STUDIES ON THE EFFECT OF COLD ON CACAO SEEDS

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To my beloved wife and children

BIOGRAPHY

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INTRODUCTION

The cacao seed is a non-dormant seed that loses its viability shortly after being harvested unless placed under conditions which permit its germination. This loss of viability after relatively short periods of time has made it impossible for geneticists and plant breeders to store seeds for use in long term experiments. Furthermore, long distance shipments of seeds for planting purposes must be done by air freight which is rather uneconomical. For reasons such as these, several methods have been devised in an attempt to store cacao seeds without loss of viability. These methods, however, either maintain viability for relatively short periods, or, on the other hand, permit germination of the seeds. The need exists, therefore, for the development of a system for long term storage of cacao seeds.

One of the methods which was found to be unsuccessful in maintaining viability in cacao seeds for any length of time was cold storage. The importance of conducting an investigation to determine the reasons for the failure of this method is obvious. Conclusive evidence as to why loss of viability occurs following subjection of the cacao seed to low temperature conditions would have far reaching effects. With such data it might be possible to devise methods to prevent this loss of viability thus permitting long term storage by chilling. In addition, the possibility exists that these methods might also be applicable to other cold sensitive seeds. Further investigation could then be directed at the determination, and possible elimination, of those factors that confine certain crops to tropical regions and others to temperate regions.

In the present work, the role of cold has been investigated as regards its effect on respiration, as well as its effect on the tissues of the cacao seed. In addition, cold resistance of various clones of cacao has been tested.

It must be noted here that the term "embryo" will be used in the manner defined by Ibáñez (16) as "the small white body which represents the axis, excluding the cotyledons".

LITERATURE REVIEW

1. The storage of cacao seeds

Pyke (25) has stated that the cacao "bean" is a non-dormant seed which is ready for germination as soon as the fruit is ripe. Evans (6, 7), however, has stated that the cacao seed is viable for a considerable period of time before the pod is fully ripe.

Germination capacity appears to be short lived, for according to Hansen and Hunter (12), viability is lost within 10-15 days after the seeds are harvested, unless they are placed under conditions which permit their germination.

One of the earliest methods of storage was to maintain the seeds within the pod. Pyke (25) demonstrated that seeds contained in immature pods are usually still viable after storage at 21ºC - 27ºC for 8-10 weeks.

Thompson (30) has reported that some 5,000 cacao seeds sent from Ghana, West Africa, to Malaya, Borneo and Sarawak, germinated satisfactorily. Charcoal was used as the packing material in all but two cans which were packed with damp vermiculite.

Alvim (1) has recommended that seeds for transportation be dipped in a suspension of hydrated lime which helps to dry up the mucilaginous layer. The testas are then removed. Seeds thus prepared are treated with a suitable fungicide then packed in polyethelene bags. A modification of this method using sawdust instead of lime to facilitate removal of the testas has been described by Hardy (13). Another method has been described by Erickson (5). In this process the seeds are washed, treated with

Fermate, and packed in perforated boxes, layers of seeds alternating with layers of charcoal. However, this method permits germination of the seeds and there is much seed loss through root damage.

Evans (6, 7) suggests mixing the seeds with ground charcoal containing 30% water and placing the mixture in perforated containers. This system, however, like that of Erickson (5) leads to seed germination and root damage.

2. The effect of cold on cacao seeds

It has been found by Boroughs and Hunter (2) and Boroughs and Labarca (3) that subjection to cold conditions causes a loss of viability in seeds of cacao.

The work of Ibáñez (14, 15) has demonstrated that cacao seeds lose their viability subsequent on immersion for 10 minutes in water at 4ºC. This loss of viability is, however, reversible if such chilled seeds are immersed afterwards in water at 37ºC. for 10 minutes. Under such conditions at least 85% of the seeds were restored to viability producing healthy plants. After 15 minutes of such a cold treatment, however, no length of time of post-treatment could prevent ultimate death of the seed.

Subsequent work by Ibáñez (16) demonstrated that the site of the cold effect was in the cotyledons and not in the embryonic tissue. Respiration rates in embryos of cacao remained unchanged regardless of cold treatment, while cotyledonous tissue showed a large increase in endogenous respiration after cold treatment. In addition, embryonic tissue growing in sterile medium independent of cotyledon material, developed as well after chilling as the normally treated material. No attempt was

made, however, to grow these embryos into full sized plants. In all cases, growth over 2 cm, leaf and chlorophyll production, being taken as criteria for viability. At the present time it is uncertain as to whether this growth is due to mitotic division processes or solely to cell enlargment in embryonic leaves and roots.

