Evaluation of the Potential
for the Genetic Improvement of Salt Tolerance
in Chile Pepper (<u>Capsicum</u> <u>annuum</u>) using Wild
Germplasm and Cell Selection Procedures

by

Steven D. Tanksley
Assistant Professor
Department of Horticulture

and

Gregory C. Phillips
Assistant Professor
Department of Horticulture

DISCLAIMER

The purpose of WRRI technical reports is to provide a timely outlet for research results obtained on projects supported in whole or in part by the institute. Through these reports, we are promoting the free exchange of information and ideas and hope to stimulate thoughtful discussion and action which may lead to resolution of water problems. The WRRI, through peer review of draft reports, attempts to substantiate the accuracy of information contained in its reports, but the views expressed are those of the author(s) and do not necessarily reflect those of the WRRI or its reviewers.

Contents of this publication do not necessarily reflect the views and policies of the U.S. Department of the Interior, nor does mention of trade names or commercial products constitute their endorsement or recommendation for use by the U.S. Government.

ABSTRACT

At present, 1.4 million acres or 1.8% of New Mexico's land area is being used for irrigated agriculture. However, further expansion is limited mainly by the lack of adequate fresh water supplies and few crops capable of utilizing the available brackish water. If existing crops of economic importance to New Mexico could be engineered to produce under culture with higher levels of salinity, the land area under cultivation could be significantly increased and water resources better utilized.

Chile pepper, Capsicum annuum, is a vegetable crop of major importance to New Mexico's economy. Results from experiments conducted during this project indicate that chile is very sensitive to the effects of saline irrigation. Salt concentrations as low as 775 PPM can reduce plant growth in commercial chile varieties now being grown in the state. Germination is severely inhibited at levels above 7000 PPM salt.

In screening diverse Capsicum germplasm we have discovered races that appear to germinate better at salt levels prohibitive to commerical varieties. These lines have been crossed to commercial cultivars for potential use in the New Mexico State University (NMSU) chile breeding program. In addition, a large collection of Mexican Capsicums has been obtained and partially increased. This material represents the largest collection of wild peppers with close affinity to the garden pepper, C. annuum. Preliminary screening for genetic variability using enzyme electrophoresis indicates that this material possesses a tremendous amount of genetic variation that may prove useful in the search for salt tolerance in pepper.

Repeatable methods for cloning chile plants by tissue culture have been developed that ultimately may lead to an in vitro cell selection procedure for obtaining salt tolerant pepper lines. The advantage of the cell selection procedure over classical hybridization approaches is that the cell-selected lines are derived from commercial cultivars. Thus, derived variants may be immediately useful as commercial cultivars. The response to salt stress of seedlings placed in tissue culture--but not cell or callus cultures--showed good correlation with the response of whole plants to salt stress. This in vitro seedling procedure is simple to apply and may have a role in a screening program. However, these results also suggest that in vitro selection may require organized as opposed to unorganized tissues to accomplish genetic improvement for salt tolerance in peppers.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND LITERATURE REVIEW

On a global scale, the most deleterious soil component restricting plant growth is salt (Epstein 1976). Saline habitats include wet marshes, seashores, oceans, and semiarid deserts. Only 30% of the earth's surface is land and one-third of that is semiarid or arid. Many of these zones are characterized by highly saline soils and underground water supplies. In certain areas, brackish water is already being utilized through improved agrotechnical and drain methods and research is being conducted to study the long-term effects of saline irrigation (O'Connor 1980). Most species, however, cannot withstand even moderately high salinity (Mass and Hoffman 1977).

In the past, major research efforts have been directed toward circumventing salinity by reclamation--an approach that is becoming increasingly expensive and energy inefficient. Even with these measures, most high-yielding crop cultivars are unsuited to the marginal conditions of newly claimed or reclaimed agricultural land (Wittwer 1978). Efforts to improve land conditions must therefore coincide with measures to improve crop salt tolerance through genetic modification. Epstein et al. (1980) point out the need to genetically modify existing crop plants for culture under saline conditions: "Increasing salinity of soil and water threatens agriculture in arid and semi-arid regions. By itself, the traditional engineering aproach to the problem is no longer adequate. Genetic science offers the possibility of developing salt-tolerant crops, which, in conjunction with environmental manipulation could improve agricultural production in saline regions and extend agriculture to previously unsuited regions."

New Mexico possesses approximately 25×10^{12} cubic meters (20 billion acre-feet) of underground water of which more than three-quarters is classified as saline or brackish (dissolved solids 1,000 PPM). At present 1.4 million acres or 1.8% of New Mexico's land area is being used for irrigated agriculture (Berger 1980). However, further expansion is limited mainly by the lack of adequate fresh water supplies and few crops capable of utilizing the available brackish water. If existing crops of economic importance to New Mexico could be engineered to produce under culture with higher levels of salinity, the land area under cultivation could be significantly increased.

Chile pepper, Capsicum annuum L., is one of the oldest crops in New Mexico with a history dating back several centuries. Brought from Mexico by the Spanish conquistadors in the sixteenth century (Cotter 1982), chile has long been the focus of attention by New Mexicans. In recent years, the production of chile in New Mexico has increased considerably. The acreage planted to chile nearly tripled from 1970 to 1979. In 1980, chile's $26 million cash value to the state ranked it the number one horticultural crop in the state (Cotter 1980, Fishburn 1981). If one counts the revenues from jobs related to processing chile, the economic impact of chile is even greater. Considering the rising national demand for chile products, including Mexican food, paprika, and natural red food colorings, the chile acreage in New Mexico could continue to increase. Such a situation would be desirable from the viewpoint of growers and processors alike as chile traditionally has had one of the highest cash value returns of any crop.

2

The genetic approach to saline culture of crop plants has proved productive in the few crops where it has been applied. For example, a simple selection program in barley has resulted in profitable production of this crop under irrigation with sea water (35000 PPM) (Epstein 1980). In tomato, a solanaceous species similar to chile in many ways, crossable species have been identified that possess high levels of salt tolerance (Rush and Epstein 1976, Tal et al. 1979). Using backcross, interspecific breeding with one of these species, Lycopersicon cheesmanii, tomatoes have been bred that tolerate irrigation with 70% seawater--a level lethal to standard tomatoes. In wheat, germplasm screening has resulted in the identification of 34 lines of spring wheat capable of producing grain under irrigation with 50% seawater--a level of stress lethal to commercial wheat (Epstein et al. 1980). Recent studies have revealed that some related wheat species possess even higher levels of salt tolerance and efforts are now being focused on transferring "salt tolerance genes" from those species into wheat (McGuire and Dvorak 1981).

Tissue and cell culture techniques provide an additional genetic avenue to achieving salt tolerance in commercial crops. As many as 10 million cells in as little as 100 ml can be subjected to recurring salt stress. Using this approach, Nabors et al. (1975) and Nabors (1976) obtained salt tolerance in cell cultures of Nicotiana tabacum both by treatment with a mutagen (ethane methane sulfonate) and by spontaneous adaptation to 0.5 and 0.8% sodium (NaCl). Plants recovered from these cultures retained their salt tolerance through the F1 and F2 sexual generations (Nabors et al. 1980). This was an important demonstration of genetically transmissible salt tolerance isolated through cell selection. Dix and Street (1975) selected for NaCl tolerance in cell cultures of Capsicum annuum (bell pepper varieties) and Nicotiana

3

sylvestris (wild tobacco). Resistant lines tolerated up to 20,000 PPM NaCl and some lines retained the resistance in the absence of the selection pressure. However, plants were not regenerated from these cultures.

Croughan et al. (1978) selected for 1% NaCl tolerance in alfalfa cell cultures by spontaneous adaptation. These selected cells required NaCl for growth. Plants have been regenerated from these lines (Stavarek et al. 1980) but neither field evaluation nor genetic analysis has been reported. Tyagi et al. (1981) recovered tolerance up to 2% NaCl in cell cultures of a haploid line of Datura innoxia. Regenerated plants yielded new cell cultures that retained the resistance trait with phenotypic stability of regenerated plants being retained for at least three years.

The objectives of this study were:

1. To determine the response of chile pepper to varying levels of salinity at different stages of the plant life cycle.

2. Based on the results of objective 1, to devise an efficient screening procedure by which to evaluate pepper germplasm for salt tolerance.

3. To obtain and screen a broad base of pepper germplasm for salt tolerance.

4. To begin transferring tolerance into commercial cultivars of chile pepper.

5. To develop tissue and cell culture procedures that might allow selection for salt tolerance at the cellular level.

EXPERIMENTAL RESULTS

Part One: Seedling and Whole Plant Studies

All salt solutions regardless of concentration consisted of 85% NaCl and 15% CaSO4. Experiments 1-4 were designed to define the response of commercial chile to salinity stress. All seeds were of the widely grown variety NuMex RNaky (CA 133). Subsequent experiments involved germplasm screening and gene transfer studies.

Effect of Salinity on Seed Germination

Experiment 1. Salt treatments are listed in table 1. Two petri dishes of 81 seeds each (on germination paper) were subjected to each salt treatment in an incubator at 25° C. The experiment ran for 21 days with records of radical emergence and seed germination (expanded cotyledons) taken daily.

Conclusion. Germination under the defined conditions is not occurring at or above 24,000 PPM (table 1). Germination, as determined by the rate of germination (number of days to 50% final germination), first shows signs of inhibition at 6,000 PPM.

Experiment 2. This experiment was a follow-up on experiment 1 and was designed to discover precisely what level of salt inhibits germination and what level of salt prevents germination. Salt concentrations ranged from 0 - 24,000 PPM. One hundred seeds in single germination boxes were subjected to each salt level. The experiment ran for 22 days after imbibition with all treatments being maintained in an incubator at 25° C. Daily records were taken of the number of seeds that: (1) had sprouted radicals, and (2) had germinated to the stage of cotyledon expansion. The experiment was terminated 22 days after initiation and the data summarized (table 2).

5

Table 1. Effect of salinity level on seed germination -- Experiment 1

PPM Salt	Radical Emergence % (mean/std.err)	Germination % (mean/std.err)	No. Days to 50% Final Germination
0 (control)	93/3.5	95/5.0	9.2/0.1
800	93/3.5	87/7.0	8.1/0.4
1600	89/2.5	85/4.0	9.8/0.2
2400	91/0	90/1.5	8.3/0.1
3200	91/2.0	89/4.0	8.2/0.3
4000	95/0.5	92/2.0	8.6/0.2
6000	86/2.5	83/0	10.1/0.4
8000	92/2.5	90/3.5	10.4/0.4
12000	84/4.0	48/8.0	17.0/0.2
16000	34/7.0	0/0	--
24000	4/3.5	0/0	--
32000	0/0	0/0	--

Table 2. Effect of salinity level on seed germination -- Experiment 2

PPM Salt	Radical %	Emergence % ctrl.	Germination %	% ctrl.	No.Days to 50% Final Germination
0 (control)	85	100	79	100	7.5
4000	91	107	91	115	7.3
5000	88	104	85	108	7.8
6000	89	105	87	110	8.3
7000	92	108	88	111	8.7
8000	91	107	80	101	9.9
9000	94	111	81	103	11.9
10000	86	101	62	78	12.3
11000	86	101	41	52	14.7
12000	82	96	30	38	15.9
13000	84	99	33	42	17.0
14000	80	94	17	22	19.2
15000	81	95	9	11	17.3
16000	72	85	3	4	16.5
17000	56	66	5	6	20.4
18000	44	52	0	0	-
19000	67	79	0	0	-
20000	47	55	0	0	-
21000	33	39	0	0	-
22000	17	20	0	0	-
23000	13	15	0	0	-
24000	4	5	0	0	-

Conclusions. Germination is first inhibited at 5,000-6,000 PPM (figure 1). A reduction in the total number of seeds germinated occurs around 10,000 PPM and no germination is obtained at or above 18,000 PPM (figure 2).

