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JAMES LESLIE MUSTOE

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MORPHOLOGICAL STUDIES OF *TRICHOCEREUS SPACHIANUS* CULTURED ON SIX SUGARS *IN VITRO*

APPROVED BY

Norman Boke

Eddie Carol Smith

James R. Estes

Leonard P. Wilson

Paul G. Risser

DISSERTATION COMMITTEE

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MORPHOLOGICAL STUDIES OF *TRICHOCEREUS SPACHIANUS* CULTURED ON SIX SUGARS *IN VITRO*

CHAPTER I

INTORDUCTION

Tissue culture techniques have become a valuable tool in studies of plant growth and development. With our knowledge of plant nutrition it is possible to culture cells, tissues, and organs of many plants for indefinite periods of time. The cells and tissues of some species are easily grown on a relatively simple synthetic medium. Those from other species may require combinations of complex growth factors and the tissues of still other species are cultured with much difficulty or not at all.

To the physiologist and developmental morphologist tissue culture techniques have been extremely useful in studying certain aspects of plant nutrition, the biogenesis of numerous cellular compounds, and of various processes involved in tissue and organ differentiation. For the geneticist and cytologist *in vitro* cultures have been used to nurse excised hybrid embryos to maturity when they would otherwise have aborted because of incompability with maternal tissues. Cell and tissue cultures have proven of much utility in studies of chromosome morphology, polyploidy, somatic mutations, and tumor formation and

physiology.

Attempts to culture plant parts and cells date back at least to 1878 when Vochting sought an experimental proof of the theory of cellular totipotency. He dissected plants into smaller and smaller fragments while keeping these growing. He found a polarity to be characteristic of every fragment. Thus the distal portions of stem fragments produced leaves and proximal portions produced roots. The morphogenetic pattern was a function of the "organism as a whole" yet the cell appeared to be totipotent, its actual expression being the result of influences from the outside.(White, 1962).

The history of successful experiments with plant cells and tissues begins in 1934 with White's report of excised tomato roots and with three separate reports in 1939 from Gautheret, Nobecourt, and White of the establishment of cambial explants of carrott and tobacco. A more complete history will be found in White's _The Cultivation of Animal and Plant Cells_ (1962).

Reports of tissue cultures of cacti are limited. Nitsch (1951) grew _Opuntia monacantha_ Haw. on a medium fortified with thiamine, pyridocine, nicotinic acid, cysteine, saccharose, and tomato juice, the latter in concentrations of 0 to 50%. In nine weeks he obtained a 12½-fold increase in fresh weight and determined the optimum tomato juice concentration to be between 10% and 25%. King (1957) tried to grow plants from five genera: _Echinocerues_, _Cereus_, _Wilcoxia_, _Nopalea_, and _Pereskia_ on a medium of 20% coconut milk, 5 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 10% sucrose but had only limited success. Gautheret (1959) reports some work of Morel with _O. monacantha_ crown

gall but gives no details. Steinhart (1962) used Trichocereus spachianus (Lemaire) Riccobono for a study of alkaloid synthesis. She used various media which were fortified with glucose or sucrose; indole-3-acetic acid or 2,4-D; nicotinic acid, thiamine, pyridoxine, and an aqueous extract of stem material; kinetin, yeast extract or casein hydrolysate; or a mixture of several of these components. She obtained best results on a medium containing 10% coconut milk (by volume) and 2.5 mg/liter 2,4-D.

My work dealt with growth of T. spachianus on a chemically defined medium and a study of themorphological changes, both in weight and callus organization, produced by various sugars which were supplied in different concentrations as the carbohydrate source.

CHAPTER II

MATERIAL AND METHODS

Trichocereus is a genus of 19 species in the tribe Cereeae of the Cactaceae. *Trichocereus spachianus* is a native of western Argentina and is commonly grown in succlent gardens as an ornamental and for use as grafting stock. Mature plants consist of clusters of unbranched or occassionly branched stems 6-8 cm in diameter which may attain a height of 1.5 m or more. Its spines are 1 cm long, stiff, sharp, amber-yellow to brown and spreading from aeroles spaced about 1 cm apart (Britton and Rose, 1963). The experimental material I used was grown in the departmental greenhouse. Stem segments 4-6 in. long were collected as needed and surface sterilized with a 1:1 mixture of commerical Chlorox and Photoflo (1%) for 15 min, after which they were allowed to air dry in a sterile transfer chamber for 15-20 min. Tissue culinders were removed aseptically with a number 3 cork borer (inside diameter 0.235 in.) and cut into ½-in. segments which were stored in a sterile petri dish until being placed in the growth tubes.

