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Physiological and biochemical changes in cocoa seed *(Theobroma cacao L.)* caused by desiccation

Perubahan fisiologi dan biokimia benih kakao (Theobroma cacao L.) *akibat desikasi*

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Ringkasan

Benih kakao tergolong rekalsitran, benihnya sensitif terhadap desikasi dan apabila disimpan pada kondisi yang menyebabkan kehilangan air, benih akan kehilangan viabilitasnya. Viabilitas benih kakao hanya dapat dipertahankan beberapa hari saja dalam keadaan terbuka pada suhu kamar. Hal ini merupakan kendala dalam penyimpanan dan pengiriman benih kakao. Tujuan penelitian ini adalah untuk menetapkan pengaruh desikasi terhadap karakter fisiologis dan biokimia benih kakao. Benih ICS 60 (kakao lindak) dan DR2 (kakao mulia) diletakkan dalam cawan Petri kemudian disimpan pada suhu 25oC dan Rh 55-75% selama empat hari. Percobaan dilakukan dengan rancangan petak terpisah, petak utama adalah kandungan air awal dan kritikal. Sebagai anak petak adalah jenis kakao, masing-masing diulang empat kali. Peubah fisiologis yang diukur adalah viabilitas benih mencakup kandungan air benih, potensi tumbuh maksimum, daya berkecambah, kecepatan tumbuh, bobot kering kecambah normal, dan laju pertumbuhan kecambah normal. Di samping itu juga dilakukan pengamatan pola pita protein benih yang dianalisis dengan SDS-PAGE. Kandungan asam absisik (ABA) dan gula stahiosa, raftnosa, glukosa, fruktosa, arabinosa, silosa, serta sukrosa dalam benih yang ditetapkan dengan HPLC Integritas membran benih ditetapkan berdasarkan daya hantar listrik air perendaman benih yang diukur dengan konduktometer. Hasil yang diperoleh menunjukkan bahwa adanya interaksi yang nyata antara desikasi dengan seluruh tolok ukur fisiologis. Desikasi menyebabkan penurunan daya berkecambah, bobot kering dan laju pertumbuhan kecambah normal, potensi tumbuh maksimum dan kecepatan tumbuh. Sedang untuk, kandungan ABA, sukrosa, arabinosa dan rafinosa mengalami peningkatan. Di samping itu desikasi menyebabkan dibentuknya protein baru dengan BM 32,5; 47,0 dan 51,0 kDa (DR2); 47,0 dan 51,0 kD (ICS 60). Beberapa protein yang hilang oleh pengaruh desikasi yaitu dengan BM37, 0 (DR2), 19, 0 dan 37, 0 kD (ICS60). Benih ICS60 lebih tahan terhadap desikasi dibandingkan dengan benih DR2.

Summary

Seed of cocoa is recalcitrant and sensitive to desiccation. In open condition at room temperature, the viability of cocoa seed ultimately lost for several days. These characters are a problem for seed storage and delivery. The objectives of this study are to investigate the effect of desiccation on physiological and biochemical characters of cocoa seed. Seeds of ICS 60 (bulk cocoa) and DR2 (fine cocoa) were placed on Petri dishes and stored at 25oC, Rh 55-75% for four days (critical water content). The experiment was conducted with split plot analysis, (1) The main plot was the storage condition initial and critical seeds water content. (2) The sub plot was the variety of cocoa, with four replications of each treatment. The effect of desiccation on seeds viability was tested, based on seed water content, maximum growth potential, seed germination, germination rate, dry weight of normal seedling, and seedling growth rate. Besides, the changes of seed proteins band pattern were also analysed by SDSPAGE. Abscisic acid, stachyose, raffnose, fructose, arabinose, xyllose, and sucrose seed content were determined by HPLC. The integrity of seed membrane based on the leakage of electrolytes from seeds was measured with a CM 100 multicell conductivity meter. The results showed that there is an interaction with highly significant correlation between desiccation and all of the physiological and biochemical parameters. Desiccation caused the decrease of seed germination, dry weight and growth rate'of normal seedling, maximum growth potential, and germination rate and while the leakage of electrolytes, ABA, sucrose, arabinose and raffinose increased. Besides, desiccation was also caused the formation of new proteins with MW 32.5, 47,0 and 51,0 kDa (DR2); 47,0 and 51,0 kD *ICS 60)* . On the other hand, several protein were disappeared i.e. MW 37,0 (DR2), 19,0 and 37,0 kD *(ICS60)*. Seeds of *ICS 60* are more tolerant to desiccation than seeds of DR2.