Experiment 3. The objective was to evaluate the germination response of chile in a soil medium under varying levels of saline irrigation. The levels of salinity used were: 0; 400; 800; 1,600; 3,200; 7,200; 11,200; 16,000; 19,200; 23,200; 28,000 PPM. Fifty R Naky seeds were sown in individual compartments of germination flats in a greenhouse. Day length was approximately 10 hours. Daytime temperature averaged 27° C, and night temperatures 15° C. Two flats were used for each salt treatment for a total of 22 flats. All flats were randomized with respect to bench position in the greenhouse. The level of soil in each compartment was a minimum of 1 cm below the top to allow for irrigation and to promote daily leaching. The soil and fertilizer application was the same as in experiment 4.

Flats received daily waterings with corresponding saline solutions. The number of seeds germinated in each treatment were recorded at the termination of the experiment and total plant weight (fresh and dry, roots and shoots) were recorded for each flat. Electrical conductivity was measured on a bulk soil sample taken from one flat of each saline treatment by the New Mexico Soil and Water Testing Laboratory.

Conclusion. Based on the percent germination, inhibitory effects are observed at 3200 PPM and germination ceases at 7,200 PPM or above (table 3). This is in sharp contrast to the tests in petri dishes where germination
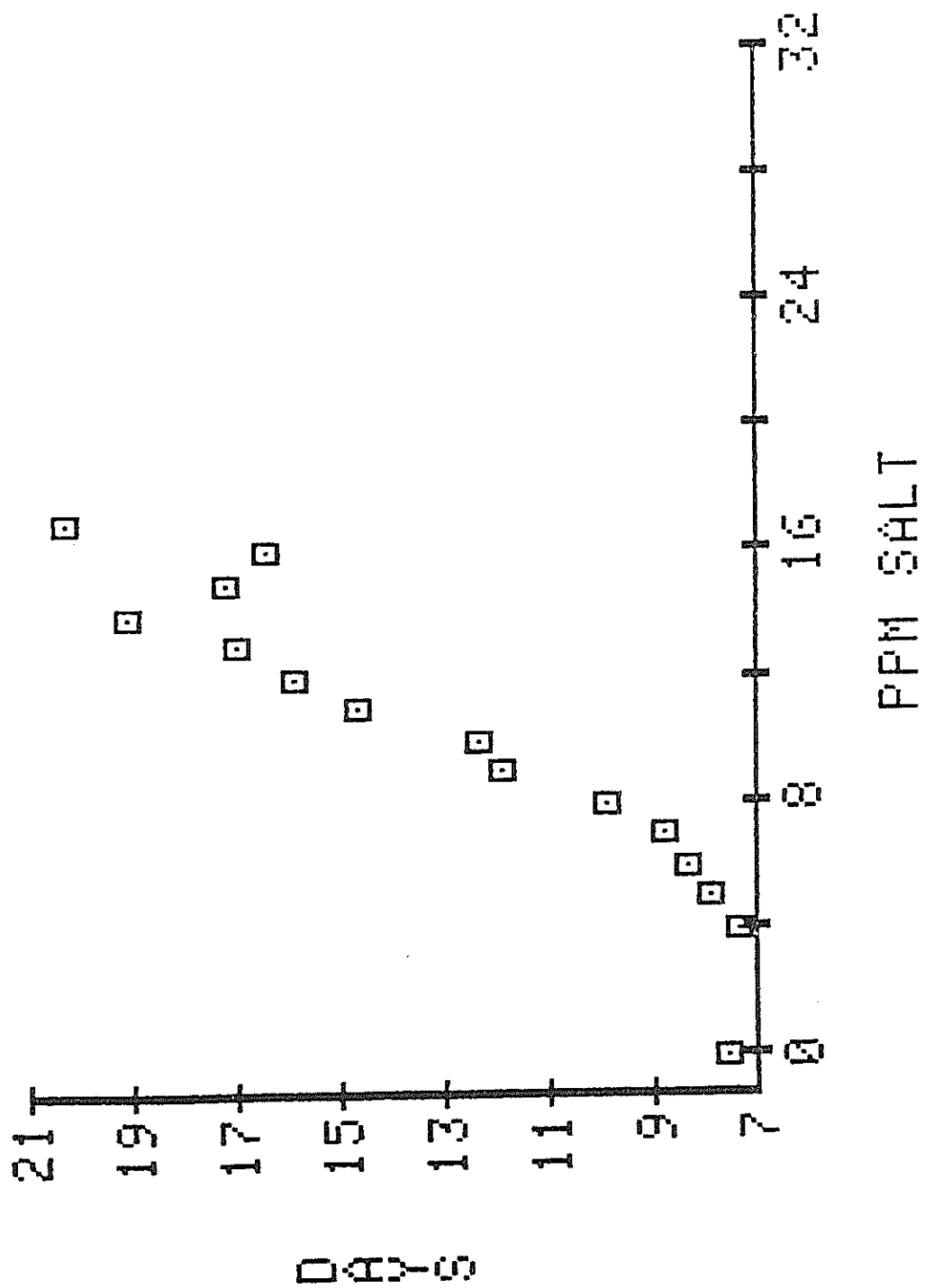
FIGURE 1. Germination rate versus PPM salt

INCUBATOR (25 C)
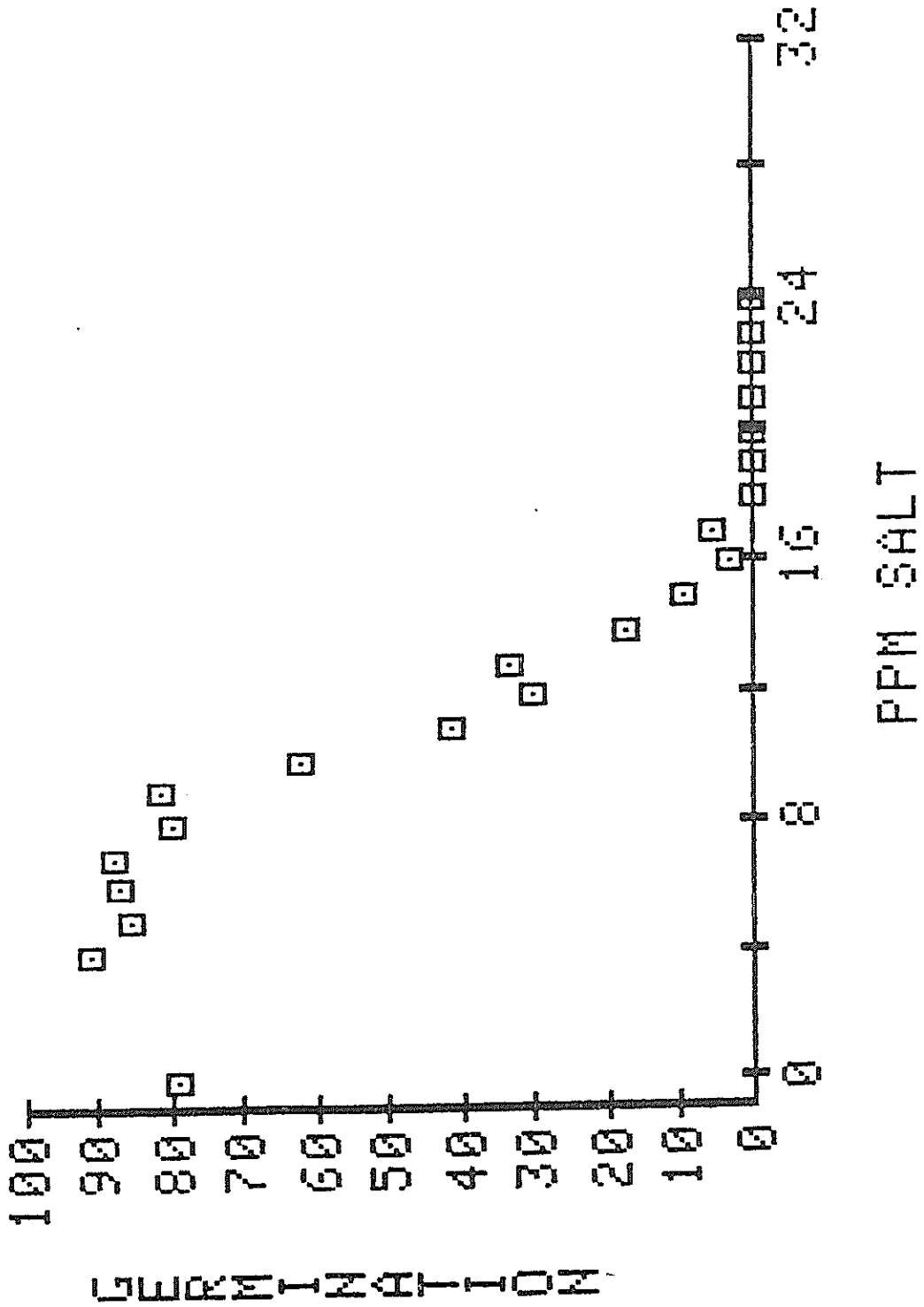
FIGURE 2. Germination frequency versus PPM salt

10

Table 3. Seed germination under soil conditions under varying levels of
salinity

PPM Salt	Soil E.C.	% Germination
0	0.98	70
400	2.36	56
800	3.12	62
1600	6.46	75
3200	9.10	24*
7200	20.29	0
11200	32.20	0
16000	33.44	0
19200	45.04	0
232000	50.79	0
280000	54.96	0

* Based on readings from 1 flat only

still was obtained at levels as high as 17,000 PPM (experiments 1 and 2). The presence of the soil medium obviously has an exacerbating effect on germination under saline irrigation. This study suggests that screening in petri dishes may yield misleading data because of the absence of the soil medium. Hence, it was decided that any germplasm screening would be conducted in a soil medium in a greenhouse atmosphere (see experiment 5 -- germplasm screening).

Experiment 4. This experiment was conducted in a greenhouse with average daily temperature of 33° C and night temperature of 15° C. The day length was approximately 12 hrs. R Naky seedlings were grown under normal greenhouse conditions and transplanted into 6" pots when they had four true leaves. Ten days later when most plants had 10 true leaves, the experiment was initiated. At this time 7 of the plants were sacrificed to determine initial mean fresh and dry weight before the salt treatments began.

