Exothermic Events in Cocoa (*Theobroma Cacao* L.) Seeds Associated with Low Temperature Damage

Y.L. HOR¹ AND ?.C.² STANWOOD

¹Department of Agronomy and Horticulture, Faculty of Agriculture, Universiti Pertanian Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan.
²National Seed Storage Laboratory, Colorado State University, Fort Collins, Co 80523, USA.

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**ABSTRAK**

Analisis terma pembeza (differential thermal analysis) untuk mengesankan fasa peralihan (pepejal kepada cecair dan cecair kepada pepejal) kandungan tisu embrio dan kotiledon biji benih koko telah dikaji. Tisu-tisu embrio berkelembapan antara 27% dan 64% menunjukkan satu exoterm antara —14°C dan —38°C. Eksoterm yang disebabkan oleh air sejuk-beku ini tidak didapati daripada tisu embrio yang kering, iaitu yang mempunyai kelembapan kurang daripada 27% - 15%. Tisu-tisu kotiledon yang berkelembapan antara 15% dan 27% juga menunjukkan satu exoterm yang sama antara suhu —19 °C dan —25 °C. Di samping itu, satu eksoterm tambahan yang tidak disebabkan oleh air sejuk-beku juga didapati antara suhu +15°C dan +11°C dari setiap tisu kotiledon, tanpa mengira kandungan kelembapannya. Pengesanan fasa yang dilakukan pada suhu yang tinggi ini (+15° C to +11° C) menyebabkan kerosakan yang serius dan memusnahkan percambahan biji benih, walaupun embrionya tidak dicederakan. Kepentingan kejadian eksoterm terhadap kehilangan percambahan biji benih koko semasa penyimpanan konvensional dan kriogenik dibincangkan.

**ABSTRACT**

Embryonic axes and cotyledon tissues from individual cocoa (*Theobroma cacao* L.) seeds were subjected to differential thermal analysis (DTA) to detect phase transitions (liquid to solid and solid to liquid phases) of tissue constituents. Embryonic axes at 27% to 64% moisture content (fresh weight basis) exhibited a single exotherm between -14° C to -38°C. The exotherm caused by the presence of freezeable water and was absent in tissues dried below 27% to 15% moisture. Cotyledon tissues at moisture contents between 15% to 27% exhibited a similar aqueous exotherm between —19°C to —25°C. In addition, a non-aqueous exotherm was also detected between +15° C to +11° C in all tissues, irrespective of moisture content. The phase transition which occurred at this temperature (+15°C to +11°C) was sufficiently damaging to cotyledon function so as to prevent seed germination, although the axis remained unaffected. The importance of these exothermic events to seed death during conventional (abovefreezing) and cryogenic (sub-freezing) storage of cocoa seeds is discussed.

**INTRODUCTION**

Cocoa (*Theobroma cacao* L.) seeds are recalcitrant and unable to withstand even moderate dehydration (Barton 1965; Swarbrick 1965). Germination is destroyed when seeds are dried below 17% moisture content (Mumford & Brett 1982). Like other recalcitrant seeds, cocoa also is very sensitive to cool temperatures (0°C to 15°C). Germination is rapidly reduced when seeds are stored at 15°C (Hor et al 1984). Sensitivity to cool temperature and desiccation makes cocoa seed difficult to preserve even for short periods of time.

The sensitivity of cocoa seeds to low temperature is a function of temperature and time of exposure. For example, exposure of cocoa seeds to 12 °C for 8 and 32 min resulted in 88% and 56% germination respectively; while at 8° C, the corresponding percentages were 36% and 0% (Boroughs & Hunter 1963). The increased rate of germination loss at lower temperatures could be related to more rapid cooling of seed tissues to a critical temperature. Ibanez (1963) reported that
the lethal effect of exposing cocoa seeds to 4°C for 10 min was overcome by an immediate heat treatment at 37°C for 10 min. If seeds were exposed to 4°C for 15 min or more, no amount of heat treatment could reverse the cold injury. It was further suggested that cold injury was localized in the cotyledons which eventually impeded transfer of nutrient to the embryo axis (Ibanez 1964).

Survival at low temperature is an important requirement for successful storage of cocoa seeds in both conventional and cryogenic systems. The mechanism of temperature damage is therefore further examined here by evaluating exothermic events in cocoa seed tissues during cooling. Phase transitions occurring in the constituents of both tissues were separately monitored using differential thermal analysis (DTA) to define the role of the cotyledon and axis more clearly. The latter were then compared with seed and embryo survival at low temperatures to confirm the lethal effects of cold injury sustained by the cotyledon and the embryonic axis.