[*Keywords: Theobroma cacao,* desiccation tolerance, recalcitran seed, seed's-biochemical characters, seed's-physiological characters]

Introduction

Cocoa seeds are recalcitrant seeds. Roberts (1973) described the recalcitrant seeds with the characteristics of being shed at a relatively high water content and not tolerating the loss of any substantial proportion of this water without adverse effects culminating in viability loss. According to Chin (1980), Chin *et al.* (1989), and Bonner (1996) the seeds highly susceptible to desiccation injury, high water content, and thus is not storable and has a very short lifespan. Recalcitrant seeds are damaged by dehydration, may also be chillingsensitive, and generally cannot be stored effectively for useful periods. They will not withstand removal of water to the level of critical seed water content (Chin *et al.*, 1989; Copeland & McDonald, 1995; Bonner, 1996).

To understand the response of seeds to dehydration it is necessary to understand the phenomena occurring at different hydration levels and the mechanisms and processes counteracting possible deleterious effect of water removal. According to Pammenter & Berjak (1999) a number of processes or mechanism have contributed to desiccation sensivity of seed. The mechanisms that have been implied to `'date include the following: intercellular physical characteristics, particularly reduction of the degree of vacuolation, the amount and nature of

accumulated insoluble reserves, "switching off' of metabolism, accumulation and roles of putatively protective molecules, including late embryogenic abundant proteins (LEAs), sucrose and certain oligosaccharides or galactosyl cyclitols, intracellular dedifferentiation, which effectively results in the minimization of surface areas of membranes and probably also of the cytoskeleton.

Vertucci & Farranf (1995) reported that at different levels of tissue hydration different biochemical and physiological processes can occur. The fact that in recalcitrant seeds lethal damage can occur in the critical water content where free radical-mediated processes are supposed to occur. According to Obendorft (1997) desiccation tolerance in development process and maturation of seed involve many components such as nonreducing sugars, or cytosols (Horbowicz & Obendorft, 1994; Fu *et* al., 1997), heat-stable hydrophilic proteins (Blackman *et* al., 1995; Fu *et* al., 1997), and free radical systems (Leprince *et al.*, 1994). Kermode (1990) reported that desiccation has an important role in the changes of seed activity programs, from seed development to seedling geminations. During the desiccation period, several of the proteins have a potential in preventing the intracellular components. Jia-Rui *et al.* (1997) found that heat-stable protein especially dehydrin, late embryogenesis abundance (LEA), storage protein, and inhibitor protein are associated with desiccation sensitivity.

Cocoa seed is desiccation sensitive, they cannot be stored under the conventional conditions of low water content and below-freezing temperatures. Germination ofhydrated seeds in storage at ambient temperatures can also be a problem. Fungal contamination can lead to rapid deterioration, particularly at high water content. However, even in the absence of, or much reduced microbial contamination, recalcitrant cocoa seeds ultimately lose viability. The reason for the loss of viability of cocoa seeds in storage is not known. So far, the knowledge of the mechanisms of cocoa seed response to desiccation is very limited. These results are encouraging in terms of extending the lifespan of cocoa with short-lived seed, raising the chances of successful long-term storage and long-distance transport associated with the seed trade.

The objective of the present research is to examine loss of seed moisture and viability in intact seeds during desiccation, and to establish the relationship between desiccation and physiological as well as biochemical changes in seeds of two cocoa cultivars.