The following salt concentrations were used to irrigate the potted chile: 0 (control); 775; 3,100; 7,000; 11,000; 15,500; 18,500; 22,500; 27,000; 31,000 PPM. Each concentration was applied daily to two groups of six plants each. The only exception to this rule was for the control concentration, 0 PPM, which was applied to four groups of six plants. The groups of six plants each were randomized with respect to location on the benches to minimize the effect of micro environments, which may exist in the greenhouse. The mean of data collected from each group of six was treated as a single data point and used for further calculations. Statistics were computed on the basis of a completely randomized design. The soil mix was 4 parts farm soil (sandy clay loam), 2 parts peat moss, and 1 part sand. Fertilizer (33N - 46P - OK) was

mixed with the soil at the beginning of the experiments after which no further applications were made. The soil level in the pots was a minimum of 2 cm below the top of the pot to allow flooding during irrigation, promote daily leaching, and prevent differential salt accumulation at various depths in the soil. All plants were harvested 1 month later when the controls had reached anthesis. The percentage of plants surviving in each group was noted. Fresh and dry weights were recorded for the foliar and root portions of surviving plants. The mean weight of the plants before the beginning of the salt treatments, as estimated from those plants sacrificed prior to the salt treatments, was subtracted from each weight to give estimates of accumulated weights (table 4).

To estimate final soil salinity, electrical conductivity (mmhos/cm) was measured on a single soil sample from each of the treatments (table 4).

Conclusions. Fresh and dry weight accumulation of both roots and foliage show a decline at the first level of salinity (775 PPM) (figures 3, 4). Roots are more affected than shoots at increasing levels of salinity as indicated by the increase in the foliage:root ratio (figure 5). Survival is not decreased until much higher levels (15,500 PPM); 27,000 PPM or higher result in 100% mortality of plants.

These data indicate that chile is very sensitive to water salinity and that even under modest levels (750 - 3,100 PPM) a significant decrease in dry matter production and thus probably yield can be expected.

Experiment 5 -- Germplasm screenings. Because of the differential results obtained with germination in soil versus on germination paper (experiments 1-3), it was decided to conduct initial germplasm screenings in soil in the greenhouse. Plastic flats with 50 single cells each were filled with soil (same mix as in experiment 4). Two seeds were sown in each cell.

13

Table 4. Response of whole plants to varying levels of saline irrigation under greenhouse conditions

Water PPM	Soil E.C. (mmhos/cm)			Accum.* Foliage Wt. (gm) Fresh	Dry	Accum.* Roots Wt. (gm) Fresh	Dry	Foliage:Root Ratio Fresh	Dry	Survival (%)
0	4.0	x	=	19.4	2.45	5.11	0.53	3.70	4.53	100
		Sx	=	1.7	0.22	0.70	0.04	0.23	0.15	0
775	3.3			14.5	1.89	3.16	0.39	4.42	4.61	100
				2.0	0.34	0.66	0.02	0.02	0.49	0
3100	14.4			7.1	.90	0.96	0.20	4.82	4.51	100
				0.7	.01	0.17	0.04	0.32	0.16	0
7000	18.6			5.1	.60	0.43	0.13	4.90	4.22	100
				0.4	.00	.00	0.01	0.01	0.06	0
11000	21.5			4.1	.41	0.27	0.04	6.64	6.33	100
				0.5	.09	0.02	0.04	0.40	0.44	0
15500	26.9			2.9	.35	-0.37	0.01	7.54	6.58	75
				0.2	.00	0.04	0.01	1.40	0.76	25
18500	29.3			2.2	.34	-0.63	-0.02	10.25	8.65	75
				0.1	.04	0.01	0.00	0.22	0.49	8
22500	30.7			1.6**	.26	-0.87	-0.03	17.55	10.22	33
27000	32.6			--	--	--	--	--	--	0
31000	37.9			--	--	--	--	--	--	0

* Mean weights of corresponding plant parts before initiation of saline irrigation has been subtracted to give estimate of accumulated weights. Mean fresh and dry weights were determined from seven average sized plants which were sacrificed before the experiment began. Those control values are: roots (fresh) = 1.169 gm/plant; roots (dry) = 0.108 gm/plant; shoots (fresh) = 2.359 gm/plant; shoots (dry) = 0.333 gm.

** Based on measurements from a single treatment group
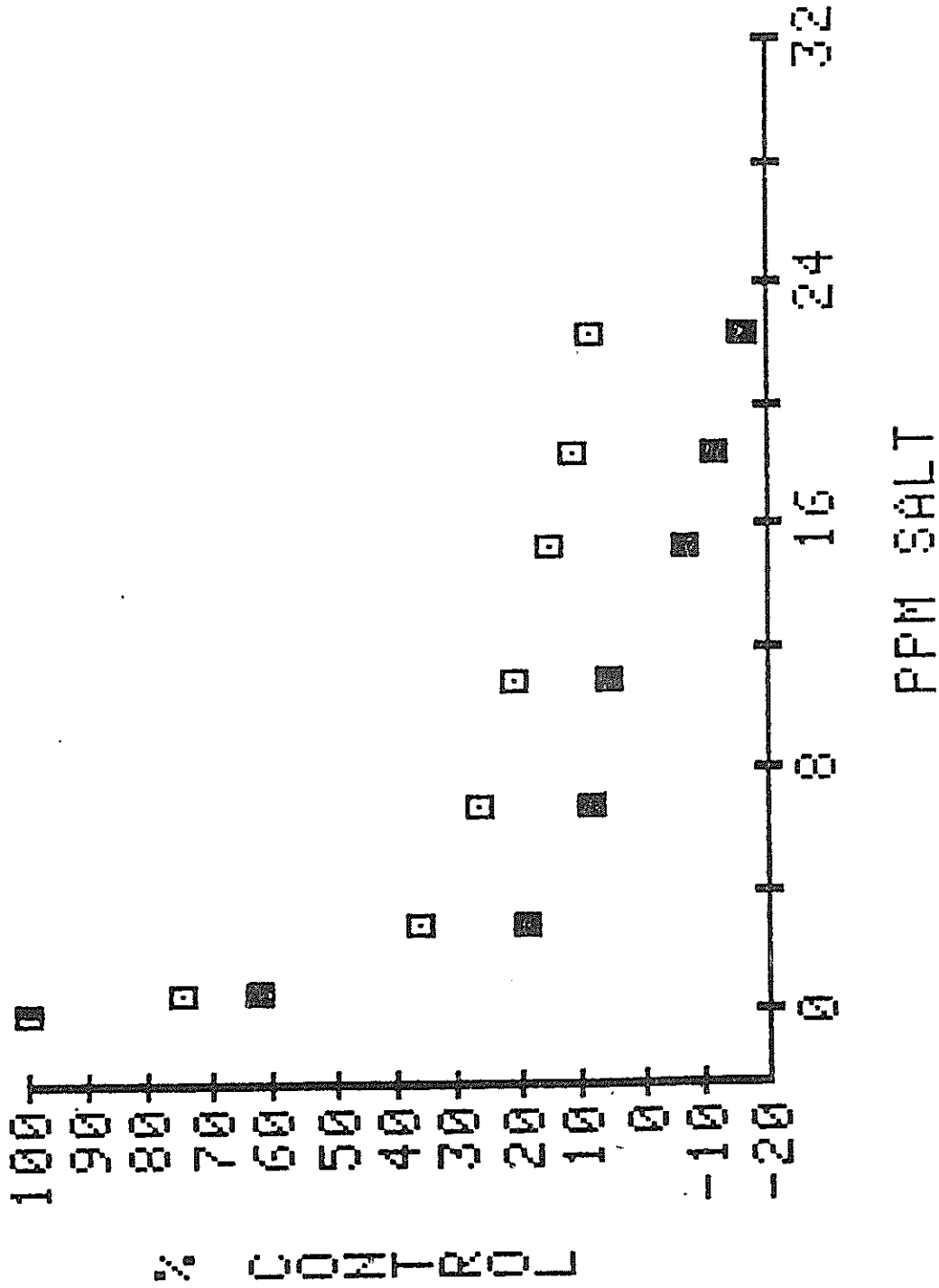
FIGURE 3. Fresh weight accumulation versus PPM salt (open squares = foliage, closed squares = roo

15
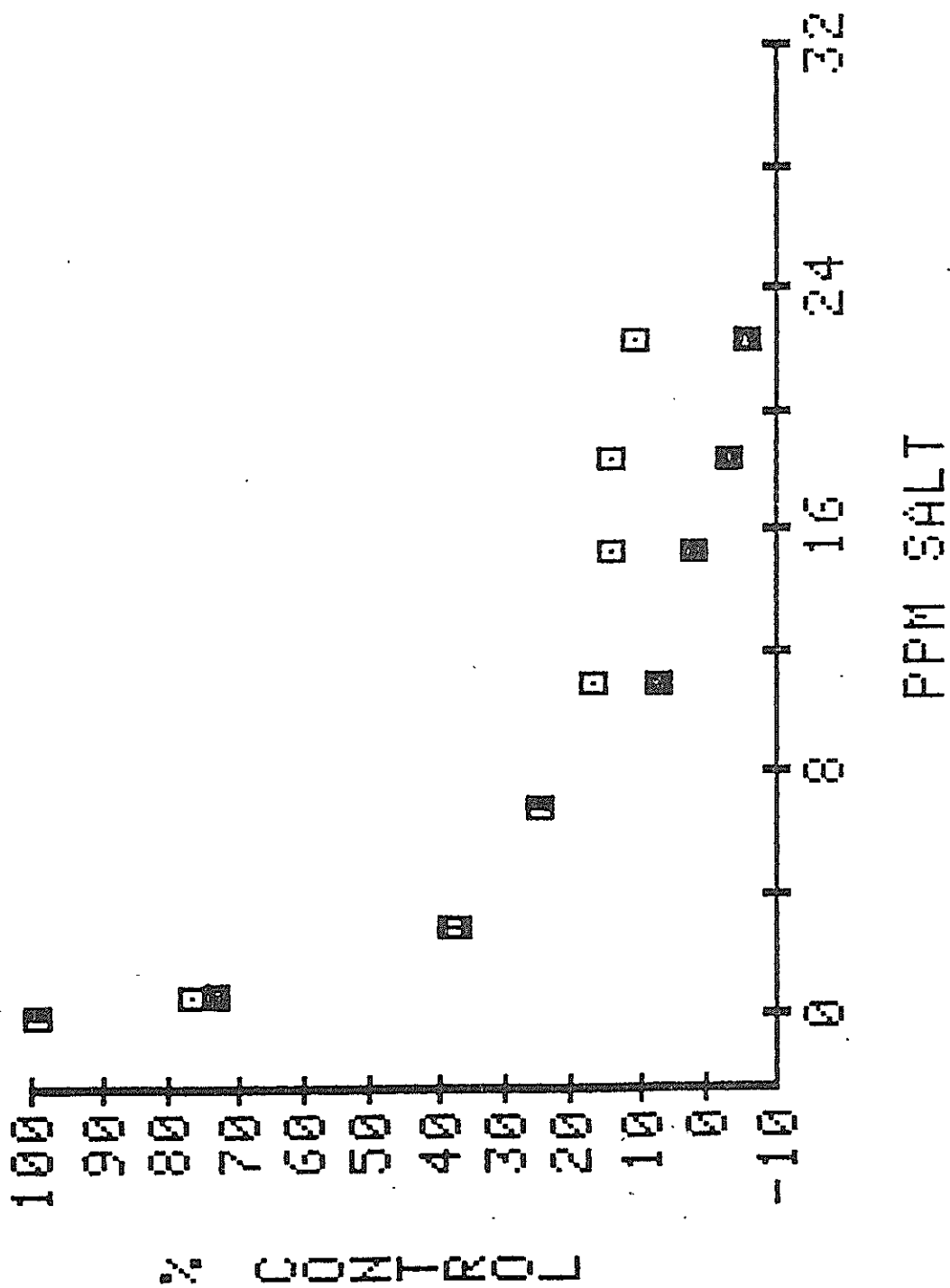
FIGURE 4. Dry weight accumulation versus PPM salt (open squares = foliage, closed squares = roots)