The basic culture medium used was Brown's modification (1967) of the medium developed by Murashige and Skoog (1962) for the growth of tobacco. Brown's modification was for gymnosperm cambial culture and is designated medium "G." It is a chemically defined medium composed of: Skoog's major and minor elements, nicotinic acid (0.5 mg/liter), thiamin-HCl (0.1 mg/liter), pyridoxine-HCl (0.1 mg/liter), asparagine (100 mg/liter), 2,4-D (5.0 mg/l), kinetin (1.0 mg/liter),

myo-inositol (100 mg/liter), gibberellic acid (0.1 mg/liter), sucrose (20,000 mg/liter), and agar (8000 mg/liter) with the pH adjusted to 5.7-5.8 with NaOH or HCl. Twenty ml of medium was dispensed into 25-mm test tubes which were plugged with cheesecloth-wrapped cotton and autoclaved at 15 psi for 15 min after which they were stored in a refrigerator until used.

After inoculation the tubes were kept in a growth room for 28 days at 25 C under 400 ft-c of light on a 12 hr photoperiod. Examination of growth curves derived from fresh and dry weights of _T. spachianus_ grown on medium "G" using 3% sucrose as the carbohydrate source reveals that maximum weight, both fresh and dry, is achieved after about 33 days of growth. A growth period of 28 days was selected because it lies in the logarithmetic phase of rapid growth, near the maximum weight. At the end of the growth period the material was collected and fresh and dry weights were determined. The calluses were dried at 110 C until constant weights were obtained (usually 2-3 days) and results were analyzed with Student's t-test (Mendenhall, 1967).

Fresh material was sectioned and examined with bright field and polarizing optics for changes in starch grains, cell walls, intercellular spaces, relative cell size, crystals, vascular tissue, and lignification that might have resulted from culturing. Fresh material was stained with a dilute aqueous solution of IKI and with phloroglunicol (Jensen, 1962). Material was killed and fixed in CRAF V for 24 hr, dehydrated in tetrahydrofuran (Leuty, 1964), embedded in paraffin (Paraplast 56-57 C), and sectioned at 10-12 nm.

Staining was with safranin and fast green (Sass, 1958).

Sugars tested were sucrose, glucose, fructose, a 1:1 mixture of glucose and fructose, maltose, and galactose in concentrations of 0, 10, 20, 30, 40, and 50 grams per liter (0-50%).

CHAPTER III

OBSERVATIONS

Trichocereus spachianus exhibited both morphological and weight responses to the six sugars used as carbohydrate sources. Both fresh- and dry-weight increases were closely related to the amount of callus produced as a result of the relative amounts of energy available from the sugar supplied as the carbohydrate source.

Growth Curve

The logarithmetic phase of the growth curve ended about day 33 for both fresh and dry weights. Fresh weights began to decline on day 44 about as rapidly as they had gained earlier. Dry weights remained constant after day 33.

Dry Weights

The mean dry weights for each of the experimental treatments are presented in table 1. With the exception of galactose, the highest dry weights occured at either 2% or 3% levels with weight decreasing at 1% and at 4% and 5% levels. Table 2 is a comparison of the highest means derived from table 1. Two percent glucose is significantly greater than 3% sucrose at the 0.1 level and greater than 2% fructose, 5% galactose, 2% maltose at the 0.05 level but is not significantly different from 3% mixture at either level. A comparison of glucose and mixture at 2, 3, and 4% levels shows no significant differences in any of the concentrations (table 3).

A comparison of the highest means with values from material grown on the same medium without any carbohydrate source (table 2) indicates that the amount of growth on all sugars was significant at the 0.05 level. Similarly, a comparison with the initial (starting) dry weight reveals that all treatments produced a significant increase in weight. However, the medium without any sugar inhibited the growth of the explant (inhibition being defined as any condition of culture which results in a loss in weight from that of the initial explant). This inhibition, or loss in weight, is to be expected as the explant is living off stored food materials.

Fresh Weights

Dry weights range between 4 and 5% of fresh weights with no evident trends being developed either within a sugar or from sugar to sugar at a given concentration (table 1). The lowest percent value (1.5%) which indicates the greatest difference between fresh and dry weights was for 2% glucose. However, the 3% mixture, which produced a dry weight very near that of 2% glucose, had a value of 4.5%, which is three times as great as that for 2% glucose.