Materials and Methods

Cocoa ICS 60 and DR2 seeds have used in this experiment were obtained from the Indonesian Coffee and Cocoa Research Institute, Jember. The seeds were extracted from mucillage pulp with CaCO, (25 g/kg seeds) for 1-2 min., and than washed with water. The hydrated seeds were mixed with Delsen MX-200 fungicide (3 g/kg seeds) before being used for tests on desiccation, while seeds for physyiological and biochemical analysis were not treated with fungicides. Fresh mature seeds having moisture content of 35.7 (fresh weight basis).

Desiccation treatment and storage

Fresh seeds,DR2 and ICS 60 were placed on Petri dish and stored in an Air Conditioned room at 25°C and Rh 55-75% for four days. For physiological and biochemical analysis, untreated cocoa seeds were used. Every day of the storage period, 25 seeds each in triplicate were withdrawn for germination and evaluation.

The experiment was conducted with experimental split plot design. The main plot was the storage condition at two levels i.e. control and desiccation in an AC room for four days. The sub plot was the variety of seed, at two levels ICS 60 and DR2. The 16.treatments were replicated four times with 25 seeds treatment, so that a total of 704 seeds were used.

Germination assessment

Random samples of 25 seeds from each treatment were soaked in water for one hour before planting out on wet fine river sand for seed germination in a plastic box of 32.5 x 22.5 cm. To control the incidence of seed diseases associated with the cocoa, the medium was sprayed with Dithane M-45 (Maneb) at the rate of 28.5 g mixed in 4.54 L emulsion. The plastic box with seeds were put in a greenhouse. Seeds were considered germinated when the roots had extended to 2 mm and as established seedling when the first two true leaves had emerged. Germination and establishment trials continued for 45 days after planting, although maximum germination generally occurred between 14 and 21 days (ISTA, 1993).

Water relations

The moisture content of fresh seeds were determined gravimetrically on at least three replicates by drying in an oven for 48 h at 103°C. For each replication 1.5 to 3.5 g representing about 10 seeds were cut into pieces of roughly 4 mm' before drying. All moisture contents are expressed on the basis of fresh weight. The moisture content of seeds in each treatment described above was determined according to the following method assuming that the seed samples have initially the same water content. The weight of each seed sample was recorded at the beginning and repeated again at the time of sampling for the germination experiment. Based on the known moisture content of fresh seeds and the loss of mass after storage for a specific period, the seed moisture content was thus calculated. Water content are expressed a dry mass basis (g H,O (g dry mass)$^{-}$'; g/g).

Germination tests were carried out according to the official testing prescriptions (ISTA, 1993), i.e. growth potential, germination percentage, growth rate, dry weight of normal seedling, and growth rate of seedling.

Electrolyte leakage (EL)

The leakage of electrolytes from seeds was measured using a CM100 multi-cell conductivity meter. The conductivities of the leachate from ten replicates of each treatment soaked in 3 ml, distilled water were measured after 18 h. Leakage was expressed as the percentage increased in conductivity over control values after distilled water blanks had been subtracted. Conductivities were expressed as micro Siemens per gram of fresh weight (Og^{-}').

Extraction and separation of *proteins*

Dried seeds sample were weighed, ground to a fine powder and suspended in cold extraction buffer (15 mL, Tris-HCl buffer, pH7.0, 100 mM Tris-HCI pH 7.4, 10 mM MgCl,, 1 mM 2% R-mercaptoethanol and 1 mM EDTA) , at the ratio of 1 mL, buffer: 10 mg tissue. After incubation in ice for 10 min. the homogenate was centrifuged at 16.000 x g for 10 min and the

protein content of the supernatant was determined (Bradford, 1976). While bovine serum albumine (BSA) was used for standard curve.