16
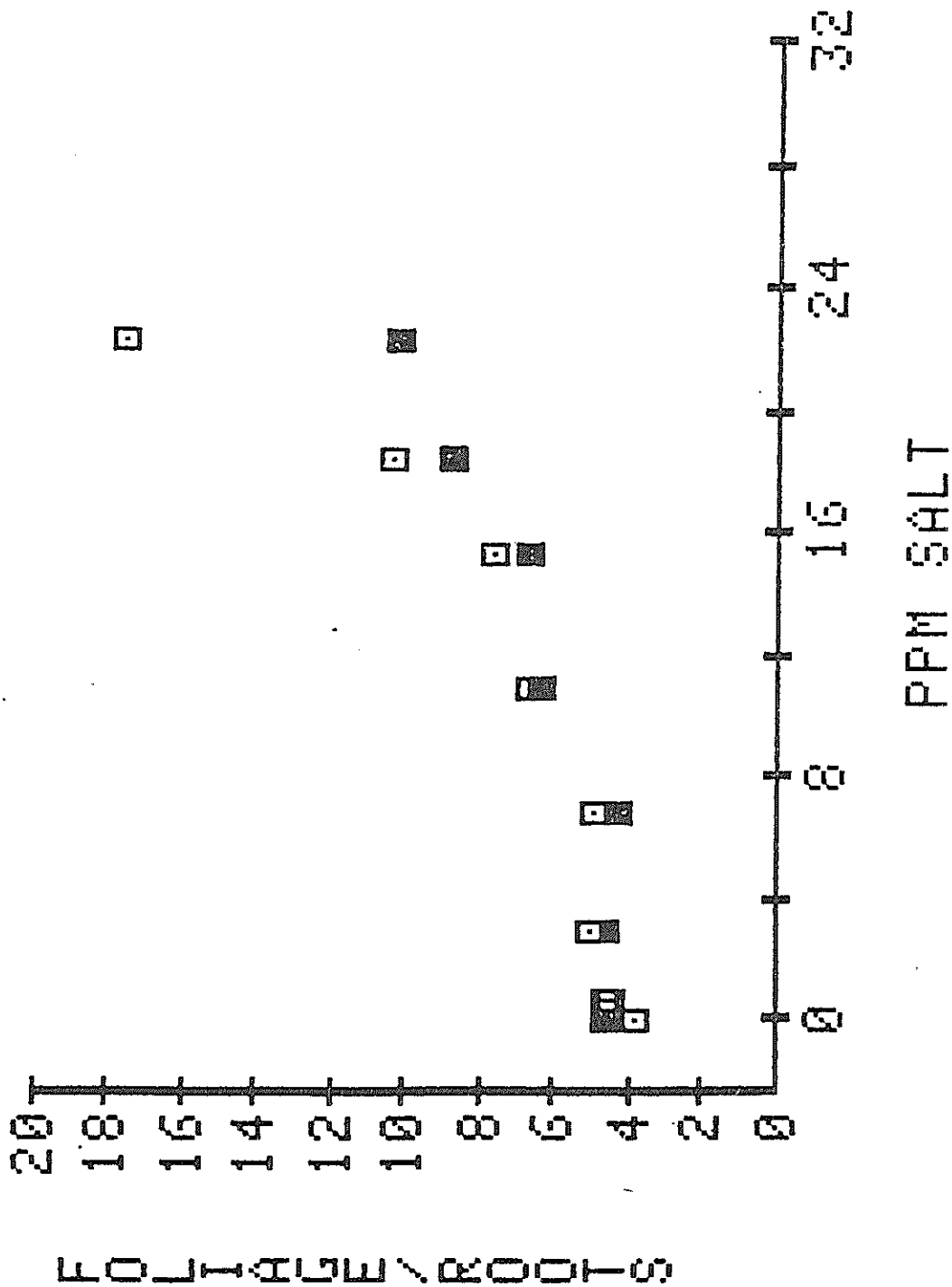
FIGURE 5. Foliage: root weight ratio versus PPM salt (open squares = foliage, closed square roots)

17

For each accession, two flats were sown--one control (0 PPM irrigation water) and one treatment (7,200 PPM).

Flats were irrigated daily or every other day, depending upon the weather conditions, for a total of 35 days. Daily records were taken of the number of seeds germinated in each flat. Seeds were counted as germinated when the cotyledons could be observed emerging through the soil. For the majority of lines, seeds that germinated under the salt treatment did so only after a delayed period (table 5). Most germinated seeds died before or shortly after the cotyledons expanded. Commercial New Mexico varieties (Nu Mex R Naky - CA 133, and NM 6-4 - CA 8) performed poorly under the stress. The few seeds of these varieties that did germinate died before the cotyledons were expanded. CA 4 seeds germinated quickly under the stress and survived long enough to fully expand their cotyledons but failed to survive the stage of producing the first true leaves. However, the rapid rate of germination and stage to which they did survive suggests that this accession possesses increased salinity tolerance as compared to commercial varieties. A single plant from P.I. 164454 survived after germination long enough to produce true leaves.

Additional screening for survival only. Approximately 50 additional accessions were screened for survival only, under the same salinity regime outlined above. Seedlings from five additional accessions (PI 137444, PI 140375, PI 164311, PI 164835, PI 164847) survived to produce true leaves and were saved for further crossing and selection.

Production of selfed lines and hybridizations of selected lines with CA133. Selected plants from PI 127444, PI 140375, PI 164311, PI 164835, PI 164847 and PI 164454 were grown to maturity and self pollinated to obtain seed. In addition, selected plants were hybridized with CA133 (Nu Mex R Naky)

18

Table 5. Results from screening of germplasm for salt tolerance (C = control
-- 0 PPM, T = treatment -- 7200 PPM, A = C. annuum, B = C. baccatum, C = C.
chinense, F = C. frutescens, P = P. pubescens)

Accession	Species	Germination total	% ctrl.	No. days to 50% germ. ctrl.	trt.	ratio	survival*
CA 46 (c)	A	110		21	30	0.70	0
(t)		2	1.8%				
CA (c)	A	137		16	36	0.44	0
(t)		3	2.2%				
CA 49 (c)	A	45		28	-	-	0
(t)		0	0.0%				
PI 439364 (c)	B	118		19	-	-	0
(t)		0	0.0%				
PI 439414 (c)	C	73		33	-	-	0
(t)		0	0.0%				
PI 164961 (c)	A	130		18	38	0.47	0
(t)		5	3.8%				
CA 230 (t)	P	103		31	-	-	0
(c)		0	0.0%				
PI 435918 (c)	C	117		26	-	-	0
(t)		0	0.0%				
CA4 (c)	C	105		16	15	1.07	0
(t)		9	8.6%				
CA 181 (c)	P	18		31	-	-	0
(t)		0	0.0%				
PI 260536 (c)	B	131		21	39	0.54	0
(t)		1	0.8%				
CA 139 (c)	A	104		22	27	0.81	0
(t)		18	17.3%				
PI 260535 (c)	B	137		21	35	0.60	0
(t)		1	0.7%				
CA 80 (c)	A	110		21	36	0.58	0
(t)		10	9.0%				
PI 159264 (c)	C	134			26		
(t)		1	0.7%				
CA 133 (c)	A	117					
(t)		7	6.0%				
PI 164454 (c)	F	142					1
(t)		6	4.0%				
CA 131 (c)	A	131			34		
(t)		10	8.0%				
CA 134 (c)	A	142					
(t)		1	0.7%				
PI 260438 (c)	A	55					
(t)		0	0.0%				
Sandia (c)	A	114					
(t)		1	0.9%				

for production of F2 and backcross generations for salt selections and potential transfer of "salt tolerant genes" into commercial cultivars.

Mexican Capsicums. Mexico is the center of origin and diversity for cultivated pepper (C. annuum). In addition to the material screened, a collection of 191 Mexican Capsicums was obtained from Dr. Jose Laborde, Unit of Genetic Resources, Celaya, Mexico (figure 6). The material is being increased to produce sufficient numbers of seeds for salt screening. Preliminary electrophoretic surveys indicate that this material possesses more genetic variability (and perhaps more genetic potential) than any other Capsicum collection that has previously been available to pepper geneticists. Some of these collections of wild peppers come from the states of Sonora and Chihuahua, where natural environments may have already selected for salt tolerant chile types. We are hopeful that such germplasm will yield even better results than the materials already screened.
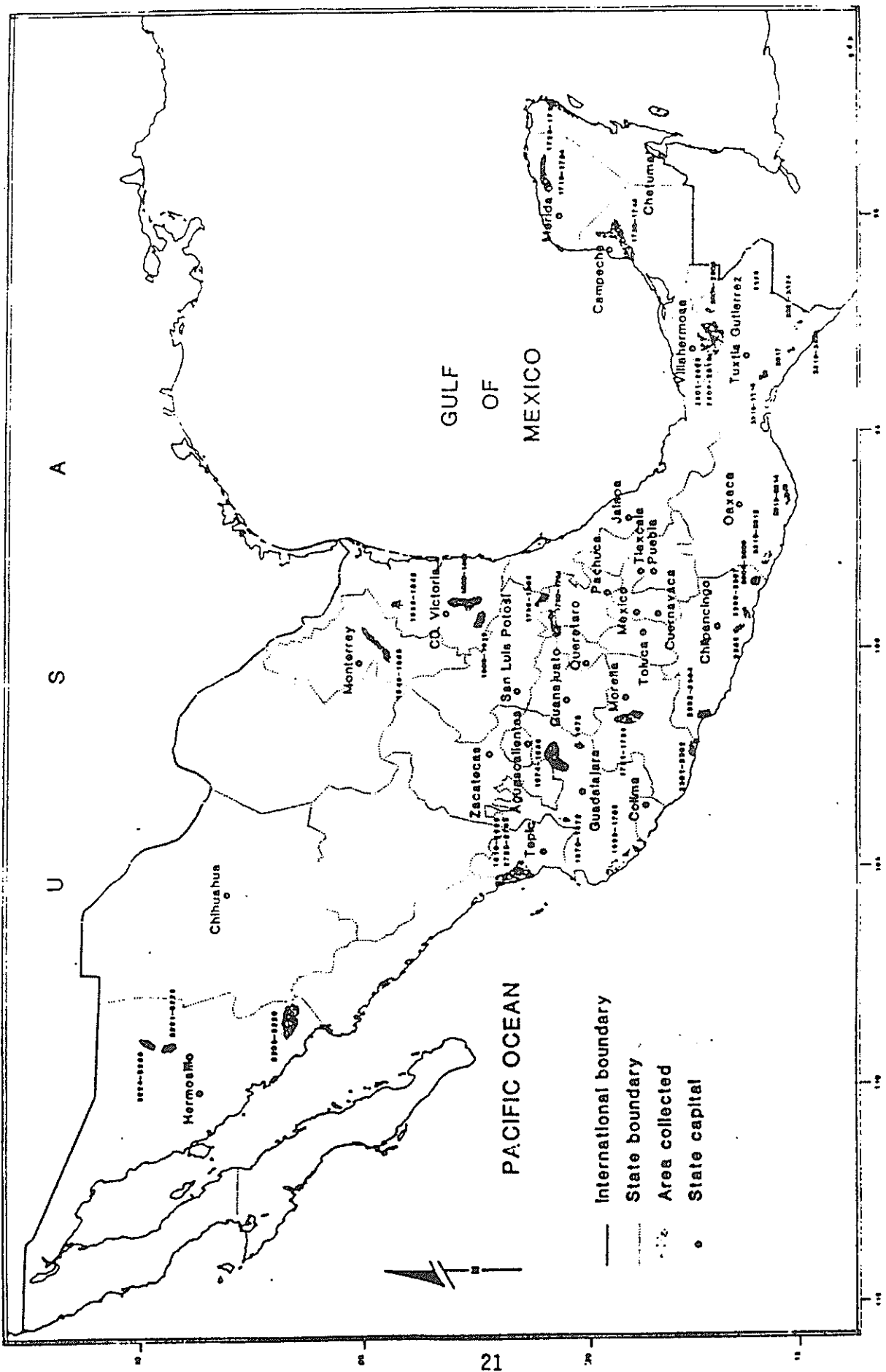
Figure 6. *Capsicum collecting in Mexico*