That cold affects mitotic processes has been demonstrated by Moh (21) and Moh and Alán (22) using Phaseolus Vulgaris L. A temperature of 4ºC completely arrested the chromosome movement at metaphase. Recovery from this low temperature blocking effect on the mitotic processes was effected when the treated bean radicles were returned to room temperature (23ºC). This blocking mechanism of low temperature on bean mitosis is not fully understood, but Moh and Alán (22) have suggested that low temperature may inhibit the spindle formation at metaphase leading to an inhibition of chromosome movement.

Ibáñez (14) has demonstrated that pigment leakage occurs from cold killed tissue but not from seeds that have been restored to viability by post-treatment for 10 minutes in water at 37ºC. Ibáñez and Casas (18) have stated that the brown colour of the pigment released is probably due to polyphenol leakage from the cotyledon cells and subsequent oxidation by polyphenol oxidase. The presence of the polyphenols and their oxidases in cacao have been reported by Forsyth (8, 9, 10) and Griffiths (11). Roelofsen (26) has stated that the polyphenols are apparently localized in the tannin cells of the seed.

It is interesting to note the absence of inhibitory effect on embryo survival and growth by the pigments released by cold killed seeds as demonstrated by Ibáñez (16).

Ibáñez (15) has said that these data suggest the occurrence of a biophysical change in the cotyledon cells. This theory seems feasable, and gains support from Kramer (20), who has stated that permeability changes in cytoplasmic membranes may be caused by environmental factors such as low temperature conditions. In addition, it has been demonstrated by studies carried out by Casas and Ibáñez (4), that little or no damage occurs to the vascular tissue between the cotyledons and embryo following subjection of the cacao seed to cold.

MATERIALS AND METHODS

A. Respiratory changes in cacao seed cotyledon coincident with seed death

Seeds of Theobroma cacao, clone UF-613, were taken from mature pods. Mucilaginous substance and testas were removed and the seeds treated in the following way:

Normal control seeds were immersed in water at room temperature (25ºC) for 5, 10, 15, and 20 minutes. Seeds subjected to cold treatment were immersed in water at 4ºC for periods equal to those listed above for the normal control. In addition, a group of cold treated seeds received post-treatment for 10 minutes in water at 37ºC.

The exposure times were chosen due to previous work by Ibáñez (15), which showed that a treatment of 5 minutes at 4ºC. had considerable killing effect on seed viability, while a 10 minute treatment was almost completely effective in killing the seed. These processes, however, were reversible by post-treatment at 37ºC. A treatment of 15 minutes at 4ºC. killed the seed and post-treatment at 37ºC. did not reverse this effect. The chilling process was carried further in the 20 minute treatment.

After cold treatment the embryonic tissue was removed from the seeds. The cotyledon was then cut with a small diameter cork borer to yield pieces of approximately 200 mg in weight. These samples were then placed in Warburg flasks (Bronwill model UV-85) containing the following ingredients: 0.1 ml potassium phosphate buffer pH 7.0, 0.1M; 200 mg cotyledonary material; 0.2 ml 10% KOH (in the centre well to serve as a trap for carbon dioxide); and water to a final volume of 3 ml. The final pH of the mixture was 7.0.

The assembled flasks and manometers were equilibrated to temperature (30ºC.) for 10 minutes. Manometer readings were made at zero time and at appropriate time intervals thereafter. All calculations were made in μ litres of oxygen absorbed per gram of tissue, per milliliter of solution in the Warburg flask. These techniques are detailed by Umbreit, Burris, and Stauffer (31).

Statistical analysis was carried out on the treatment averages by the method of analysis of variance, differences being measured by the test of Least Significant Difference. This method is detailed by Snedecor (29).

B. Effect of cold on mitosis in root tips of cacao embryos grown in sterile culture

A number of seeds from newly harvested mature pods of Theobroma cacao, clone UF-613 were procured. Mucilaginous substances and testas were removed. These seeds were then divided into two groups. One group was immersed in water at room temperature (25ºC.) for 20 minutes, the other was immersed in water at 4ºC. for an equal length of time.

One lot of seeds from each group was germinated under normal conditions in petri dishes containing moistened filter paper. These were the viability controls.

Embryos were then removed from the remaining seeds for growth in sterile culture.

The method used for removal and sterile cultivation of the embryos was that developed by Ibáñez (17). This method involved the use of a modification of the culture medium of Rudolph and Cox (27), sucrose being replaced by destrose as the main carbon source. A further

modification was introduced in that carbon black was added to one-half of the culture medium. This served to prevent access of light to the embryo radicles.