Cell Measurements

The measurements taken from fresh cells are given in table 4. Cell sizes were remarkably uniform except in material grown on the mixture of glucose and fructose in which the explant cells, the elongated cells, and the small cells of the callus were larger than similar cells from other treatments. Cells of the "frosting" were smaller in material grown on the mixture. Material grown on galactose

showed more enlargement than did the explant cells on other sugars. Cell sizes indicate that weight increases, i.e. growth, resulted from an inrease in the number of cells present and, except for material grown on fructose, not in cell size.

Morphological features of the callus

The explant developed a reddish-brown pigment in all treatments. The pigment appeared to be localized in cell walls, freely diffusing into the aqueous mounting medium. Steinhart (1962) observed development of a red pigment which became black with aging of the cultures. She attributed it to the action of polyphenoloxidases since addition of tyrosine, casein hydrolysate, and amino acid mixtures; indole acetic acid and naphthaleneacetic acid; glutamine or high levels of sugars, especially sucrose, tended to increase the rate of pigmentation and decrease the extent of callus initiation. No separate experiments were undertaken to examine the pigmentation; however, increased concentrations of any one sugar did not appreciably change the amount of coloration.

Investigation of fresh sections under the polarized light revealed the presence of doubly refractive ergastic bodies of three types: numerous small prismatic crystals were present in the cytoplasm of explants and callus cells; large druses and smaller raphides were present only in material grown on galactose; numerous small bodies which were assumed to be starch grains, exhibited a Maltese cross and stained purple with a dilute aqueous solution of IKI. Starch grains were limited to 3, 4, and 5% glucose, 4% sucrose, and all treatments

with the mixture and fructose.

Trichocereus spachiamus grown on media supplemented with glucose, sucrose, maltose, or a mixture of glucose and fructose was remarkably uniform in appearance. Externally, the callus was greenish-gray, tending to become red near the end of the growth period. The callus appeared frosted with white, which microscopic investigation revealed to be caused by clumps of large, spherical cells about 250 nm in diameter (table 4). These cells were loosely arranged on the surface of the callus (Fig. 2). Cell walls of both explant and callus were uniformally thin and non-lignified (as evidenced by negative reactions to phloroglucinol and a green color when stained with safranin and fast green). Intercellular spaces were generally triangular and uniform in size and occasionly filled with the red pigment found in the cell walls. Cells of the explant showed little change except for a decrease in diameter in material grown on all media except galactose and the mixture (Fig. 4, 6).

No evidence of meristems was found; however, certain areas seemed to produce abundant callus while others did not. In some areas callus developed as in "fans" supported by long stalks of callus tissue embedded within the explant (Fig. 7). These bore no relation to vascular bundles as none were present in the cortex used. Elongated cells occured between the "fans" and these were, in turn, covered with smaller callus cells, creating a zonation around the explant consisting of: (1) elongated cells, approximately 684 X 321 nm; (2) smaller callus cells, approximately 69 nm in diam., and finally, (3) the "frosting" (Fig. 3, 5).

Tissues grown on media supplemented with fructose and galactose were very similar. Callus growths on 1% fructose resembled those grown on glucose; however, callus production on fructose media decreased as the concentration increased and galactose supported very little callus at any concentration. With the exception of 1% fructose, which produced a red explant and gray callus, both explant and callus (if any) were red. Zonation and callus on 1% fructose were similar to those on glucose; however, the organization and "frosting" were lost with higher levels of fructose and were not present at all in material grown on galactose. Small crystals of the same type noted for material grown on glucose were present on fructose and galactose.

Numerous small, oval to spherical bodies were observed in fructose-grown material, especially in the peripheral cells. These stained purple with a dilute aqueous solution of IKI but were not birefringent and did not look like starch grains.

Tissues grown on galactose media showed no signs of growth; however, the explants enlarged about ¼ in. in length and slightly in diameter. No starch grains were present and, in addition to the small crystals, many large druses averaging 234 nm in diam. and smaller raphides averaging 175 X 50 nm were present in galactose-grown material. No other material had large crystals of these types. The explants were heavily pigmented with red, which was localized in cell walls.

Tissues grown on medium without any sugar were red, slightly enlarged over the initial explant, and with little or no callus growth. No starch and very few crystals were present. Initial explant material was parenchyma with average diameters of 225 nm (Fig. 4). The walls were thin and intercellular spaces triangular.

