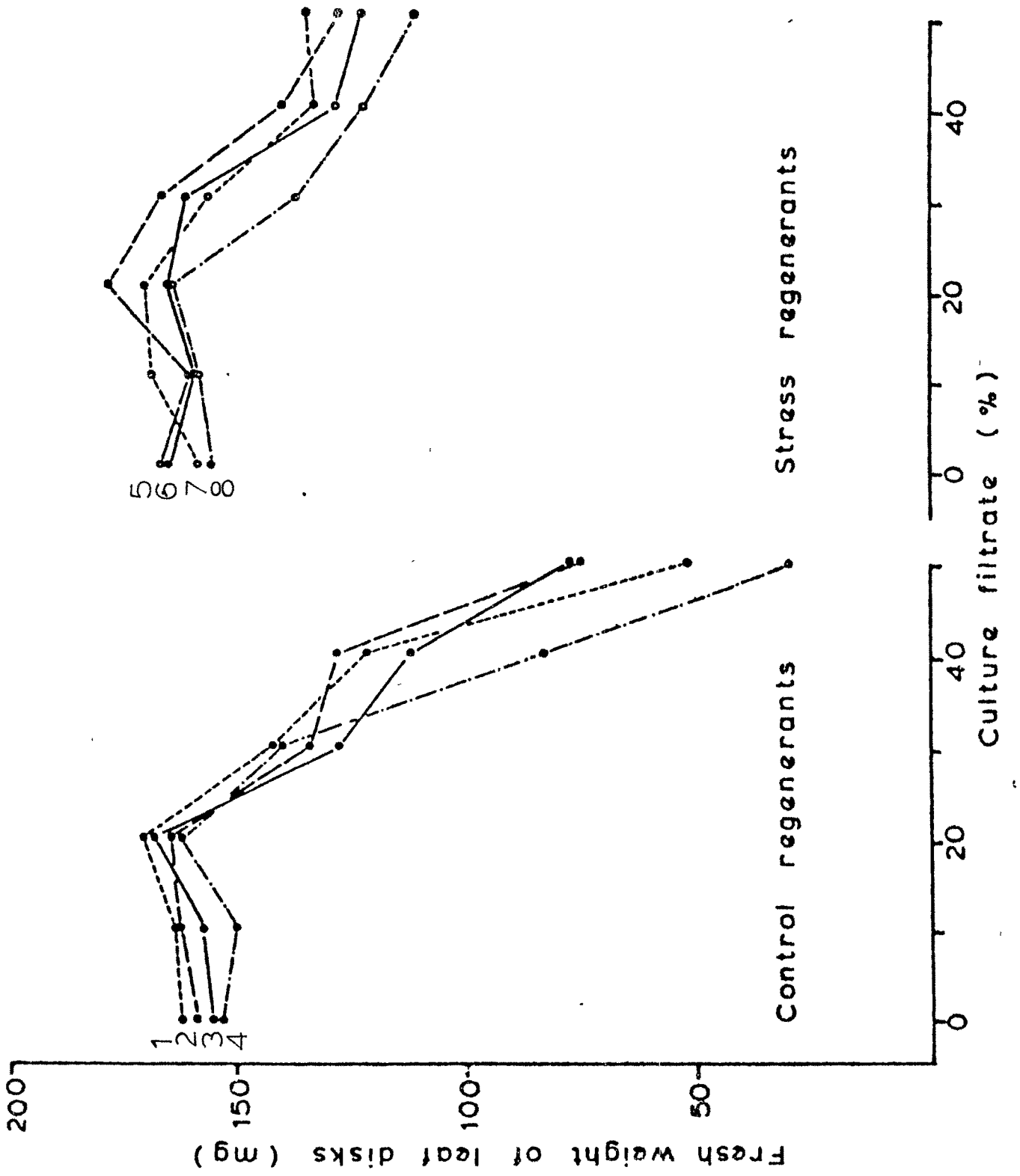

A


250
CORNI
B

Fig.5-9 Control study to see whether the resistancy obtained is due to somaclonal variation or not.