The embryos were transformed to screw cap vials containing the culture medium and these and the controls were allowed to grow at room temperature (25ºC.) for 6 days. The period of 6 days was chosen for two reasons. Firstly, it has been stated by Monge (23), that a minimum percentage of mitosis takes place after 3 days of germination of cacao seeds, attaining a maximum at 4 days. Secondly, it has been found by Ibáñez (14) that after 3 days chilled cacao seeds may be judged living or dead.

Following growth for 6 days, root tips were removed and fixed with Randolph's Modified Navashin Fluid ("Craf" fixative). This fixative was found to be effective by Muñoz (24) in his work on chromosomes of cacao root tips. Dehydration was effected with tertiary butyl alcohol followed by embedding in paraffin. Longitudinal sections were cut in 10 μ thickness and stained with gentian violet. These techniques are detailed by Johansen (19) and Sass (28).

Following mounting in permount and drying of the slides the sections were examined microscopically.

C. Cytological changes induced by cold in cacao seed cotyledon

A number of seeds from newly harvested mature pods of Theobroma cacao, clone UF-613 were procured. Mucilaginous substances and testas were removed. These seeds were then divided into two groups. One group was immersed in water at room temperature (25ºC.) for 20 minutes, the other was immersed in water at 4ºC. for an equal length of time. Each seed was cut transversely, and from one of the cut faces a 3 mm thick

transverse section of cotyledon was removed. Each of these sections was divided into two equal portions and fixed in FAA solution. Dehydration was effected with tertiary butyl alcohol followed by embedding in paraffin. Section of 10 μ thickness were cut and stained with fast green. These techniques are detailed by Johansen (19) and Sass (28).

Following mounting in permount and drying of the slides the sections were examined microscopically.

D. Resistance of various clones of cacao to cold treatment

A number of seeds from newly harvested mature pods of clones UF-168, UF-221, UF-296, UF-613, UF-650, UF-667, UF-668, UF-676, ICS-29, ICS-39, R-2, R-9, R-10, IMC-67, SPA-9, IAL-407 were procured. Mucilaginous substance and testas were removed. These seeds were then divided into seven equal lots. One lot from each clone was assigned to one of the following treatments in water at 4ºC.: 0,5, 10, 15, 20, 25, and 30 minutes. At the end of the specified time interval, the lots of seeds were removed from the water and germinated at room temperature (25ºC.) in petri dishes containing moistened filter paper. Following a 6 day germination period the seeds were judged living or dead.

RESULTS

A. Respiratory changes in cacao seed cotyledon coincident with seed death

Figure 1 demonstrates graphically the respiration tendencies of the cotyledon material after the various cold treatments and under normal conditions.

Figure 2 demonstrates graphically the respiration tendencies of the cotyledon material after the various cold treatments and post-treatments and under normal conditions.

These results represent the corrected averages (Appendix C) of 5 individual experiments.

It should be noted in Figure 1 that while there is some difference in respiration tendencies between normal tissue and tissue chilled for 5 minutes, and there is an appreciable increase in respiration tendencies of tissues chilled for 10, 15 and 20 minutes over the respiration of the normal system, a boiled control showed no activity. An analysis of variance and test of significance (Appendices A, B, D) has shown a highly significant difference between the respiration tendencies of normal tissue and tissue chilled for 5 minutes, and a significant difference between the respiration tendencies of tissue chilled for 5 minutes and tissue chilled for 10 minutes.

In Figure 2 respiration tendencies of normal and 5 minutes at 4ºC. + 10 minutes at 37ºC. are practically indistinguishable from one another, while respiration tendencies of 10, 15 and 20 minutes at 4ºC. + 10 minutes at 37ºC. show an appreciable increase over the normal