Part Two: Tissue and Cell Culture Studies

Sensitivity of Chile Cells Exposed to Salt in Cell Culture

Experiment 1. A standard culture medium (Gunay and Rao 1978) was used to establish stock callus (aggregated cell-tissues) cultures from NuMex R Naky hypocotyl seedling explants. This callus was used to establish stock cell suspension cultures (dispersed cells). The standard medium contains approximately 4,200 PPM dissolved solids supplied by an array of nutrient salts providing all essential elements. The literature indicates cell selection for enhanced salt tolerance is feasible using cell cultures. The first step in this approach is to describe cell sensitivity to salt. Moreover, cell-plant correlations for salt sensitivity may provide useful insights. However, it is appalling to see in the literature that such cell sensitivity curves to salt have never been adequately performed. A combination of 85% NaCl and 15% $CaSO_4$ was added to the standard culture medium to evaluate cell sensitivity (4 replications; experiment repeated once). Data were recorded after 20 days of incubation (table 6).

Conclusion. This experiment indicates that NuMex R Naky cells are not very sensitive to salt (figure 7). Dry weight increased with added salt. Only very high additions of salt resulted in decreased fresh weight of cells. This approach does not appear very reliable for screening for salt sensitivity or tolerance in chile. If cell selection is to be pursued, levels of 32,000 PPM added salt or greater will be required.

22

Table 6. Growth of NuMex RNaky cells in response to added salt.

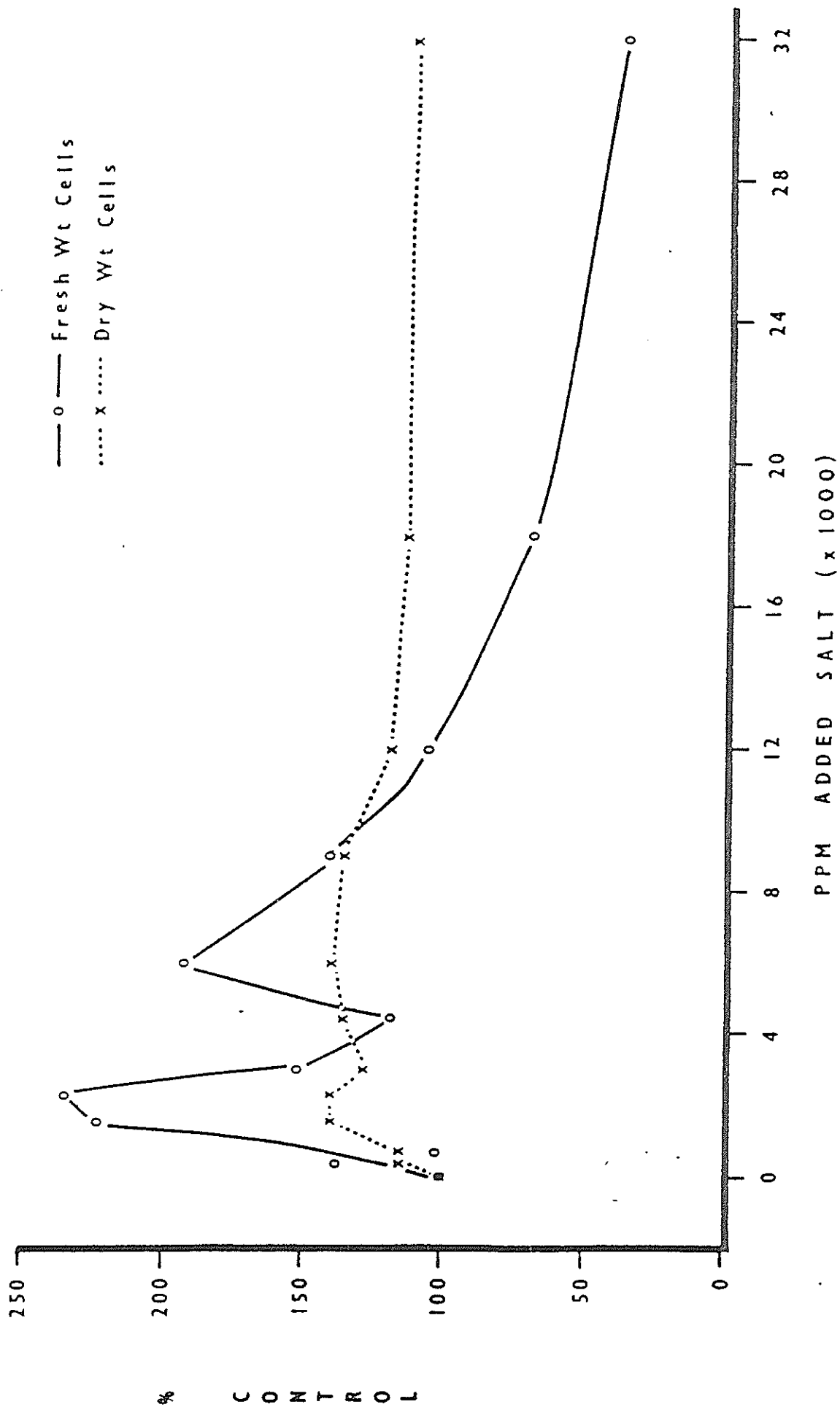| Added Salt, ppm Dissolved Solids	Fresh Weight, % Control	Dry Weight, % Control
0 (Control)	100.0	100.0
375	137.0	119.4
750	101.5	119.4
1500	222.2	138.9
2250	234.1	138.9
3000	152.6	127.8
4500	117.8	136.1
6000	192.6	138.9
9000	140.7	136.1
12000	105.9	119.4
18000	68.9	113.9
32000	37.0	113.9

Figure 7. Sensitivity of chile cell suspensions exposed to salt
additions to standard medium after 3 weeks incubation

24

Sensitivity of Chile Callus Exposed to Salt in Callus Culture

Experiment 2. Stock callus cultures of NuMex R Naky hypocotyl explants were exposed to the salt combination described above. Each treatment was replicated 4 times. Two versions of the experiment were evaluated, one using the standard callus culture medium, and another using the same medium with only one-quarter strength nutrient salts. Data were recorded at 30 days (table 7).

Conclusion. This experiment indicates that moderate levels of added salt actually stimulated fresh and dry weight of NuMex R Naky callus compared to the control (figures 8, 9). This phenomenon is most pronounced when the nutrient salts were reduced, and the added salt combination appeares to "substitute" for nutrient requirements in a sense (figure 9). Dry weight is reduced to 50% or less of the control only when 28,000 PPM added salt is present, although fresh weight is reduced to 50% or less of the control at 10,000 (figure 9) or 20,000 PPM (figure 8) added salt depending on the level of nutrient salts present. The phenomenon of fresh weight exhibiting greater salt sensitivity than dry weight reflects similar trends observed in whole plant analyses. However, this test system still fails to provide a sensitive screening system for chile compared to whole plant screening procedures.

Sensitivity of Chile Seedling Explants Exposed to Salt in Callus Induction Culture

Experiment 3. This experiment was conducted similarly as that described above except 2-week-old seedlings were sectioned into root, hypocotyl and shoot tip explants and placed onto standard media to induce callus growth. Visual scoring indicates all explant sources responded comparably. However, initial masses of the explant sources vary consideralby, so all tissues

Table 7. Growth of NuMex RNaky callus in response to added salt

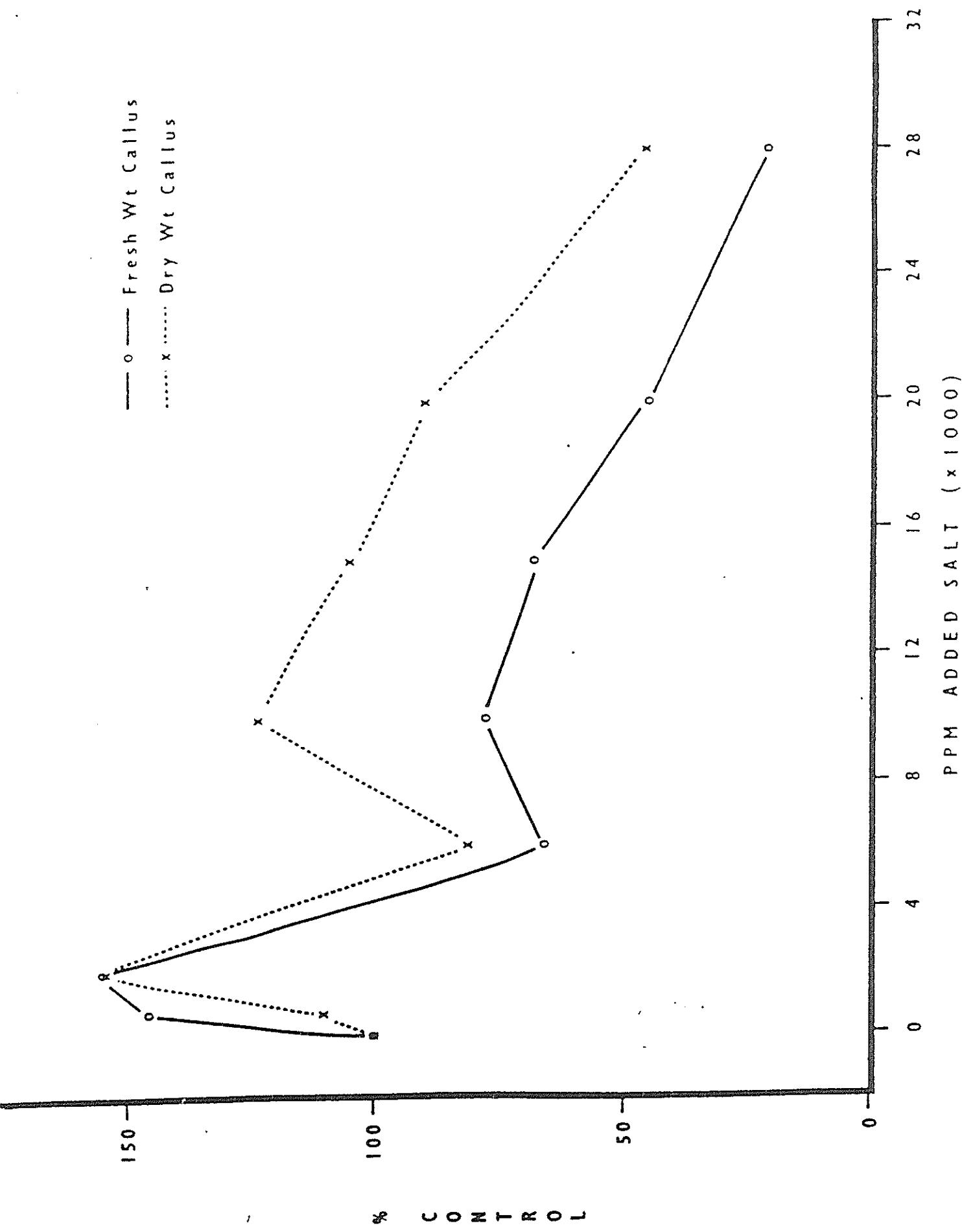| Basal Medium	Added Salts, ppm Dissolved Salts	Fresh Weight, % Control	Dry Weight, % Control
Standard	0 (Control)	100.0	100.0
	750	146.0	110.2
	2000	155.0	154.2
	6000	66.0	81.4
	10000	77.5	123.7
	15000	68.0	105.1
	20000	45.1	89.8
	28000	21.1	45.8
One-Quarter	0	88.5	120.3
	750	230.5	201.7
	2000	66.9	86.4
	6000	59.8	100.0
	10000	48.5	93.2
	15000	42.6	89.8
	20000	23.6	66.1
	28000	13.1	50.8

Figure 8. Sensitivity of chile callus exposed to salt additions to standard medium after 4 weeks incubation.