1 - 4 Control regenerated plants

5 - 8 LD50 selected regenerants


Fresh weight of leaf disks (mg)
200
150
100
50
1
2
3
4
5
6
7
8
Control regenerants
Stress regenerants
0
20
40
Culture filtrate (%)

weight. However, in no concentration, the disks showed browning and death.

5-C.13 Direct selection of resistant plants through anther culture :

Anthers containing uninucleated pollen were inoculated on NS medium supplemented with 15% coconut milk, 1% activated charcoal and various doses of cell free CF from 0 to 50%. In a second study of dose sensitivity of CF, activated charcoal was not added. The anthers were incubated in dark. After 30 days of incubation per cent productive anthers (PA) and number of plants produced per productive anther (NPPA) were determined. Results are presented in Table 5-4 and Fig.5-10, 5-11.

The control (medium in which charcoal was added) showed 32% of PA and 4.17 NPPA. Addition of 12.5% CF gave 12% PA and 2.5 NPPA and at 25% CF, the figures were 6% and 1.33 plants respectively. Complete inhibition of androgenesis was noticed when the CF was increased to 25% w/v of the medium.

In the case where activated charcoal was not added total inhibition of haploid production was noticed at and above 25% level of CF itself. Here, in control 6% PA and 7.7 NPPA was registered, whereas, at 12.5% CF the PA was 2% and NPPA was 1.

Table 5-4: Effect of different concentration of CF on anther culture in presence and absence of activated charcoal

Conc. of CF.	Total number of anthers inoculated	Number of anthers producing plants		Total number of plants produced		% Productive anthers		Number of plants produced per anthers	
		A	B	A	B	A	B	A	B
0	50	16	3	67	23	32	6	4.17	7.1
12.5	50	6	1	15	1	12	2	2.5	1
25	50	3	-	4	-	6	-	1.33	-
37.5	50	-	-	-	-	-	-	-	-
50	50	-	-	-	-	-	-	-	-

A : Medium with 1% activated charcoal.

B : Medium without activated charcoal.

Fig.5-10 A. Haploids transplanted to pot.

B. Anther culture in the presence of fungal CF :

left = control

right = treated with 12.5% CF


A
B

Fig.5-11 Effect of CF conc. on anther culture (culture medium added with activated charcoal).

% Productive Anthers
50
25
0
% Culture Filtrate
12.5
25
37.5
50
Number of Plants produced per Productive Anther
1
3
5

The sublethal concentrations of CF were, therefore, considered as 25% in presence of charcoal in the medium and 12.5% in its absence. The plantlets obtained at these phytotoxic levels were not healthy when compared to the plants of control origin (Fig.5-5). They looked etiolated and there was no growth of the leaves. These plants did not survive even when transferred to CF free medium.

5-D DISCUSSION

The possibility of cellular breeding to provide a valuable adjunct to disease resistance was investigated by Brettell and Ingram (1979). Mutation induction followed by selection in cultured cells was the one discussed among the experimental approaches by them. This has been followed by Grout and Weatherhead (1980), Strauss et al. (1980) and Ramnath et al (1983) for selecting various resistant lines.

Chemical mutagens were used by Heinz et al., in 1977 (methyl methane sulfonate on sugarcane callus for raising eyesopt disease resistance), Ramnath et al. in 1983 (N-methyl-N' nitro-N-nitroso guanidine on S. khasianum anthers for selecting Fusarium wilt resistant lines) etc.

However, frustration was faced by some. Chimerism was a formidable problem when vegetative tissues were mutagenized by x-irradiation. (van Harten and Broertjes, 1981). Sometimes it is quite apparent that mutagenized as well as non-mutagenized cells could give similar variations (Flick, 1983). As it is

noted that the non-mutagenized cells too can give similar variations (somaclonal variations), a specific mutagenic treatment is not necessary (Larkin and Scowcroft, 1981). Moreover, the drugs that are used to bring about the resistant lines themselves are considered as mutagens (Strauss et al., 1980).