One-dimensional gels containing 12% sodium dodecyl sulphate, polyacrilamide (SDS-PAGE) were prepared for separation of proteins in seeds. Fifteen to 80 gg of protein previously solubilized with 2% SDS and heated 5 min. at 100° C were loaded into each well (Laemmli,1970). The gels were run with 40 mA constant current for 4-5 h and subsequently stained with AgNO, according to Oakley *et al.* (1980).

Analysis of sugar content

Seed sugar content was analysed according to the method of Snyder *et al.* (1988). Fresh samples (3 g) from each treatment were weighed, ground to fine powder in liquid N2 with a mortar and pestle, and sugar was extracted with destilled water. For quantitative analysis of sugars, the extract was centrifuged, and the supernatant was used for HPLC. The stachyose, raffmose, glucose, fructose, arrabinose, xyllose, and sucrose contents were measured with HPLC (Shimadzu LC-6A). The chromatography was achieved using column p-BondapackC,$_B$ (Waters Co. Ltd. 3.9x 300 mm) and acetonitrile : aquades (60:40) as an eluent. The condition for HPLC analysis was as follows: the flow rate of mobile phase was 1.0 mL/min, detector Refractive Index Detector (RID), and attenuation 1-2, column temperature 40°C, and the compounds were detected by UV detector at 254 nm.

Results and Discussion

Desiccation sensitivity

During desiccation, seed viability of both cultivars decreased rapidly in line with dec-reasing water content up to the critical value. Based on 50-60% seed germination (at temperature of 25°C and Rh 55-75% for 4 days of storage), showed that the effect of desiccation on viability and seed water content have a highly significant correlation (Table 1). The same results were also found on cocoa cultivars, except on the maximum growth potential of seedlings. While the interaction between seed water content and cocoa cultivars was highly significant only for dry weight of normal seedlings, growth rate of seedlings, and water content of seeds (Tabll 1). The interaction between water content and cocoa cultivars showed that critical water content for DR2 was 9.94% and 18.95% for ICS 60.

The response of seed from two cocoa cultivars to desiccation up to critical water content showed significantly difference ($P<0.05$) based on seed water content, seedling maximum growth potential, seed germination percentage, seedling growth rate, dry weight of normal seedling, seedling growth rate and decreased leakage conductivity. The rate of seed viability loss of DR 2 was much higher compared to that of ICS 60 seed (Table 1).

Table 1. The effect interaction of cocoa cultivar and water content on several seed viability parameters and electrolyte leakage.

No.	Parameters	Cocoa cultivars			
		DR 2		ICS 60	
		O -day	4-day of desiccation	O -day	4-day of desiccation
1.	Seed water content (%)	4 3.85 d	19.94 b	35.67 c	18.95 a
2.	Germination percentage (%)	73.57 b	42.71 a	90.00 b	46.72 a
3.	Dry weight normal seedling (g)	9.80 b	2.83 a	9.80 b	2.83 a
4.	Seedling growth rate (mg/Ns)	425.00 c	245.00 a	438.00 c	388.00 b
5.	Maximum growth potential (%)	100.00 b	54.00 a	100.00 b	52.00 a
6.	Electrolyte leakage (Ohm/g)	5.91 a	16.77 b	8.65 a	56.55 c
7.	Germination rate (%Ns/etm)	6.75 b	2.75 a	7.83 b	3.28 a

Note : *) Transformation arcsin ;¯transformation percentage.
**) Numbers in rows following by the same letters were not significantly different, according to Duncant test (P<0,05).
- Ns: Normal seedling

This difference suggested a strong effect of desiccation on DR2 seed mesocarp which was softer than ICS 60 cultivar. The results showed that the sensitvity of seeds to desiccation depended slightly on the cultivar. The water content of DR 2 seed rapidly decreased, which resulted in the loss of germination ability. According to Lin (1996) the sensitivity to dehydration depends on the species and quality of seeds.