MATERIALS AND METHODS

Cocoa seeds of Upper Amazon origin were extracted from the pods and de-pulped using clean sawdust. The cleaned seeds were air-dried at 20 °C to various moisture contents before differential thermal analysis (DTA) of the excised axial and cotyledonary tissues were conducted. Tissues located approximately in the center of the cotyledon were used, but whole embryo axes were evaluated. Fresh weights of individual tissues were obtained before analysis. Each tissue was then firmly wrapped around a thermocouple (Type T, 22 gauge) using thin aluminium foil. The thermocouple was enclosed in a polypropylene cryovial that fitted closely into an aluminium block. Each block accommodated five thermocouples of which the middle probe (with no tissue) was used as a reference sensor. The freezing chamber (Cryo-Med, model 972) accommodated three blocks for a total of 15 thermocouple probes. The chamber was cooled with liquid nitrogen (-196°C) at a rate of -1°C/min from a starting temperature of +20°C to a final temperature of -70°C. Control of the freezing protocol and data acquisition was accomplished using the computer system described by Becwar**, (1983).

After freezing and rewarming, the moisture contents of the axial and cotyledon tissues were determined by drying in a 103°C oven for 16 h. Moisture contents were expressed on a fresh weight basis.

Three replicates of 120 seeds each were exposed to 20°, 10°, and -30°C for 0, 2, and 24 h to monitor the effect of temperature reduction on seed germination and embryo axis viability. After exposure, 100 seeds were germinated in sand (International Seed Testing Association 1985), and embryo axes of the remaining 20 seeds were excised. The latter were surface sterilized in 0.2% w/w chlorine for 5 min, rinsed six times in sterile water, and cultured in Murashige and Skoog (1962) agar under 12 h light. The agar was supplemented with 5 mg per liter of IAA (indol-3-acetic acid) and kinetin (6-furfuryliminopurine). Embryonic axes were considered viable when well developed roots and shoots were formed.

RESULTS AND DISCUSSION

Exothermic Events in Excised Embryonic Axes

Excised cocoa axes at moisture contents between 27% to 64%, exhibited a single exotherm at
EXOTHERMIC EVENTS IN COCOA (T. CACAO L) SEEDS ASSOCIATED WITH LOW TEMPERATURE CHANGE

Fig. 2: Changes in exotherm temperature of embryonic axes in response to dehydration. When freezable water was present (unshaded) the exotherm temperature is linearly related to the tissue moisture content which was significant at the 0.01 level of probability.

subfreezing temperatures between -14°C and -38°C (Figs. 1 and 2). The exotherms decreased in height as seed moisture was reduced. Exotherms were not observed when the embryonic axes were dried below 27% moisture. Decrease in magnitude of the exotherm with dehydration and its eventual absence at low moisture levels indicates that the exotherms are caused by freezing of water within the embryonic axis. The exotherms were also within the range of exothermic temperatures reported for freezable water for other seed tissues (Becvar et al. 1983). As only a single exotherm was observed for each embryo axis, no other cell constituents exhibited a detectable phase transition within the temperature range studied (+20°C to -70°C).

Individual exotherm was narrowly spiked and peaked vertically before falling more gradually to give a slightly skewed profile (Fig. 1). Therefore, all freezable water in the embryonic axis was frozen instantaneously after supercooling to -14°C or lower. Embryonic axes dried below 27% did not exhibit exotherms in their DTA profiles indicating that this contained no freezable water.

When freezable water was present, decrease in tissue moisture caused a reduction in the exotherm peak and also a reduction of the exotherm temperature (Fig. 2). Regression between exotherm temperatures and embryonic axes moistures was linear ($r^2=0.86$, $p = 0.01$), with a regression function $Y = -49.14 + 0.51X$. This reduction in exotherm temperature is likely to be related to increased freezing point depression brought about by solution concentration as a result of moisture loss from the tissue.

Exothermic Events in Excised Cotyledons

Unlike the embryonic axes, excised cotyledons showed two exotherms, one occurring at sub-zero temperatures and the other at temperatures above 0°C (Figs. 3 and 4). The subfreezing exotherm was similar to that detected in the embryo axes. It was narrowly spiked and observed between -19°C to -25°C at moisture contents of 15% to 27% (Figs. 3 and 4B). Exotherms were not detected in cotyledons at moisture contents below 15%. This suggests that the exotherm is similar to that detected in the embryonic axes, and is due to freezing of water in the cotyledon tissues.