Selection of populations of cultured cells for disease resistance necessarily requires that resistance and susceptibility be expressed in vitro. Hence, Helgeson et al. (1976) envisaged screening of tobacco callus clones with zoospores of Phytophthora parasitica var. nicotianae. In cases where pathogenicity is modified or co-determined by a toxin then the toxin itself can be isolated and used in host culture media.

Plant pathogens also produce many other phytotoxic substances whose role in disease development is less clear. Some phytotoxins, (like tabtoxin from Pseudomons tabaci; Carlson, 1973a) produce only part of the disease syndrome or are produced in quantities difficult to relate to disease development. Selection for resistance to these kinds of toxins will probably give at best only partial resistance to disease. This was proved well by the two toxins, ethyl acetate factor and the aqueous factor of the CF of F. oxysporum and its effect on tobacco plants (chapter 4). Hence, for selecting wilt resistant tobacco cell lines CF of F. oxysporum was used in the present approach. Moreover, utilization of CF for selection study was encouraged by the pathological data

reported in chapter 1. As CF could induce all the symptoms akin to the fungus, it is considered that resistance to CF could be predicted as resistance to the pathogen also.

Though there are many reports using callus as the unit of selecting material (Helgeson et al., 1972, 1976; Gengenbach and Green, 1975; Behnke 1980a, b; Ramnath et al., 1983; Hartman et al., 1984) the origin of chimeric tissue is obvious. By using single cells or unit of cells from the suspension culture (Malmberg and McIndoo 1984; Widholm, 1976; Atsumi, 1980; Heinz et al., 1977; Connell, 1985 etc.,) uniform exposure to stress can be achieved. Hence, I used cell suspension culture to select resistant clone to the CF.

When tobacco cells were exposed to CF, the low concentration of CF had promotary effect on their growth. CF above 25% w/v concentration was inhibitory. Thus the CF contains not only toxic substance but also growth stimulatory material.

The mean colony diameter was found inversely proportional to the number of colony formed on plates. Cells of control plates as well as those plated in lower CF concentration gave maximum number of cell colonies, which resulted in the depletion of nutrition. This reduced the rate of cell division in them. When the colony number became less they freely absorbed the nutrients from the surrounding medium.

Hence, the colonies became larger due to rapid cell division. Another possibility could be that once the cells become resistant to the toxin/s the cells could begin to respond to the growth promoting substances present in the CF. Gengenbach and Green (1975) working with maize-*Helminthosporium* system, Behnke (1980b) in potato-*Fusarium* system, Ramnath et al. (1983) in *S. khasianum*- *F. oxysporum* system and Hartman et al. (1984b) in alfalfa-*F. oxysporum* system conducted similar dose response studies in vitro. But none could observe such catagorical growth stimulating and growth inhibitory behaviours of CF.

Resistant selection to antimetabolites e.g., aminoacid analogue is best carried out at the concentration which either kills or totally inhibits the growth of all wild type cells. However, most of resistant selections to the pathogen toxin or their CF were achieved either at sublethal level or by selecting cultures to stepwise and gradual increase in the toxin concentration (Gengenbach and Green, 1975; Brettell et al., 1980; Strauss et al., 1980; Ramnath et al., 1983; Hartman et al., 1984b). The reason is that concentrations which are too high may mean that potentially resistant variants are also unable to grow and are thus lost. On the other hand doses which are too low should also be avoided, because cells escaping for purely physiological reasons could outnumber true mutants. In the present selection approach we did not use stepwise increase of CF. Instead resistant cultures were selected at LD50, LD70 and LD90 in a single step.

Throughout our study, the density of the cells was maintained constant as 6-7 X 10^3 cells ml^{-1}. Strauss et al. (1980) also suggested that the cell density in dose response determinations must be the same as in the selection experiments, as drug sensitivity is strongly dependent upon the mass of drug-detoxifying substances present in the cells.

Further, previous workers achieved regeneration of selected clone only in non-selective medium. Whereas, we maintained selection pressure even during regeneration.