27
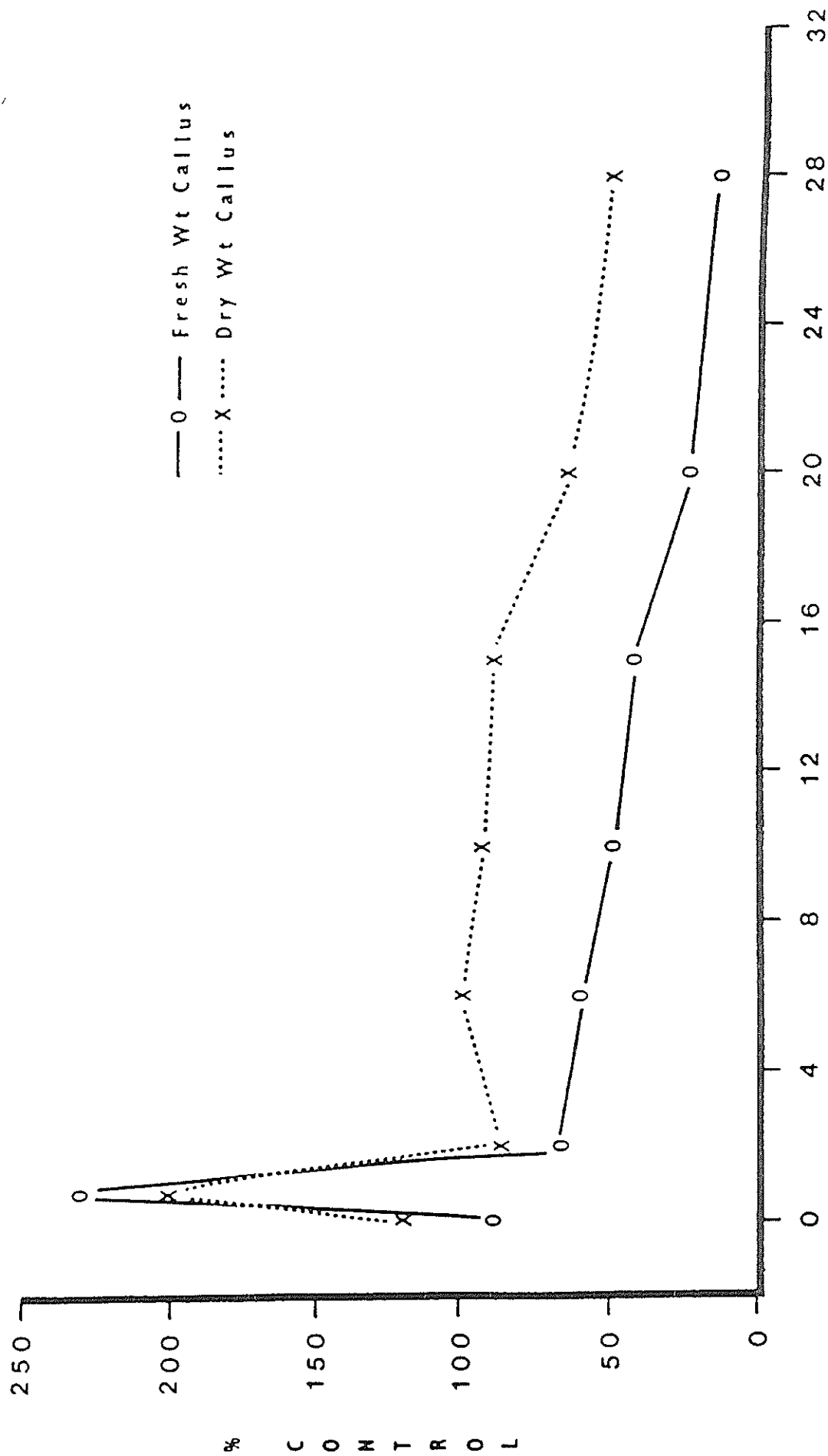
Figure 9. Sensitivity of chile callus exposed to salt additions to one-quarter-strength medium after 4 weeks incubation.

28

derived from a single seedling were bulked for analysis. Data were recorded at 30 days (table 8). The experiment was repeated once.

Conclusion. This experiment also exhibits stimulation of fresh and dry weights when low to moderate levels of added salt were present (figures 10, 11). However, with both versions of the nutrient medium, both fresh and dry weights were reduced to 50% or less of the control when 6,000 PPM or greater added salt was present. Apparently, seedling explants, being organized tissues, exhibit salt sensitivity somewhat comparable to that seen with whole plant screening systems. On the other hand, unorganized cell proliferation systems--whether callus or cell suspensions--seem to deal with the added salt in a different manner than organized tissues, such as seedling explants in tissue culture or whole plant test systems. Either version of this experiment appears to be a reliable indicator of salt sensitivity or tolerance for chile, although the responses were more dramatic using the one-quarter strength nutrient salt base.

Screening of chile accessions for salt tolerance can be accomplished rapidly using this in vitro method. In as little as 30 days, seedling explants on one-quarter strength nutrient medium plus 6,000 PPM added salt exhibited at least 50% growth arrest. For more stringent selection conditions, this same medium plus 15,000 PPM added salt will almost completely arrest growth. These test systems require very little space or time, so large populations of seedlings can be screened for moderate or high salinity tolerance. Moreover, this system need not be destructive; for example, only the cotyledons of seedlings could be used to accomplish the screening, so elite plants can be retained. Further tests will more accurately establish

29

Table 8. Growth of NuMex RNaky seedling pieces in response to added salt in callus induction culture

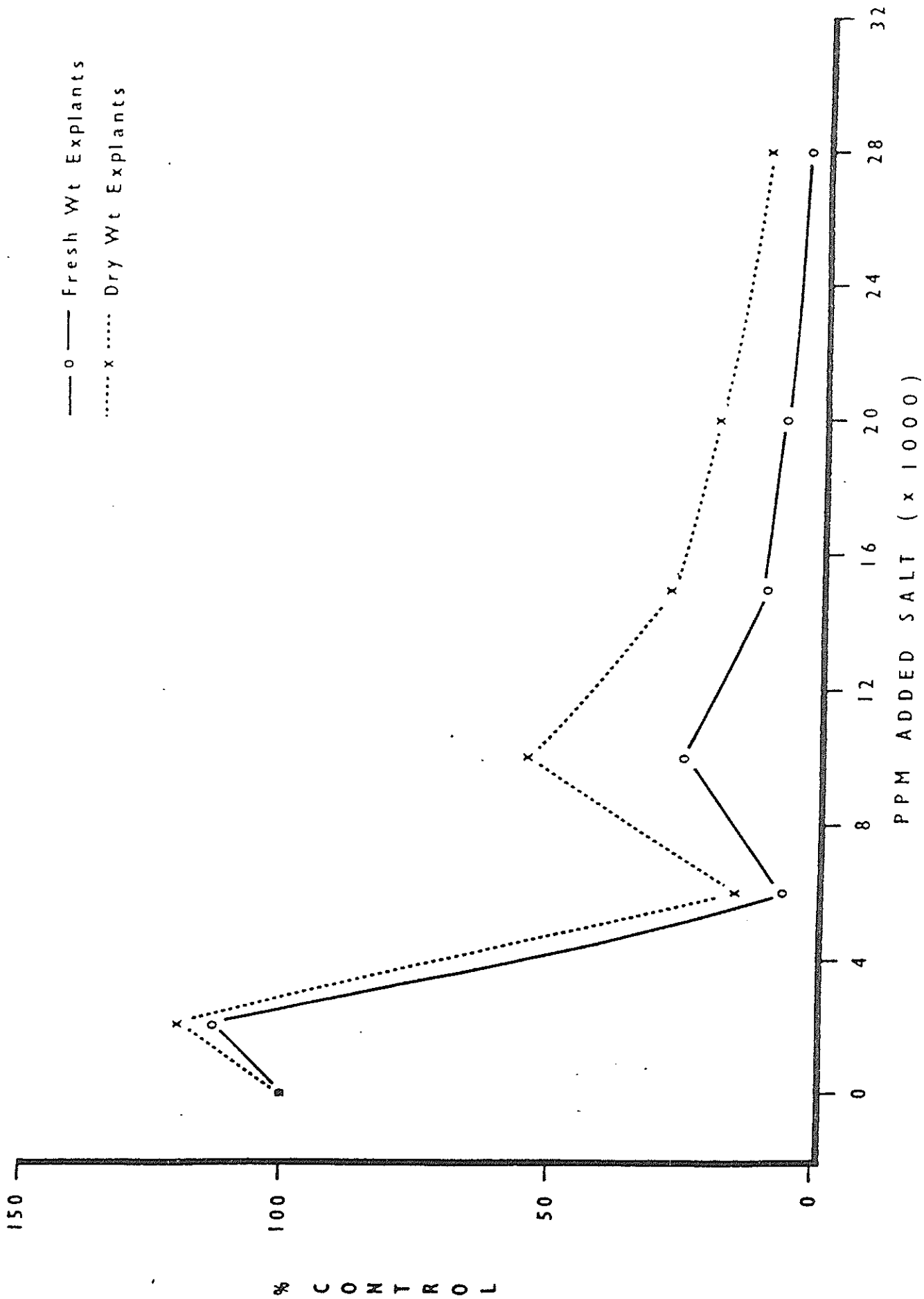| Basal Medium	Added Salt, ppm Dissolved Solids	Fresh Weight, % Control	Dry Weight, % Control
Standard	0 (Control)	100.0	100.0
	750	(Missing)	(Missing)
	2000	112.8	119.3
	6000	6.3	12.6
	10000	25.2	54.8
	15000	9.8	28.1
	20000	6.9	19.3
	28000	2.9	11.1
One-Quarter	0	65.0	94.8
	750	54.5	79.3
	2000	117.1	151.1
	6000	15.5	43.7
	10000	23.9	45.9
	15000	0.7	2.2
	20000	1.2	16.3
	28000	1.3	5.2