Dehydration was also induced deterioration of cell membranes, as indicated by a high increase in leakage of solutes (Table 1). According to Kermode (1990) the integrity membranes in seeds is of crucial importance to the maintenance of viability. Some changes in membrane structure may be provoked as a consequence of desiccation.

ABA content

The response of seeds to desiccation could be seen from the differences of ABA content at critical water content condition. The results showed that ABA content in ICS 60 seeds was higher than in DR2, which also has the capability to prevent seed viability and vigor at a higher level.

Farant *et al.* (1996) found the difference in seed biochemical of *Avicennia marina* and *Barringtonia racemosa* after seed water content was decreased. However, ABA content increased which promoted dehydrin protein formation in *B. racemosa* seeds, but it was not found in *A. marina* seeds. According to Kermode (1997), ABA compound is capable of protecting

intracellular components against desiccation, considering that the ABA and the heat stable proteins may have some protective function.

ABA has long been involved in the development of desiccation tolerance in seeds and revival of plants (Vertucci & Farrant, 1995). Among the attributed roles in desiccation tolerance are sign transducer for induction ofgenes and LEA proteins and the general induction of metabolic quiescence (Kermode, 1990).

Sugar content

Another mechanism of cocoa seed response to desiccation was through the formation of a certain sugar (Figure 1 & 2). The concentration of carbohydrates in ICS 60 especially sucrose, raffinose, fructose, and arabinose increasing. However in DR2 seeds, only glucose and fructose were increased. Gradual water loss my allow protective changes to occur and hence increase the seeds resistance to disruption by dehydration. Sucrose and raffmose may play a key protective role to prevent the osmotical pressure in seed cells and increase seeds tolerance to desiccation. According to Kermode (1997) the accumulation and the increase of the concentration of raffinose family oligosaccharide (RFO_S) and galactosyl cyclitol has a correlation with the ability of seeds to germinate after desiccation. The loss of seed viability was assumed initiated by the loss of RFO_S and galactosyl cyclitol and increase of reduction sugar in cotyledon, hypocotil and radicula tissues. Pammenter and Berjak (1999) found that sucrose, raffmose and stachyose may play a key protective role by accumulating under water deficit conditions and functioning to replacing water in the phospholipid of the membranes cell thus stabilizing membranes and other sensitive systems.

According to Obendorft (1997) soluble carbohydrates are one of the multiple components required for the acquisition of desiccation tolerance. Sucrose and raffmose series of oligosaccharides have been extensively studied in relation to seed desiccation tolerance. The stabilization of membranes appears to be the prominent role for sugars in conveying desiccation tolerance in seeds and a model membrane systems (Sun *et al.,* 1994). Desiccation tolerance appears to be a gradual process, and accumulation of sucrose correlates with the early stages of desiccation tolerance when measured as leakage (Sun *et al.,* 1994). However, sucrose alone is not sufficient for desiccation tolerance when measured as the ability to germinate after drying. According to Obendorft (1997), Blackman *et al.* (1992); Horbowicz & Obendorft (1994), desiccation tolerance in mature seeds involves multiple components, including accumulation of nonreducing sugars and/or cytolitols, heat-stable hydrophylic proteins (Blackman *et al.,* 19965) and free-radical scavening systems (Leprince *et al.,* 1994). Maize and other grass seeds accumulate soluble carbohydrates i.e. sucrose to oligosaccharides and raffmose with high concentrations in association with loss of starch in embryos (Black *et al.,* 1996; Brenac *et al.,* 1997). According to Steadman *et al.* (1996) a low ratio of sucrose to oligosaccharides is correlated with long term storability of seeds.