In our study, a problem of vitrification with the LD50 regenerated plants was often observed. In the literature, various authors have implicated vitrification due to high concentrations of cytokinins and/or auxins (Beauchesne, 1981; cf. Vieitez et al., 1985), the relative humidity in the culture vessel and the water status of the medium (Ziv et al., 1983). The water potential of the medium was affected particularly by the concentration of agar (Debergh et al., 1981; Leshem, 1983) and the production of ethylene (Kevers et al., 1984). We did not observe this problem on regenerated plants of non-selective medium. Thus CF of F. oxysporum seems to be responsible for vitrification in vitro regenerants of tobacco.

By increasing the concentration of the agar from 0.8% to 1% this problem was eliminated just by following the suggestion of Debergh et al. (1981).

On screening the LD50 regenerated plants with leaf-disk bioassay, it was noticed that they showed a lot of variation in wilt resistancy from -5 to 86.5% fresh weight increase as the per cent of control. This indicates that mosaics of genetic and physiological resistancies occurred in the cells during the selection pressure for more than two and a half months. Hence, those clones acquired physiological resistance to the phytotoxin were lost, when the pressure was removed while transplanting to pot. And those LD50 clones showed more resistance might have been mutated. Hence, the acquired resistancy was not lost during transplanting. And along with these there were also seen many intermediate resistancies. Moreover, the leaf-disk-assay was found highly correlated when some of the plants treated with the lethal concentration of F. oxysporum. The plants confirmed as susceptible in leaf-disk study using CF were lost and the resistants were grown well in the fungal treatment.

Moreover, the pathogen tried to colonize in the host root, resulting in the root portions becoming brownish. And even one leaf each in two of the resistant plants showed wilting. Later these clones did not allow the fungus to colonize further in the host. They produced new healthy roots from the lower region of the stem. This can be attributed to the expression of hypersensitive ('R' gene) reaction in cultures. In 1965, Ingram and Robertson reported that potato in vitro cultures from resistant plants supported less growth

of Phytophthora infestans than cultures from susceptible plants. Similarly, Warren and Routley (1970) obtained resistancy with tomato cultures to the same pathogen.

Genetic transmission of the wilt resistancy in the resistant tobacco subclones is currently being tested. However, three lines of evidences indicate that a heritable resistance has been selected in vitro.

First resistant cell colonies appeared during LD50 stress were twice subcultured in the similar selective medium as done by Ramnath et al. (1983), Hartmen et al. (1984b) etc.,

Secondly even the regeneration was achieved in the resistant callus masses in the same selection pressure. But Behnke (1980 a,b), Sacristan (1982) and Hartman et al. (1984b) regenerated plants from the resistant cultures only in non-selective medium.

Thirdly plants confirmed as wilt resistants by leaf-disk study were highly correlated with whole plant infection in Fusarium culture. This was true for selection of Brassica napus resistant to Phoma lingam (Sacristan, 1982), maize plants resistant to Helminthosporium maydis race T (Gengenbach et al., 1977) and alfalfa resistant to F. oxysporum (Hartman et al., 1984b).

The above confirmations are strongly indicative of a mutagen rather than an epigenetic change. However, these can

be considered for the time being as only resistant variants until the inheritance data is collected, because proof of a mutation needs sexual transmission of the selected trait as pointed out by Maliga (1978).

To confirm whether the resistant variation obtained was due to somaclonal variation or not, the plants regenerated from control cultures were tested (as suggested by Larkin and Scowcroft, 1981) against the CF of the fungus. The data showed no variation to disease resistant ~~was~~ had ? occured by the culture cycle as the leaf disks of all were inhibited by the CF like the parental plants. Hence, this indicates that the resistancy ~~y~~ e obtained during LD50 stress might be due to CF only.

However, the changes in nematode resistance (from resistance to susceptible) of S. sisymbriifolium and Patriot tomato plants regenerated from tissue culture appear to be induced by the phytohormone constituents of the culture media (Fassuliotis and Bhatt, 1982). The plants regenerated from maize cultures, selected from originally toxin sensitive to Tms material, were highly resistant to T-toxin, but showed a reversion to male-fertility (Gengenbach et al., 1977; Brettell et al., 1980 a,b). Later it was confirmed that male fertility and toxin resistance are inseparable and are inherited together maternally.