The second exotherm was broader, and occurred at temperatures above 0°C (Figs. 3 and 4A). It commenced at cool temperatures between +15°C to +11°C and terminated at +10°C to +5°C. This cool temperature exotherm was present in all

Fig. 3: DTA cooling (-1°C/min) profiles of cotyledon tissues at 12%, 15%, 16%, and 22% moisture contents. The exotherms occurring at temperatures above freezing are non-aqueous in origin. The sub-freezing exotherms occurred when freezable water was frozen. See Fig. 1 for further explanation.
Fig. 4: Changes in supra-freezing (A) and sub-freezing (B) exotherm temperature of cotyledon tissues in response to dehydration. The supra-freezing exotherm temperatures are non-aqueous in origin and are independent of tissue moisture content ($r^2 = 0.29$, n.s. $p = 0.05$). The sub-freezing exotherm when present (unshaded area) was not correlated with tissue moisture content ($r^2 = 0.49$, n.s., $p = 0.05$). n.s. = statistically nonsignificant.

cotyledon tissues irrespective of their moisture content. Although its magnitude varied slightly between cotyledons, it was independent (not significantly correlated) of the presence of free/able water and, therefore, is considered non-aqueous in origin.

The presence of this second exotherm in cotyledons but not in embryonic axes confirmed the occurrence of a thermal-sensitive event in cocoa cotyledons at temperatures ranging from +11°C to +15°C, that was absent in the embryonic axes. The highest temperature at which this phase transition occurred, that is 15°C is significant since it coincides with the lethal temperature at which germination is rapidly lost during cocoa seed storage (Hor etai 1984). It is postulated that the close relationship between the two events indicates that the phase transition occurring at 15°C in the cotyledon is responsible for the loss in germination of seeds stored at 15°C or lower. Cotyledon injury may be caused by phase transition of non-aqueous cotyledonary constituents that are sufficiently damaging to result in the eventual death of the attached axis and subsequent non-germination of the seed.

Low Temperature Exposure and Seed Tissue Injury
Support for the above postulate is strengthened when the effect of low temperature exposure on seed germination and embryo axis viability is examined (Table 1). At 20°C, germination and embryo axis viability were unaffected from 0 to 24 h exposure. However, 2 h exposure at 10°C, which is below the temperature at which phase transitions of the non-aqueous cotyledonary constituents were observed; seed germination was totally lost while embryo axis viability remained unaffected. These results confirm the observation that cool injury is confined to the cotyledon and does not affect the attached embryonic axis. However, if the axis remains attached during the course of germination the damage sustained by the cotyledon prevents further development of the axis, leading to its eventual death. At-30°C, both seed germination and embryo axis viability were destroyed which confirms the lethal effect of freezing on the embryo axis.

On the basis of these results, cocoa seeds are apparently subjected to two different types of injury when cooled from +20°C to -70°C. At 15°C, phase transition of a non-aqueous cotyledonary constituent occurs which results in damage to the cotyledon but does not affect the attached embryonic axis. However, the injury sustained by the cotyledon is sufficient to prevent development of the attached axis during the germination process, leading to its eventual death. As the temperature is reduced to subfreezing levels, a second injury occurs between

| Temperature Exposure Effects at 20°C, 10°C and -30°C for 0, 2, and 24 h on germination and embryonic axis viability of cocoa seeds. |
|---|---|---|---|---|---|---|
| Time (h) | Germination (%) | Axis Viability (%) |
| 20°C | 10°C | -30°C | 20°C | 0°C | -30°C |
| 0 | 99 a | 99 a | 100 a | 97 a | 95 a | 98 a |
| 2 | 99 a | 0 b | 0 b | 93 a | 98 a | 0 b |
| 24 | 99 a | 0 b | 0 b | 98 a | 93 a | 0 b |

Means within each parameter and column are separated by the Least Significant Difference (LSD) test at $P = 0.05$. 

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-14°C to -38°C depending on the moisture content of the tissue. At higher moisture contents, where freezing of the aqueous component occurs, death is presumed to be caused by intracellular ice formation in both cotyledon and axis. However, at lower moisture contents, where no freezable water is available (embryonic axis moisture is below 15% to 27%), death is primarily through dehydration since such moisture levels are well below the critical moisture content for cocoa embryonic axes (Hor et al 1984). From the perspective of cocoa seed storage, the 15°C injury of the cotyledon is important in conventional storage at temperatures above freezing. Storage above 15°C is recommended in such cases. Freezing injury at subfreezing temperatures, however, is important in cryogenic storage of excised embryonic axes. It confirms lethal ice formation at -14°C to -38°C in those axes frozen at moisture contents above 15% to 27%.

Although lethal freezing can be avoided at lower moisture contents, such moisture levels are below the critical level for cocoa axes which are killed by dehydration. Further studies are required to devise appropriate freezing protocols which can avoid freezing injury to cocoa embryonic axes at a narrow range of moisture just above the critical level.

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