TABLE 1

FRESH AND DRY WEIGHT MEANS OF DIFFERENT TREATMENTS OF SUGARS, in grams

TREATMENT		DRY WEIGHT	FRESH WEIGHT	DRY WEIGHT % OF FRESH WEIGHT
SUCROSE	1%	0.0428	1.21	3.5
	2%	0.0579	1.47	3.9
	3%	0.0726	1.62	4.5
	4%	0.0655	1.67	3.9
	5%	0.0522	1.13	4.6
GLUCOSE	1%	0.0532	1.33	4.0
	2%	0.0873	5.79	1.5
	3%	0.0800	2.28	3.5
	4%	0.0713	1.34	5.3
	5%	0.0424	0.56	7.5
MIXTURE	1%	0.0570	2.68	2.1
	2%	0.0879	3.27	2.7
	3%	0.0900	2.11	4.3
	4%	0.0718	1.14	6.3
MALTOSE	1%	0.0380	1.40	2.7
	2%	0.0603	1.97	3.1
	3%	0.0642	1.58	4.1
	4%	0.0575	1.12	5.1
	5%	0.0549	0.92	6.0
FRUCTOSE	1%	0.0438	1.42	3.1
	2%	0.0462	0.89	5.2
	3%	0.0294	0.95	3.1
	4%	0.0268	0.50	5.4
	5%	0.0291	0.42	6.9
GALACTOSE	1%	0.0136	0.53	2.6
	2%	0.0211	0.51	4.1
	3%	0.0225	0.52	4.3
	4%	0.0239	0.45	5.1
	5%	0.0257	0.43	6.0
INITIAL		0.0101	0.32	32.0
SUGAR	0%	0.0084	0.40	21.0

Highest means are underlined.

Single vertical lines indicate 0.1 significance.

Double vertical lines indicate no significance.

All other combinations are significantly different at 0.05.

FIGURE 1

GROWTH CURVE OF TRICHOCEREUS SPACHANIUS ON MEDIUM "G"