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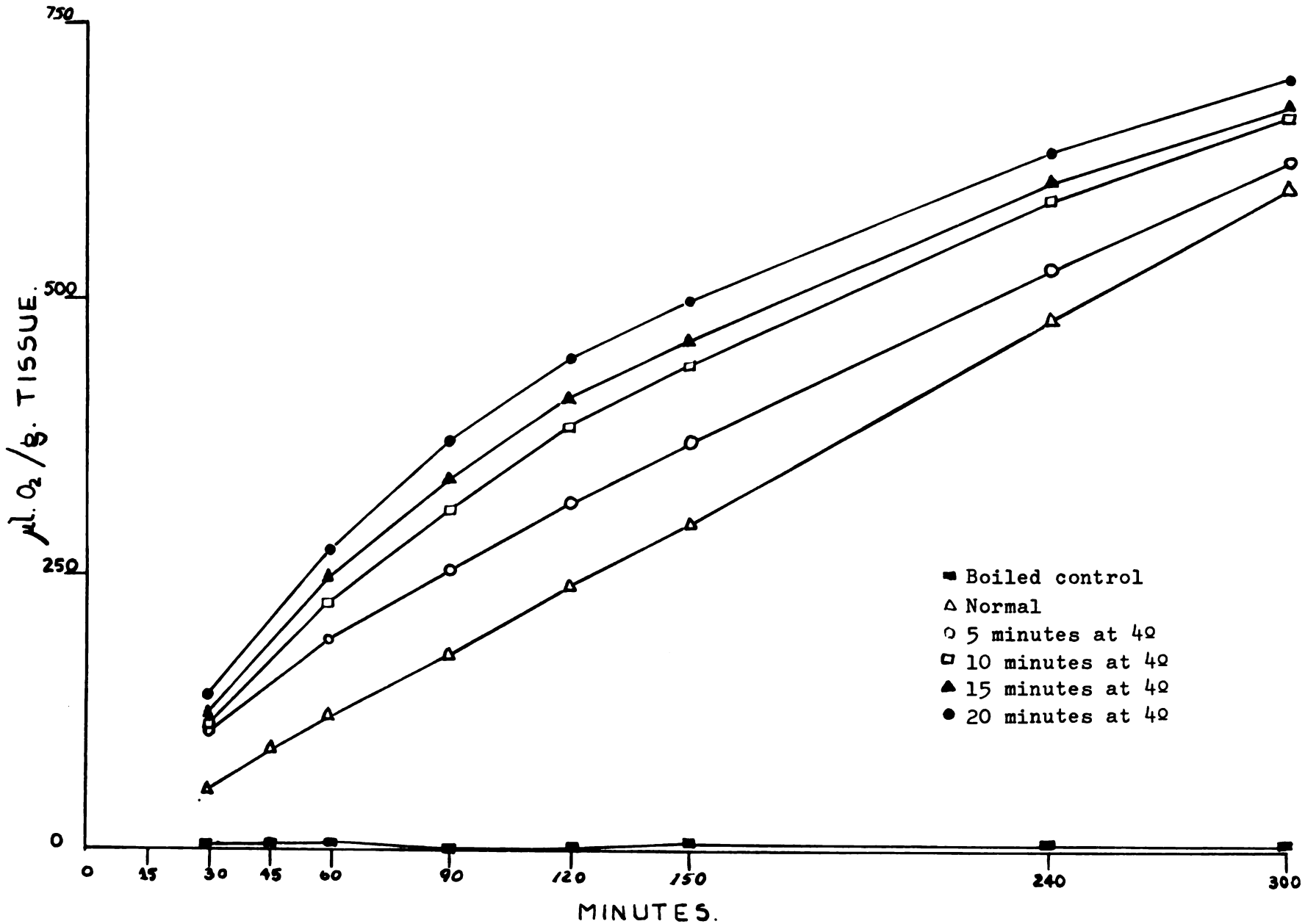

Figure 1. Graph demonstrating the respiration tendencies of cotyledon material after various cold treatments and under normal conditions.