Truly haploid cell material offers the broadest possible spectrum of resistant mutants as both recessive and

dominant traits can be expressed at this ploidy level (Strauss et al., 1980). Moreover, the inheritance of Fusarium wilt resistance in tobacco is not clearly known. Hence, the present strategy of selecting disease resistancy was extended by screening the haploids of tobacco. Here, the variations that are expected to occur could be either preexisted and segregated during gamete formation or occurred during dedifferentiation.

The plants obtained through anther culture were screened for wilt resistancy by the leaf-disk bioassay. From the data (Table 5-2), the maximum resistancy was noticed in one plant as 58.7% (fresh weight increase % control). Maximum were seen below 35%. Hence, it evidences that high level resistancy may not be visible at a high frequency (NB=number of plants screened were only forty), unless there are variations due to in vitro atmosphere. It also proves, that no in vitro variations occurred.

There is also other way to discuss in this regard. In one of the previous experiments when the effect of CF was tested on different host cultures, suggested that all the diploid cultures (whole plants, leaf disks and cells) were inhibited around 50% CF. But anther culture was inhibited, when the CF level was raised above 25% itself. So it was concluded, that the haploid tissue may be more sensitive to the phytotoxin.

But in the present experiment for screening the haploids the CF level used for leaf-disk-assay was 30%. This was the LD50 nearer dose (LD50 = 28% CF) observed for inhibiting the diploid leaf disks. This suggests that the CF level might be very high towards inhibiting haploid tissue. Hence no resistancy could be noticed. Resistance to wilt diseases depends in part on the inoculum concentration and genetic potential for virulence within the pathogen population. At very low levels of inoculum even the susceptible cultivars may show less symptoms (Bell and Mace, 1981). Their susceptibility, however, increases rapidly as inoculum levels increase; percentages of wilted plants increase; incubation periods for symptom development decrease; severity of symptoms, including mortality increases (Abawi and Lorbeer, 1972; Ashworth et al., 1972; Conroy et al., 1972; Evans and McKeen, 1975; Fenn et al., 1975; Schreiber and Stipes, 1967). Similarly Alon et al. (1974) and Raucher et al. (1974) concluded that resistant cultivars also may become progressively more susceptible as inoculum concentrations exceed certain critical levels.

May be this is the reason why the LD50 resistant plants, showed slight browning of the root portions in the beginning when they were inoculated with lethal concentration of the fungal mycelium (4%). Hence, different levels of resistances occur by using different levels of phytotoxin can lead to false interpretations (Nielson et al., 1979). Note that a change in sensitivity cannot be excluded despite a stable genetic basis

for resistance when confirmation of the resistant phenotype takes place in a type of culture different from that in which the selection was performed (Strauss et al., 1980).

Ramnath et al., (1983) standardised various cultural conditions for obtaining resistant cell lines of S. khasianum through mutation and subsequent screening against CF of F. oxysporum. Behnke (1980) aimed to obtain dihaploid calli of S. tuberosum resistant to the CF of F. oxysporum. Neither plant regeneration nor induction of new callus caused loss of the resistance.

Successful production of haploid plants has been in practice since Guha and Maheswari (1964) in many plants viz., datura, rice, tobacco, wheat etc., (Reinert and Bajaj, 1977). In tobacco, anther culture is an easy practice in our laboratory since long (Rawal, 1979). By the present studies I could get upto 34% of productive anthers by standardising with addition of activated charcoal as suggested by Agnostakis (1974). The direct selection of haploids from the tobacco anthers inoculated on medium containing CF was tried. Dose sensitive study of CF on androgenesis was conducted. The plants obtained at 25% CF were physiologically very weak and were not able to recover. If this problem is solved, resistant plants can directly be raised and tested in the field. The same reliable method can also be followed latter to pick up any other diserable trait just simply mixing the respective lethal/ sublethal drug in the medium.