Figure 10. Sensitivity of chile seedling explants exposed to salt additions to standard medium after 4 weeks incubation.
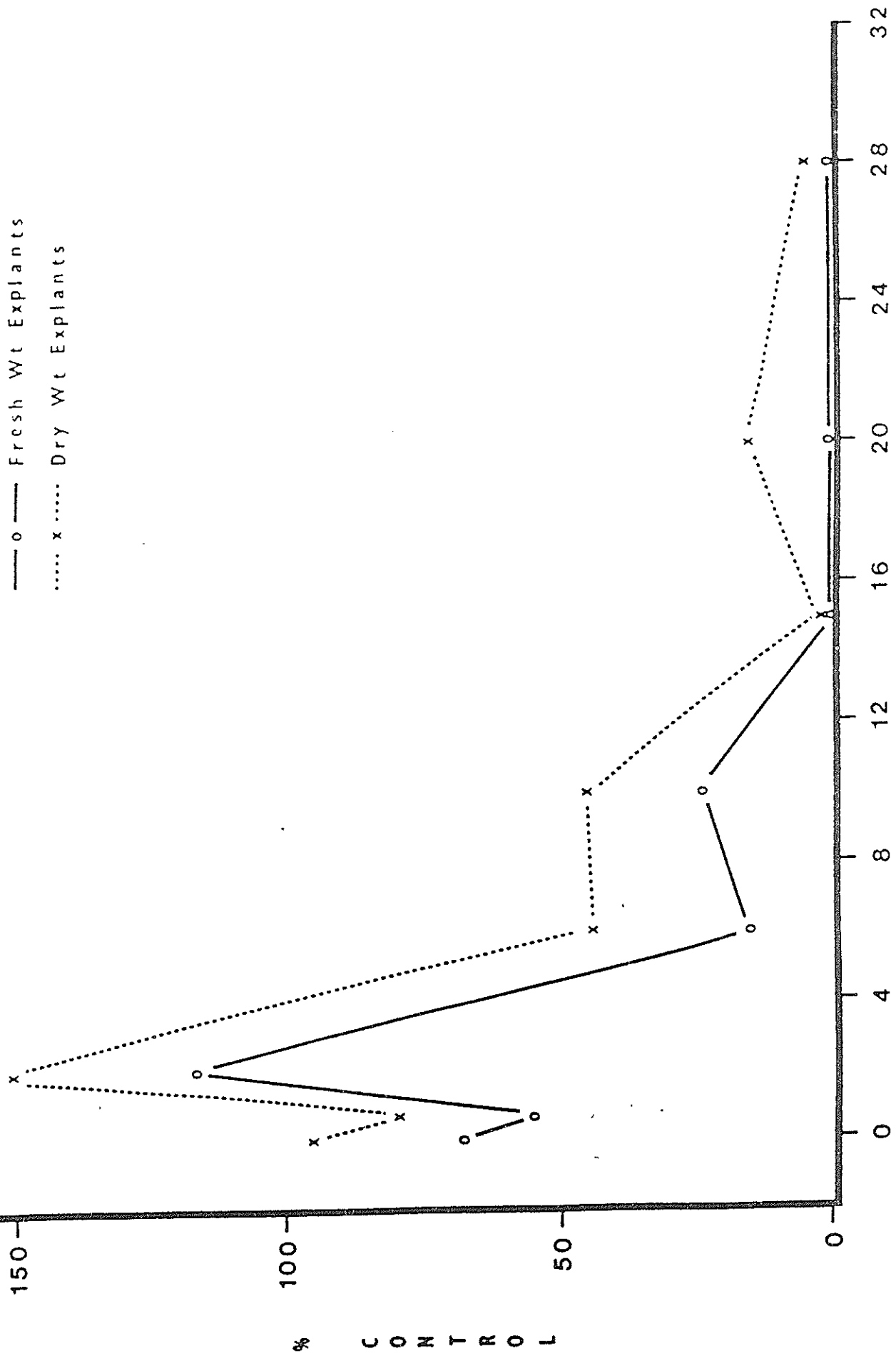
Figure 11. Sensitivity of chile seedling explants exposed to salt additions to one-quarter- strength medium after 4 weeks incubation.

the degree of correlation between this _in vitro_ system and whole plant screening systems. The growth response curves for salt tolerance generated in this series of experiments are the first of their kind recorded, to our knowledge.

Regeneration of pepper plants from tissue cultures

The literature pertaining to bell pepper tissue culture indicated that plants could be readily regenerated from callus or single cells. We repeated these experiments and were able to vegetatively propagate pepper through short-term tissue culture, but we could not repeat regeneration from callus or cells. Tissue culture propagation would be a useful tool to rapidly clone elite or difficult-to-isolate stocks, which would simplify the gene transfer program described in a previous section of this report. However, in order to pursue cellular selection and other single-cell-based approaches to genetic improvement, the capability to regenerate plants from cultured cells is essential. The following series of experiments were designed to more fully describe reliable methods of pepper plant regeneration from cells and their limitations, if any.

Seeds of _C. annuum_ cvs. 'California Wonder,' 'Yolo Wonder' (sweet bell types), 'New Mexico No. 6-4' and 'NuMex R Naky' (long green chile types) were obtained from local commerical outlets. Seeds were germinated aseptically on germination paper soaked in a liquid one-tenth-strength nutrient medium. Three- to four-week-old seedlings were used to provide explant tissues (hypocotyl sections, distal cotyledon pieces, cotyledonary nodes, and shoot-tips) for most experiments. Alternatively, plants were grown in the greenhouse for collection of 1 cm diameter leaf-disc explants from young, expanding leaves.

33

Explants were inoculated onto MS basal nutrient salts media (Gunay and Rao 1978). Some media varied in the auxins indole-3-acetic acid (0.01-10 mg/l IAA), indole-3-butyric acid (0.01-1 mg/l IBA), 2,4-dichlorophenoxyacetic acid (0.01-1 mg/l 2,4-D), 4-amino-3,5,6-trichloropicolinic acid (0.01-0.2 mg/l picloram), or the auxin inhibitor 2,3,5-triiodobenzoic acid (0.01-0.1 mg/l TIBA); or in the cytokinins 6-benzylaminopurine (0.02-50 mg/l BA) and kinetin (0.1-3 mg/l K). Media included 3% sucrose or glucose and were solidified with 0.8% plant tissue culture agar. As many as four incubation environments were used, varying in temperature (25 ± 0.2°C or 28.5 ± 0.2°C) and photoperiod length (12, 16 or 24 h). Light was supplied by Sylvania Cool-White fluorescent bulbs providing 130 $uEs^{-1}m^{-2}$ and Sylvania Ex-Cell incandescent bulbs providing 30 $uEs^{-1}m^{-2}$. Tissue cultures were transferred to fresh media every 4 weeks.

Data were recorded at the end of each culture passage in a binomial manner, scoring the presence or absence of shoots or roots. Results were assessed by standard analysis of variance for a factorial randomized complete block experimental design, utilizing the normal approximation. However, the error term used in F-tests reflects the binomial sampling variance. Least significant difference tests were applied for treatment separation, or for the description of interaction effects, where appropriate. Estimates were adjusted for unequal replication.

Two representatives each of bell pepper and chile pepper types were used in these studies on in vitro organogenesis. In each experiment, neither interaction nor main effects of the cultivars were significant at the 5% level. Therefore, cultivar data were pooled for presentation. However, the

possibility should not be ruled out that cultivar effects may interact with other variables.

A preliminary experiment evaluated growth regulator effects on seedling explants, utilizing MS media with sucrose and incubation conditions of 25°C with 16-h photoperiod (data not shown). In general, results supported the previous conclusions (Gunay and Rao 1978) that shoot and root organogenesis in 4-week-old tissue cultures is repeatable and suitable for vegetative propagation, and that IAA and BA are the best growth regulators for use in pepper tissue cultures. Our results showed significant interaction between growth regulator treatment and explant source. Meristematic explants (shoot-tips, cotyledonary nodes) generally exhibited a 2- to 10-fold greater capacity for shoot organogenesis compared to non-meristematic explants (hypocotyls, distal cotyledon pieces). The converse was true of root organogenesis. The absence of cytokinin suppressed organogenesis. Rooting occurred in the presence of any auxin with a cytokinin. Shooting occurred in the presence of cytokinin with or without IAA or IBA. Results indicated cytokinin is important for rooting and all tested auxins permit rooting, in contrast to a previous report (Gunay and Rao 1978). A striking observation during the second passage of the experiment was that root organogenesis did not occur and shoot organogenesis was rare.

In the first major experiment, environmental incubation variables were evaluated for their effects on organogenesis in long-term pepper tissue cultures. Culture media contained sucrose with IAA or IBA as auxin and BA as cytokinin. Cultures initiated from seedling explants were observed for nine monthly passages. Shoot organogenesis data are presented (table 9) showing incubation environment treatment and explant treatment separation within each

35

Table 9. Influence of incubation environment, explant source and passage number on shoot and root organogenesis in pepper tissue cultures[a]

Incubation Environment	Seedling Explants[b]	Monthly Passage Number						
		1		2	3-4	5	6-8
		% Shooting[c]	% Rooting	% Shooting	% Shooting	% Shooting	% Shooting
25°C, 12-h photoperiod	meristematic	15.0 E	0 E	0 E	0 D	0 C	0 B
	non-meristematic	0 F	0 E	0 E	0 D	0 C	0 B
25°C, 16-h photoperiod	meristematic	27.1 C	6.3 D	16.3 C	0 D	0 C	0 B
	non-meristematic	20.7 D	75.9 A	0 E	0 D	0 C	0 B
25°C, continuous light	meristematic	61.1 B	54.2 B	27.3 B	13.6 B	7.9 B	0 B
	non-meristematic	0 F	8.3 D	0 E	0 D	0 C	0 B
28.5°C, continuous light	meristematic	87.5 A	50.0 C	58.3 A	45.3 A	38.5 A	11.8 A
	non-meristematic	27.5 C	8.4 D	8.3 D	7.3 C	0 C	0 B

a Culture media are described in the text.

b Meristematic = shoot-tip and cotyledonary node, non-meristematic = hypocotyl and distal cotyledon pieces.

c Values followed by different letters within columns are significantly different at the 1% level by the least significant difference test (average number of replications was 32).

36

passage, because interaction effects were highly significant. Shooting frequencies from meristematic explants increased as temperature and photoperiod length increased, while non-meristematic explants exhibited specific responses to incubation environments. Under 12-h photoperiod at 25°C, shooting occurred at a low level only during the first monthly passage in meristematic explants. Shoot organogenesis was enhanced under 16-h photoperiod (25°C); meristematic explants responded for two months and non-meristematic exxplants responded for one month. Dramatic improvements in shooting frequencies were observed under continuous light (25°C), where meristematic explants responded for 5 months. However, non-meristematic explants failed to respond positively under this environment. Raising the incubation temperature to 28.5°C (continuous light) elicited the best shooting responses. Meristematic explants responded for 8 months and non-meristematic explants for 4 months. Shoot organogenesis frequencies generally declined rapidly over time, although the rates of decline varied with incubation environment and explant source. No shooting was observed in the ninth monthly passage (data not shown).