Protein content

Seeds responses to desiccation were examined based on the changes of protein bands (Tabe12). It was shown that the decrease of seed water content up to the critical point which promoted formation of new proteins. Decrease of seed water content caused the formation of several protein bands with the molecular weight 51, 47 and 32 kDa for ICS 60 and DR2. Beside,

these proteins, in ICS seed the protein with MW 84 kDa was also found. Based on the molecular weight, these proteins belong to the dehydrin proteins

According to Kermode (1990) the presence of dehydrin protein is associated with a high content of ABA. Desiccation tolerance which could stimulate the formation of dehydrin protein. Fan˙ant *et al.* (1996) was found that in *Barringtonia racemosa* seeds, the desiccation caused the increase of ABA content and induction of dehydrin protein with MW 16 and 43 kDa. These dehydrin proteins were one of the compound with the ability to increase seed tolerance to critical water content.

According to Jia-Rui *et al.* (1997) Blackman *et al.* (1992 & 1995) ; Thomann *et al.* (1992) there were several kinds of heat - stable proteins including dehydrin, or LEA, storage protein, and protease inhibitors related to desiccation tolerance. They were enhanced by exogenous ABA and osmotic stress. These compounds could be increased rapidly when the seeds were dried and appeared when seed germinated.

A particular set of proteins termed LEAs has been implied in an acquisition of tolerance to drying in developing seeds. The presence of these proteins has been associated with high contents of ABA (Kermode, 1990) and ABA can induce their production (Finch-Savage *et al.,* 1994).Tbe physical nature of these proteins, together with the situations under which they are expressed, has led to the suggestion that they function in the survival of water stress by acting as protectants and/or by stabilizing the subcellular structures (Dure, 1993). The presence of dehydrin proteins will play a role in protecting against the damage ofmembranes integrity and other cellular components.

Table 2. DR2 and ICS60 seed protein from the electrophoresis bands, before and after desiccation treatment.

Seed water	Cocoa	Protein molecular weight (kDa)								
content (%)	cultivar	84.0	67.0	57.5	51.0	47.0	37.0	32.5	29.1	19.0
43.85%*)	DR2	-	+	+	-	-	+	-	+	+
35.67%	ICS60	-	+	+	-	-	+	-	+	+
19.94%**)	DR2	-	+	+	+	+	-	+	+	+
18.95%	ICS60	+	+	+	+	+	-	+	+	+

Notes : *) Initial seed water content
**) Critical water content of seed

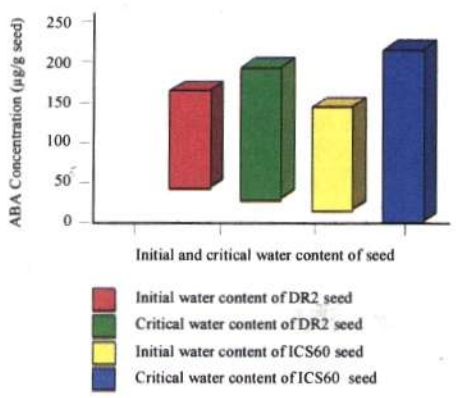

Figure 1. ABA concentrations of DR2 and ICS 60 at control and critical water content.

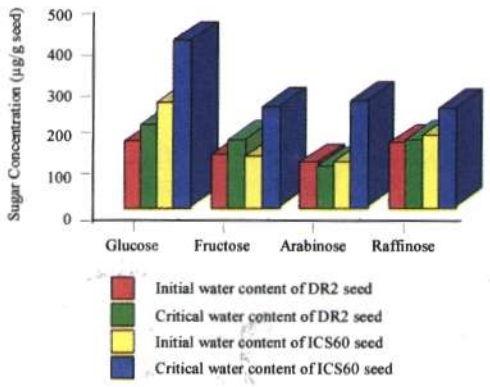

Figure 2. The concentrations of several sugar of DR2 and ICS 60 at control and critical water content.

Conclusion

Desiccation on seed cocoa caused:

I . Physiological and biochemical changes of DR2 and ICS 60 seeds. However, ICS 60 seeds have more tolerance to desiccation compared with DR2.
2. The decrease of integrity of membrane cells, and increase of glucose, sucrose, arabinose, raffmose, and ABA seed content.
3. The formation of protein with MW 51, 47, 32 and 84 kDa.

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