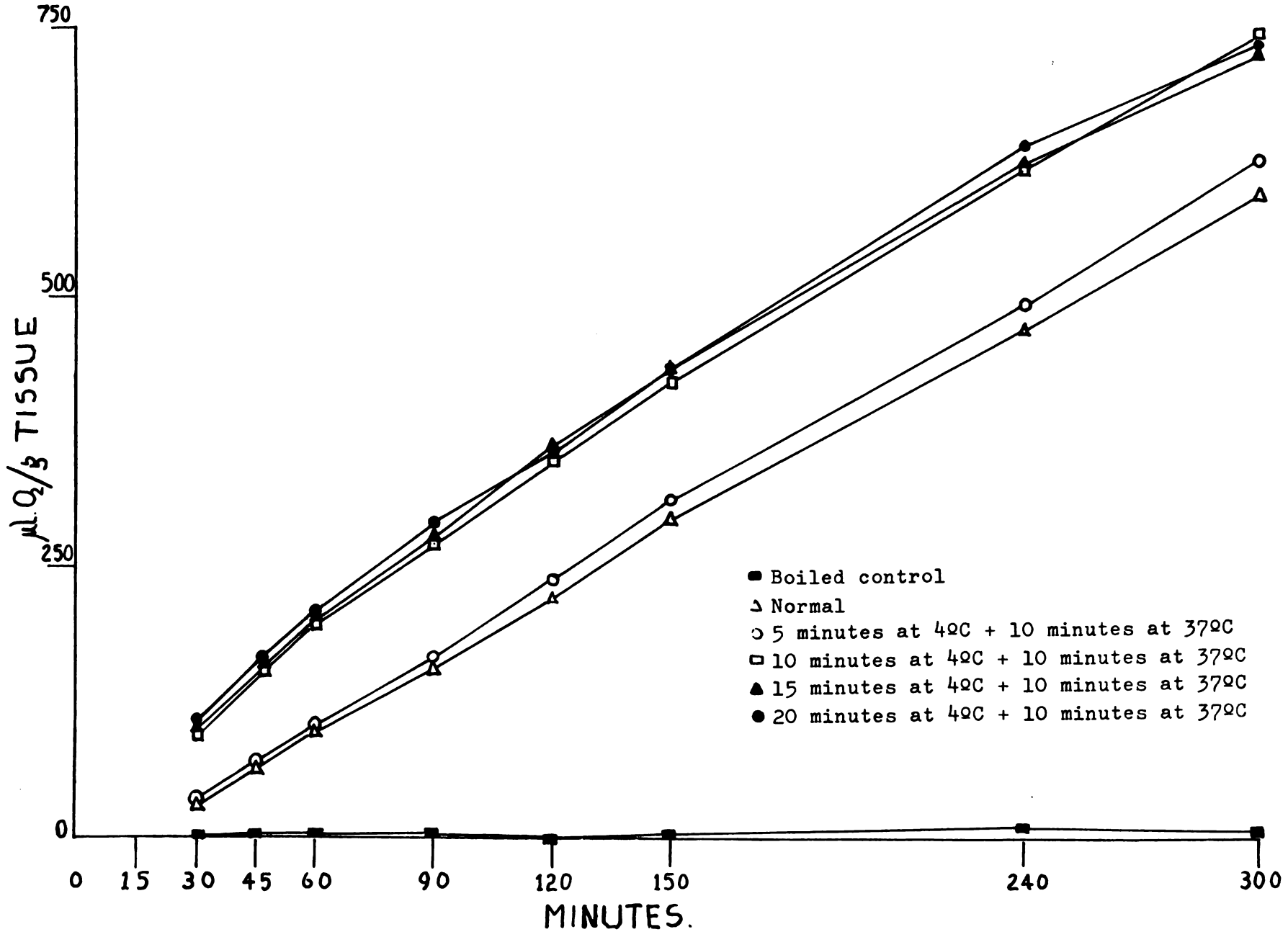

Figure 2. Graph demonstrating the respiration tendencies of cotyledon material after various cold treatments, post-treatments and under normal conditions.

system. A boiled control showed no activity. An analysis of variance and test of significance (Appendices A. B. D) has shown a highly significant difference between the respiration tendencies of tissue chilled for 5 minutes at 4ºC. + 10 minutes at 37ºC. and tissue chilled for 10 minutes at 4ºC. + 10 minutes at 37ºC.

B. Effect of cold on mitosis in root tips of cacao embryos grown in sterile culture

No sections were made of the root tips from the seeds used as controls of cold inhibition. These seeds were obviously dead following the 6 day germination period, being covered with extensive fungal growth.

Microscopic examination of root tip sections from all other treatments revealed that active mitotic division had been taking place in all roots. Cells in all stages of mitosis were observed.

Figure 3 is a photograph of a section of a cacao root tip showing cells in various stages of mitosis.

C. Cytological changes induced by cold in cacao seed cotyledon

Microscopic examination of the cotyledon sections revealed the presence in the normally treated material of numerous tannin cells. In the case of the cold treated material the polyphenols of the tannin cells had leaked away leaving tannin cell ghosts. Those few tannin cells that retained their contents were confined to the innermost areas of the cotyledon.

Figures 4 and 5 are photographs of sections of normal and cold treated cotyledon respectively. Tannin cells are clearly observable as dark areas in Figure 4.

D. Resistance of various clones of cacao to cold treatment

Germination tests revealed that the seeds from the various clones of cacao selected have almost no resistance to cold treatment. Seeds from clones UF-168, UF-296, UF-650, UF-668, R-2, IMC-67, SPA-9, and IAL-407, died following immersion in water at 4ºC. for 5 minutes or more. Seeds from clones UF-221, UF-613, UF-667, UF-676, ICS-1, ICS-29, ICS-39, R-9, and R-10, showed a slightly greater resistance to cold treatment. There were survival rates of from 60% in the case of UF-613, to 20% in the case of UF-667 following immersion in water at 4ºC. for 5 minutes.