Rooting was observed during the first monthly passage of this experiment, but not during the second month. Root organogenesis data are presented (table 9) only for the first passage, showing the interaction between incubation environment and explant source. No rooting was observed with 12-h photoperiod (25°C). Non-meristematic explants exhibited root organogenesis at highest frequency with 16-h photoperiod (25°C). Meristematic explants showed high levels of rooting under continuous light (25 or 28.5°C). These results have important implications for attempting to root various pepper explants, but are discouraging for maintaining root organogenic responses over time.

In the next experiment, seedling meristematic explants were incubated at 28.5°C with continuous light on media containing IAA and BA, and varying for either sucrose or glucose as the carbon source. Over six culture passages, the glucose treatment exhibited an average of 53.8% shooting response, while the sucrose treatment averaged 36.4%. Effects of carbon source were not significant at the 5% test level.

Another experiment, utilizing leaf disc explants collected from greenhouse-grown plants, displayed various interaction effects between carbon source and other variables (table 10). The leaf discs were incubated under two environments for two culture passages, with the same media used as in the previous experiment. Shoot organogenesis occured for only 1 month using glucose at 25°C and 16-h photoperiod, but for 2 months using either carbon source at 28.5°C and continuous light. Shooting occurred at comparable levels on the glucose treatment during the first monthly passage under the two incubation environments. Shoot organogenesis was maintained for 2 months at highest frequency using glucose with the optimum environment (28.5°C, continuous light). In practical terms, de novo adventitious budding was demonstrated utilizing a non-destructive explant sampling procedure.

A final experiment involved an assessment of shoot and root organogenesis in pepper tissue cultures, over time, under optimal cultural conditions. One growth regulator treatment included low concentrations of BA (0.02-1 mg/l) and low IAA (0.01-1 mg/l), while the other included high BA (2-50 mg/l) and low to high IAA (0.05-10 mg/l). The data (table 11) show passage treatment separation within growth regulator treatments, because interaction effects were significant. The most striking difference between the two growth

Table 10. Interactions of incubation environment, carbon source and passage
number on adventitious shooting in pepper tissue cultures

Incubation Environment	Carbon Source in MS Media [a]	% Leaf Discs with Shoot Buds [b] Passage: 1	2
25°C,			
16-h photoperiod	glucose	33.3 B	0 D
	sucrose	0 D	0 D
28.5°C,			
continuous light	glucose	34.2 B	36.0 A
	sucrose	20.7 C	20.5 C

[a] Growth regulator composition is described in the text.

[b] Values followed by different letters are significantly

different at the 1% level by the least significant

difference test (average number of replications was 69).

39

Table 11. Influence of cytokinin level on long-term organogenesis in pepper tissue cultures incubated at 28.5°C with continuous light

MS Basal Medium with Glucose or Sucrose and including (in mg l^{-1}):	Type of Shooting Response	% Seedling Shoot-tip and Cotyledonary Node Explants Responding [a]			
		Passage Number			
		1	2	3-5	6-8
(0.01-1)IAA + (0.02-1)BA	Shoot elongation	83.3 A	50.0 B	30.5 C	4.2 D
	Root organogenesis	83.3 A	33.3 B	not scored	not scored
(0.05-10)IAA + (2-50)BA	Adventitious budding	91.7 A	66.7 B	48.1 C	12.8 D
	Root organogenesis	16.7 NS	16.7 NS	not scored	not scored

[a] Values followed by different letters within rows are significantly different at the 1% level by the least significant difference test (average number of replications was 12).

40

regulator treatments was that low BA/low IAA combinations elicited shoot elongation, while the high BA/IAA combinations elicited adventitious budding. The level of IAA did not appear to be critical for adventitious budding responses. Both types of shooting response rarely occurred in the same culture. Cultures responded positively to both growth regulator treatments for eight monthly passages. Shooting responses were high during the first passage (data shown), and then declined to zero at the ninth month (data not shown). The rate of decline for shoot elongation was faster than it was for adventitious budding response. The optimal combination of growth regulators to achieve shoot elongation (data not shown) was 0.05 mg/l each of IAA and BA. The best adventitious budding was achieved on various combinations of 0.05-4 mg/l IAA with 10-50 mg/l BA (data not shown).

Root organogenesis was scored for only the first two passages of this experiment. Data (table 11) show passage treatment separation within growth regulator treatments, because interaction effects were highly significant. The low BA/low IAA treatment elicited high levels of rooting during the first passage, but then declined significantly during the second month. The high BA/IAA treatment exhibited a constant, low level of rooting over the two passages. The same combination of growth regulators that appeared optimal for shoot elongation (0.05 mg/l each of BA and IAA) also appeared optimal for root organogenesis (data not shown).

Callus was obtained in all experiments under most treatment conditions. Callus was obtained consistently at nearly 100% frequency with the use of 0.5 mg/l 2,4-D. Callus was also obtained with the use of picloram or IAA with BA, but less frequently. Unorganized calli from all sources were transferred to

41

most of the treatments used during the studies, but shoot organogenesis never was observed and root organogenesis was observed rarely. Cell suspension cultures were established using callus inoculated into liquid versions of picloram/BA media, which proliferated well and permitted recovery of callus upon plating onto agar-solidified media. No organogenesis occurred from this callus. Plants were regenerated from protoplasts in a previous report (Saxena et al. 1981), implying a single-cell-to-plant capability exists in pepper. Results of present studies argue that cell-to-plant capabilities have yet to be defined, in so far as to permit cellular selection approaches using cell suspension or callus cultures.

Conclusions. One, light and temperature treatments are important for both shoot and root organogenesis in pepper tissue cultures. Two, IAA and BA provide the optimal growth regulator combination for both shoot and root organogenesis. Low levels of each are required for shoot elongation and rooting, but high levels of BA are required for adventitious budding. Three, glucose as carbon source interacts positively with certain incubation environments and other variables for shoot organogenesis.

Long-term shoot organogenesis proceeds optimally under continuous light and warm temperature (28.5°C). Continuous light and warm temperature also proved optimal for rooting of seedling meristematic explants, but non-meristematic explants rooted optimally at 25°C and 16-h photoperiod. Vegetative propagation of pepper is feasible through tissue culture for up to 8 months under defined conditions. These plant regeneration techniques are potentially useful for limited types of somaclonal variation or transformation

42

experiments. However, additional research is needed to define conditions for regeneration of pepper plants from unorganized callus or cell suspension cultures.

SUMMARY AND CONCLUSIONS

Results from experiments conducted during this project indicate that chile is very sensitive to the effects of saline irrigation. With existing commercial chile varieties grown in the state, salt concentrations as low as 775 PPM can result in a reduction in plant growth. Germination is severely inhibited at levels above 7000 PPM salt. The most efficient means of screening large number of seed appears to be in the germination stage using a soil medium.

Screening of diverse Capsicum germplasm has resulted in the discovery of races that appear to better germinate at salt levels prohibitive to commercial varieties. These lines have been crossed to commercial cultivars for potential use in the NMSU chile breeding program. In addition, a large collection of Mexican Capsicums has been obtained and partially increased. This material represents the largest collection of wild peppers with close affinity to the cultivated garden pepper, C. annuum. Preliminary screening for genetic variability using enzyme electrophoresis indicates that this material possesses a tremendous amount of genetic variation that may prove useful in the search for salt tolerance in pepper.

Although cellular selection did not prove feasible in this study, important data were obtained characterizing responses to salt of organized and unorganized tissues in culture. One in vitro screening procedure utilizing seedling pieces may have a useful role in germplasm screening. The potential to reliably regenerate pepper plants from tissue cultures was explored, confirmed and improved for purposes of mass propagation. However, plant regeneration from single cells was not confirmed.

44

BIBLIOGRAPHY

Berger, E. J. 1981. New Mexico Agricultural Statistics 1980. U.S.D.A.
Economics and Statistics Service, New Mexico Department of Agriculture.

Cotter, D. J. 1980. A review of studies on chile. Agricultural Experiment
Station. Bulletin 673.

Cotter, D. J. 1982. The scientific contribution of New Mexico to chile.
Agriculture in the Southwest -- A Historical Symposium. pp. 16.

Croughan, T. P., S. J. Stavarek, and D. W. Rains. 1978. Selection of a NaCl
tolerant line of cultured alfalfa cells. Crop Sci. 18: 959-963.

Dix, P. J. and H. E. Street. 1975. Sodium chloride-resistant cultured cell
lines from Nicotiana sylvestris and Capsicum annuum. Plant Sci. Lett.
5: 231-237.

Epstein, E. 1976. Genetic potential for solving problems of soil mineral
stress in problem soils. Proc. Workshop, M. J. Wright, Ed. Nat. Agr.
Library. Beltsville, Maryland 1976.

Epstein, E., J. D. Norlyn, D. W. Rush, R. W. Kingbury, D. B. Kelley, G. A.
Cunningham, and A. F. Worna. 1980. Saline culture of crops: a genetic
approach. Science 210: 399-404.

Falconer, D. S. 1960. Introduction to Quantiative Genetics. The Ronald
Press Co., New York.

Fishburn, M. T. 1981. New Mexico Chile Peppers. New Mexico Department of
Agriculture.

Gunay, A. L. and P. S. Rao. 1978. In vitro plant regeneration from hypocotyl
and cotyledon explants of red pepper (Capsicum). Plant Sci. Lett: 365-372.

Mass, E. V. and G. J. Hoffman. 1977. Crop salt tolerance--current
assessment. J. Irrig. and Drainage Div., ASCE 103:115.

McGuire, P. E. and J. Dvorak. 1981. High salt-tolerance potential in
wheatgrass. Crop Sci. 21: 702-705.

Nabors, M. W. 1976. Production of mutant plants conducive to salt tolerance.
Environmental Resources Center Completion Report No. 73. Colorado State
University, Ft. Collins, CO.

Nabors, M. W., W. E. Gibbs, C. S. Bernstein, and M. E. Meis. NaCl-tolerant
tobacco plants from cultured cells. Zeitscrift fur pflanzenphysiologie
97: 13-17.

Nabors, M. W., A. Daniels, L. Nadolny, and C. Brown. 1975. Sodium chloride tolerant lines of tobacco cells. Plant Sci. Lett. 4: 155-159.

O'Connor, G. A. 1980. Using saline water for crop production in New Mexico. New Mexico Water Resources Research Institute Completion Report No. 127.

Saxena, P. K., R. Gill, A. Rashid, and S. C. Maheshwari. 1981. Isolation and culture of protoplasts of Capsicum annuum L. and their regeneration into plants flowering in vitro. Protoplasma 108: 357-360.

Stavarek, S. J., T. P. Croughan, and D. W. Rains. 1980. Regeneration of plants from long-term cultures of alfalfa cells. Plant Sci. Lett. 19: 253-261.

Tal, M., A. Katz, H. Heikin, and K. Dehan. 1979. Salt tolerance in wild relatives of the cultivated tomato: proline accumulation in Lycopersicon esculentum Mill. L. peruvianum Mill. and Solanum pennellii Cor. treated with NaCl and polyethylene glycol. New Phytol. 82: 349-355.

Tyagi, A. K., A. Rashid, and S. C. Maheshwari. 1981. Sodium chloride resistant cell lines from haploid Datura innoxia Mill. A resistance trait carried from cell to plantlet and vice versa in vitro. Protoplasma 105: 327-332.

Wittwer, S. H. 1978. The next generation of agricultural research. Science 199: 375.