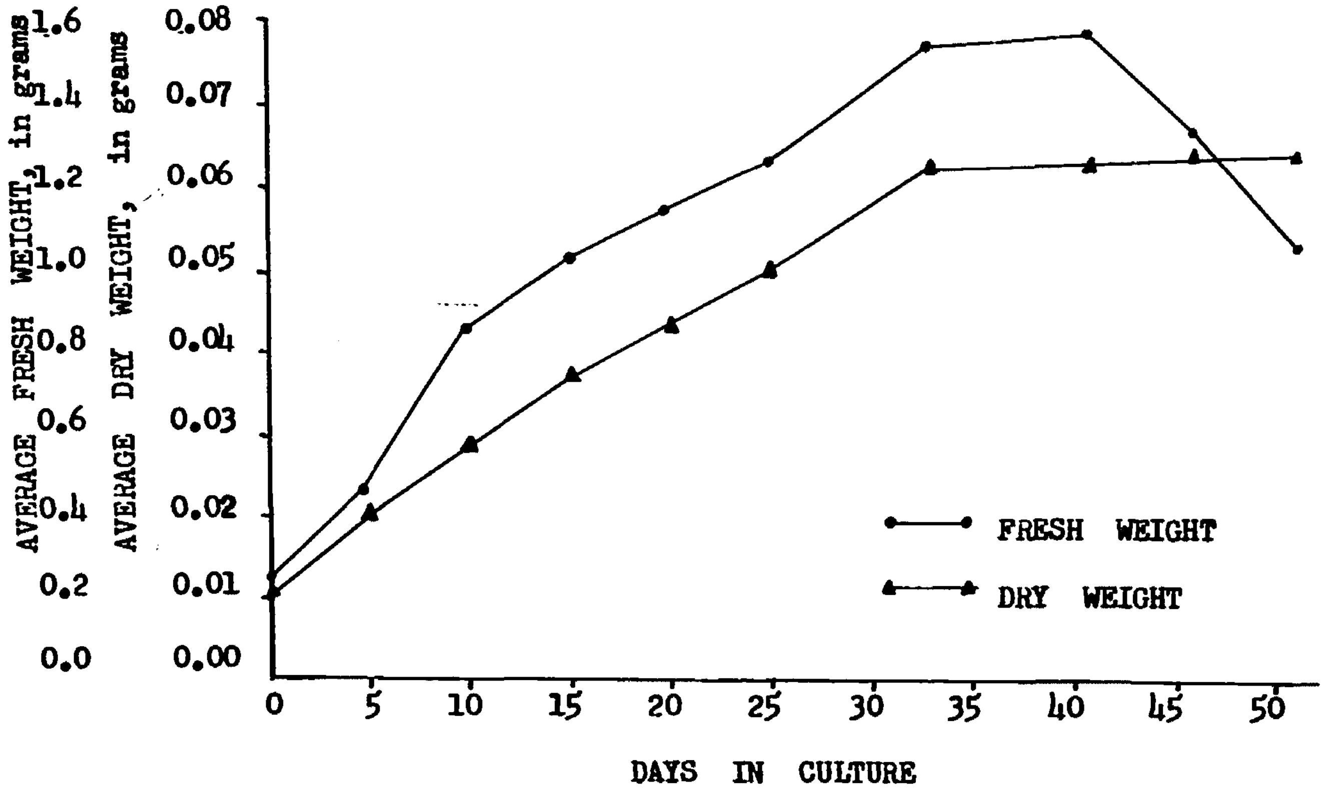

TABLE 2

COMPARISON OF HIGHEST MEANS

3%	Sucrose	0.0726
2%	Glucose	0.0873
2%	Fructose	0.0462
3%	Mixture	0.0900
5%	Galactose	0.0257
2%	Maltose	0.0603
0%	Sugar	0.0084
	Initial	0.0101

TABLE 3

COMPARISON OF GLUCOSE WITH MIXTURE

2% each:	t= 0.0498
3% each:	t= 0.9198
4% each:	t= 0.7256
0.05 level	t=1.645
0.1 level	t= 1.282

TABLE 4

MEASUREMENTS OF CELLS GROWN ON DIFFERENT TREATMENTS OF SUGARS, in nm

TREATMENT		EXPLANT	ELONGATE LONG	CELLS WIDE	SMALL CELLS	FROSTING
SUCROSE	1%	156	680	370	62	294
	2%	130	670	400	68	310
	3%	190	645	453	63	317
	4%	132	672	377	68	320
	5%	130	660	317	64	319
average		147	665	312	65	383
GLUCOSE	1%	136	690	325	75	250
	2%	143	700	370	68	294
	3%	143	680	370	60	200
	4%	143	670	371	69	264
	5%	143	680	370	68	279
average		141	684	361	68	257
MIXTURE	1%	245	820	341	83	153
	2%	236	768	229	94	134
	3%	233	788	330	67	160
	4%	234	805	308	70	165
	5%	156	785	219	--	---
average		221	793	285	79	153
MALTOSE	1%	135	656	335	63	266
	2%	151	646	254	67	272
	3%	183	622	271	76	272
	4%	147	615	284	62	264
	5%	136	587	307	63	260
average		150	624	292	66	267
FRUCTOSE	1%	143	656	362	68	294
	2%	141	650	347	65	264
	3%	147	---	---	--	---
	4%	144	---	---	--	---
	5%	142	---	---	--	---
average		143	653	354	66	279
GALACTOSE	1%	288	---	---	--	---
	2%	225	---	---	--	---
	3%	228	---	---	--	---
	4%	255	---	---	--	---
	5%	262	---	---	--	---
average		252	---	---	--	---
INITIAL		255				
SUGAR	0%	139				

FIGURES 2--8

2. Cross section of fresh material grown on 1% mixture of glucose and fructose showing cells of the "frosting". Note the loose arrangement of the cells. X 115

3. Cross section of fresh material grown on 1% mixture showing elongated cells surrounding the explant. X 66

4. Cross section of fresh material (initial) showing typical cells at the beginning of the culture period. X 115

5. Cross section of fresh material grown on 1% mixture showing the small cells of the culture which occured between the elongated cells and the frosting. X 115

6. Cross section of fresh material grown on 1% mixture showing cells of the explant. X 66

7. Cross section of paraffin-embedded material grown on 2% sucrose showing a large "fan" of callus material. X 115

8. Cross section of fresh material grown on 1% galactose showing cells of the explant. X 115

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CHAPTER IV

DISCUSSION

Steinhart (1962) tested sucrose and glucose at concentrations of 1-8% and concluded that 2% glucose was the best carbohydrate source for T. spachianus. My data support this conclusion. Relative fresh and dry weights indicate that, while dry weights of 2% glucose and 3% mixture are quite similar, some mechanism allowing for greater fresh-weight production in glucose-grown material is functioning. This might be a perimase allowing greater water uptake.

Simpkins, Collin, and Street (1970) indicate that glucose is preferentially absorbed from a mixture of one-half each of glucose and fructose by Acer pseudoplantamus (syacmore-maple). My data suggest that glucose is preferentially absorbed from such a mixture; however, growth on 3% mixture, which contains 1½ % glucose, exceeds the growth on 2% glucose and is probably greater than would have been obtained on 1½% glucose alone. This indicates some effect of the fructose in the medium, perhaps due to a limited activity of an enzyme system.