In all cases there was brown pigment release from cold killed seeds, viable seeds did not release this pigment.

Figure 3. Root tip section showing cells in various stages of mitosis.

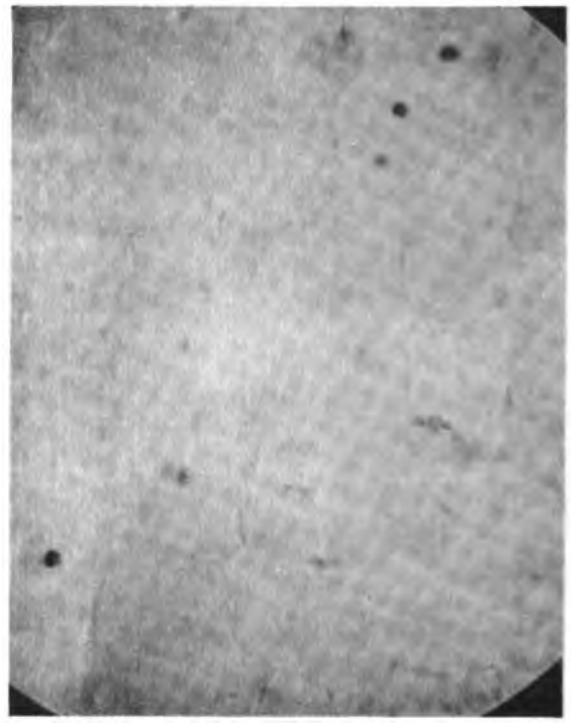

Figure 4. Section of normal cotyledon. (X 340)

Figure 5. Section of cold treated cotyledon. (X 340)

DISCUSSION

It would appear that there is some relationship between the increase in respiration tendencies following chilling of cotyledon material, and the irreversible death point of the seed after cold treatment. This is supported by the fact that restoration of viability by post-treatment for 10 minutes in water at 37ºC. results in a decrease in respiration tendencies of seeds that have been chilled in water at 4ºC for 5 minutes. This lower respiration tendency is practically indistinguishable from that of the normal system. However, the respiration tendencies of the seeds following chilling in water at 4ºC. for 10 minutes remained at a relatively high level after post treatment, even though such treatment results in a restoration of viability of the seed. This indicates that increase in respiration tendencies in cotyledon material has some relation to seed death, but it is a manifestation of processes occurring in the cell due to cold and not a direct factor in seed death.

The increase of respiration tendencies with chilling time suggests a progressive effect of cold treatment on the cotyledon. Such an effect could be attributed to a gradual change in the physiological properties of the cotyledon, possibly a change in cytoplasmic membrane permeability. Support is given to this theory by the cytological study carried out in this work, which showed that following cold treatment there is leakage of the polyphenols from the tannin cells of the cotyledons.

Mitotic studies showed that normal mitotic division was occurring in root tips of cacao embryos 6 days after they had been subjected to 4ºC. for 20 minutes. This indicates that the growth of chilled embryos

in sterile culture is due to mitotic division processes, and not solely to cell enlargement and elongation in embryonic leaves and roots.

Based on the results of the study of resistance of various clones of cacao to cold treatment, it would appear that there is little variation between cacao clones as regards sensitivity to cold. Slight differences in cold resistance could be attributed to variations in membrane permeability between seeds.

SUMMARY

The Warburg Respirometer was used to measure the endogenous respiration tendencies of cotyledonous tissue taken from normal cacao seeds which had been immersed in water at room temperature for periods of 5, 10, 15 and 20 minutes, chilled cacao seeds which had been immersed in water at 4ºC. for equal lengths of time, and cacao seeds which had been immersed in water at 4ºC. for the times given above and then given 10 minutes post-treatment in water at 37ºC.

Results showed a highly significant difference between the respiration tendencies of normal tissue and tissue chilled for 5 minutes. There was a significant difference between the respiration tendencies of tissue chilled for 5 minutes and tissue chilled for 10 minutes. Normal tissue and tissue treated at 4ºC. for 5 minutes then at 37ºC. for 10 minutes showed practically indistinguishable respiration tendencies. A highly significant difference was shown to exist between the respiration tendencies of tissue treated at 4ºC. for 5 minutes then at 37ºC for 10 minutes and tissues treated at 4ºC. for 10 minutes then at 37ºC. for 10 minutes.

Increase in respiration tendencies with chilling time could be attributed to a gradual change in the physiological properties of the cotyledon, possibly a change in cytoplasmic membrane permeability. This theory was supported by a cytological study which showed that the polyphenols of the tannin cells in cold killed cotyledonous tissue had leaked away.

Mitotic studies of embryonic root tips showed that growth of chilled cacao embryos in sterile culture was due to normal mitotic processes, and not solely to enlargement and elongation of cells in embryonic leaves and roots.

Germination tests of seeds of various clones of cacao which had been subjected to cold treatment showed that there is little variation between these clones as regards sensitivity to cold. All clones used were extremely sensitive to cold.

RESUMEN

El Respirómetro Warburg fue usado para medir las tendencias de la respiración endógena del tejido cotiledonar de los siguientes grupos de semilla de cacao: semillas normales, las cuales fueron previamente sumergidas en agua, a temperatura ambiente, por períodos de 5, 10, 15 y 20 minutos; semillas de cacao enfriadas por inmersión en agua a 4ºC. por períodos de tiempo iguales; y semillas que además del tratamiento anterior sufrieron un post-tratamiento a 37ºC. por diez minutos.

Las diferencias de las tendencias de la respiración endógena fueron altamente significativas entre el tratamiento normal y el enfriado por cinco minutos. Se encontró una diferencia significativa entre las tendencias de los enfriados por cinco minutos y los enfriados por diez. El tejido normal y aquel sometido a 4ºC. por diez minutos y luego a 37ºC. por diez minutos mostraron unas tendencias de respiración practicamente iguales. El tratamiento a 4ºC. por diez minutos y luego a 37ºC. por cinco, mostró una diferencia altamente significativa con respecto a la respiración del tejido sometido a 4ºC. por diez minutos y luego a 37ºC. por diez minutos.

El incremento de las tendencias de la respiración endógena con el del tiempo de enfriado puede ser atribuido a un cambio gradual de las propiedades fisiológicas del cotiledón, posiblemente a un cambio en la permeabilidad de la membrana citoplasmática. Esta teoría fue respaldada por un estudio citológico el cual demostró que los polifenoles, contenidos en las celulas de tanino, fueron expulsadas fuera de los cotiledones de las semillas enfriadas.

Estudios sobre la mitosis en radículas de cacao mostraron que el crecimiento, en un medio de cultivo esteril, de los embriones de semillas de cacao enfriadas es debido a un proceso mitótico normal, y no debido, únicamente, a la elongación de las células y las hojas de raíces embrionales.

Las pruebas de germinación hechas después de someter al tratamiento en frio diferentes clones de cacao, mostraron que hay una pequeña variación entre ellos con respecto a su sensibilidad al frio. Todos los clones usados fueron extremadamente sensitivos al frio.

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A P P E N D I C E S

APPENDIX A

Treatment Comparisons

1. Normal vs. Cold. 5 minutes at 4ºC ..... (N_0 vs. A).
2. Cold. 5 minutes at 4ºC vs. Cold. 10 minutes at 4ºC (A_1 vs. B).
3. Cold. 10 minutes at 4ºC vs. Cold.15 minutes at 4ºC (B_2 vs. C_1).
4. Cold. 15 minutes at 4ºC vs. Cold. 20 minutes at 4ºC (C_2 vs. D_1).
5. Normal vs. Normal + 10 minutes at 37ºC ..... (N_1 vs. N_2).
6. Normal + 10 minutes at 37ºC vs. Cold. 5 minutes at 4ºC + 10 minutes at 37ºC ..... (N_3 vs. A_2).
7. Cold. 5 minutes at 4ºC + 10 minutes at 37ºC vs. Cold. 10 minutes at 4ºC + 10 minutes at 37ºC..... (A_3 vs. B_3).
8. Cold. 10 minutes at 4ºC + 10 minutes at 37;C vs. Cold. 15 minutes at 4ºC + 10 minutes at 37ºC..... (B_4 vs. C_3).

APPENDIX B

Averages of Warburg Respirometer data in $\mu\ell$ Oxygen per 0.2 g cotyledonous tissue