Upper, Helgeson, and Haberlack (1970), using glucose and sucrose on tobacco, feel that growth is regulated by the amount of sugar present and not by the type of sugar. When sugars other than sucrose and glucose are used it becomes readily apparent that the type of sugar does make a difference in growth.

Arya, Hildebrandt, and Riker (1962) tried six sugars (sucrose, glucose, fructose, galactose, mannose, and lactose) and found all except mannose to be useable by grape stem and gall tissue induced on

grape by Phylloxena vastatrix. Simpkins et al (1970) working with Acer pseudoplantanus in liquid medium found that 2% sucrose could be replaced with 2% fructose, glucose, galactose, maltose, or soluble starch, or a mixture of 1% fructose and 1% glucose. Apparently these cells have hydrolytic enzymes active at the surfaces of the cells or excreted into the medium, which then hydrolyze the di- and polysaccharides into monosaccharides. Ball (1959) reported that galactose would not substitute for glucose or sucrose for Ginkgo embryos but that it was not toxic as reported by White for tomatoes (1940). Toxicity of galactose has also been reported by Simola (1969) for Sphagnum. High yields of T. spachianus may be obtained with either sucrose, glucose, maltose, or a mixture of one-half each of glucose and fructose; however, neither galactose nor fructose will substitute.

Glucose enters the glycolytic pathway as glucose-6-phosphate; fructose may enter as fructose-6-phosphate; both conversion may or may not be due to specific enzymes. Sucrose may be hydrolyzed to glucose and fructose, and maltose may be hydrolyzed to glucose. A comparison of treatments of sucrose, glucose, fructose, and the mixture (i.e., 2% sucrose and mixture, 1% fructose and glucose) indicates that a 1:1 mixture of glucose and fructose will produce more growth than the same molar concentration of sucrose and almost twice the growth of either glucose or fructose. Apparently T.spachianus lacks the ability to utilize certain sugars as carbohydrate sources and this inability may be due to lack of absorbtion of the sugars rather than lack of an efficent ensyme system.

Morphological changes noted are consistant with the apparent ability of the cells to utilize the carbohydrate present. The callus growth observed in material on sucrose, glucose, a mixture of glucose and fructose, and maltose is a result of growth due to the energy available from the sugar in the medium whereas the lack of growth in material on fructose and galactose indicates the lack of available energy from the medium.

While no organized meristems were observed, the surface "frosting" indicates where rapid cell division occured. The reason for the "fans" is not clear, but they might indicate areas within the explant that were especially active. The presence of starch usually indicates a high level of glucose-1-phosphate in the cells but why starch was present in all levels of fructose-grown material is unclear.

The effect of the various treatments was on the amount of growth resulting and did not effect the cell types produced or the products deposited except the pigment, starch grains, and crystals of galactose.

CHAPTER V

SUMMARY

Cortical explants of Trichocereus spachianus were grown in sterile culture on a chemically defined medium, utilizing six different sugars as carbohydrate sources. The sugars were supplied in concentrations ranging from 0 to 50 grams per liter (0-5%). Fresh and dry weights and callus development indicate that sucrose, glucose, maltose, and a 1:1 mixture of glucose and fructose will support rapid growth of T. spachianus and that neither fructose nor galactose will support rapid growth. Of the sugars tested, 2% glucose and 3% of the mixture produced the best growth.

Examination of the growth curve on medium "G," using 3% sucrose as the carbohydrate source, indicates that the logarithmetic phase of the curve ends at about the 33rd day of culture, and fresh weight decrease begins on the 40th day. A growth period of 28 days was selected because this lies near the end of the log phase of growth.

Microscopic investigations revealed that growth in culture had little effect on the type of cells produced. A red pigment was deposited in cell walls and intercellular spaces of the explant and some of the calluses. Starch grains occured in material grown on 3, 4, and 5% glucose, 4% sucrose, all treatments of the mixture, and fructose. Crystals were present in most material grown in culture as well as in initial explants.

The amount of growth suggested that T. spachianus is dependent

upon a hexose, primarly glucose, as the carbohydrate source. Callus was composed entirely of parenchyma cells produced by proliferation from surface or near-surface cells of the explant. A zonation was observed in which elongated cells were produced near the explant, then smaller cells, and finally, a "frosting" of large, spherical cells in areas of rapid cell growth.

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