TREATMENTS	TIME IN MINUTES								
	0 15	30	45	60	90	120	150	240	300
N_0	5.6	10.2	17.0	23.4	34.4	47.0	60.0	95.8	120.8
A	12.6	20.4	29.6	37.6	50.6	62.4	73.6	105.8	125.8
A_1	6.2	16.2	25.8	34.0	46.6	58.4	68.6	104.6	127.6
B_1	5.0	18.6	31.0	40.4	57.8	72.6	82.8	117.2	136.6
B_2	6.2	16.6	26.8	36.0	51.8	65.8	77.8	112.0	131.0
C_1	5.4	18.4	31.0	40.8	57.0	71.2	82.2	114.4	132.2
C_2	6.0	11.6	17.6	22.0	34.0	46.8	57.8	90.6	112.8
D_1	7.6	14.6	21.4	27.2	40.6	53.4	63.6	96.0	117.6
N_1	15.8	26.8	35.8	43.4	58.6	72.2	85.8	125.6	151.6
N_2	12.4	22.6	31.8	39.8	55.8	69.4	84.6	123.8	150.4
N_3	10.2	19.8	28.0	36.6	52.4	67.6	81.4	121.8	148.8
A_2	11.0	19.6	27.0	34.2	49.6	64.2	77.6	117.2	142.2
A_3	2.5	6.0	10.7	15.5	27.5	38.0	50.8	83.0	108.0
B_3	11.0	18.6	27.4	34.0	48.4	60.2	72.6	109.0	131.0
B_4	8.8	15.2	22.4	29.4	43.0	55.6	68.8	107.0	129.4
C_3	8.6	15.6	23.4	30.4	44.6	58.4	71.2	107.6	126.0

APPENDIX C

Correction of averages

TREATMENTS	TIME IN MINUTES								
	0	15	30	45	60	90	120	150	240	300
$A - N_0$		7.0	10.2	12.6	14.2	16.2	15.4	13.6	10.0	5.0
N_0		5.6	10.2	17.0	23.4	34.4	47.0	60.0	95.8	120.8
A		12.6	20.4	29.6	37.6	50.6	62.4	73.6	105.8	125.8
$B_1 - A_1$		-1.2	2.4	5.2	6.4	11.4	14.2	14.2	12.6	9.0
B		11.4	22.4	34.8	44.0	61.8	76.6	87.8	118.4	134.8
$C_1 - B_2$		-0.8	1.8	4.2	4.8	5.2	5.4	4.4	2.6	1.0
C		10.6	24.6	38.0	48.8	67.0	82.0	92.2	121.0	135.8
$D_1 - C_2$		1.6	3.0	3.8	5.2	6.6	6.6	5.8	5.4	4.8
D		12.2	27.6	41.8	54.0	73.6	88.6	98.0	126.4	140.6
$N_2 - N_1$		-3.4	-4.2	-4.0	-3.6	-2.8	-2.8	-1.2	-1.8	-1.2
E		2.2	6.2	13.0	19.8	31.6	44.2	58.8	94.0	119.6
$A_2 - N_3$		-0.8	0.2	1.0	1.4	2.8	3.4	3.8	4.6	6.6
F		1.4	6.2	14.0	21.2	33.4	47.6	62.6	98.6	126.2
$B_3 - A_3$		8.5	12.6	16.7	18.5	20.9	22.2	21.8	26.0	23.0
G		9.9	18.8	30.7	39.7	54.3	69.8	84.4	124.6	149.2
$C_3 - B_4$		-0.2	0.4	1.0	1.0	1.6	2.8	2.4	0.6	-3.4
H		9.7	19.2	31.7	40.7	55.9	72.6	86.8	125.2	145.8

APPENDIX D

Analysis of variance and the Least Significant Difference (LSD) test.

$$LSD = t\sqrt{\frac{2S^2}{r}}$$

where: t = 3.355 (1%)
2.306 (5%)

S = error mean square

r = degrees of freedom of error mean square

For the analysis the maximum differences between treatment averages were used, these were: -

1. N_0 vs. A - 16.2
2. A_1 vs. B_1 - 14.2
3. B_2 vs. C_1 - 5.4
4. C_2 vs. D_1 - 6.6
5. N_1 vs. N_2 - 4.2
6. N_3 vs. A_2 - 6.6
7. A_3 vs. B_3 - 22.2
8. B_4 vs. C_3 - 2.8

Results of analysis of variance

1. N_0 vs. A

LSD = 8.68^{**}

$16.2 > 8.68$ Highly significant difference

2. A_1 vs. B_1

LSD = 11.83*

14.2 > 11.83 Significant difference

3. B_1 vs. C_1

LSD = 13.82

5.4 < 13.82 No significant difference

4. C_2 vs. D_1

LSD = 8.80

6.6 < 8.80 No significant difference

5. N_1 vs. N_2

LSD = 7.54

4.2 < 7.54 No significant difference

6. N_3 vs. A_2

LSD = 20.95

6.6 < 20.95 No significant difference

7. A_3 vs. B_3

LSD = 18.23**

22.2 > 18.23 Highly significant difference

8. B_4 vs. C_3

LSD = 9.0

2.8 < 9.0 No significant difference