13

INTERNATIONAL COCOA RESEARCH CONFERENCE

CONFERENCE INTERNATIONALE SUR LA RECHERCHE CACAOYERE

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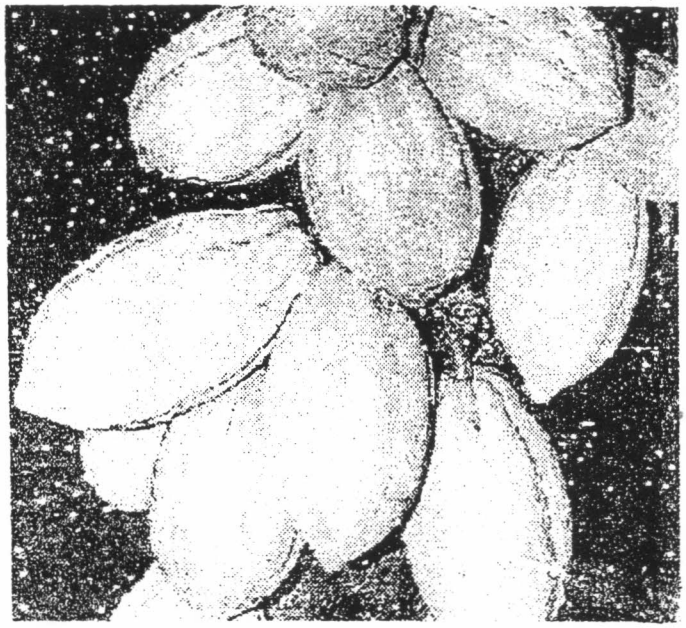

9 - 14 OCTOBER 2000

SHANGRI-LA'S. TANJUNG ARU RESORT HOTEL

KOTA KINABALU, SABAH

MALAYSIA

LIST OF PAPERS

NO. OF PAPER	TITLE OF PAPER
001	The effect of different leveis of cocos expeller cake in diets..... of sheep.
002	Etude histochimique des phenols.....apres infection par *Phytophthora Megakaria*.
003	Activies peroxydasiques et auxin.....embryogenese somatique in vitro.
004	Actividade microbiana avaliada.....cacau.
005	Reclassificacaok dos solos bon para cacau.....Bahia.....Brasileiro.
006	Response of cocoa for N,P and K fertilizer.....in Sri Langka.
007	Evaluation of yield potential of varieties.....in Sri Langka.
010	Effect of bulk sweetner and vegetable fats in developing thermoresistant ..... tropics.
011	The physico-chemical and sensory evaluation of some non-conventional.....the tropics.
012	Extraction of pulp from fresh cocoa beans for wine production.....cocoa beans.
013	Cocoa production and Socio-economic problems.
014	Comparison of approaches to determine sexual incompatibility Phonotypes in cocoa.
015	Inheritance of the grafting success in adult plants of cocoa.
017	Strategy for assessing cocoa flavour of a large number of samples for selection and breeding.
018	Use of DNA Markers for the determination of the genetic origin of cocoa.....chocolate.
019	Controle do fluxo foliar, floracao a frutificacao do cacaueiro.....reguladores.
020	Estudo comparative da producao de cacaueiros autocomparativeis e auto.....Bahia.
021	Efeito de doses crescenies de glucose nos teores de amido e acucares.....resistencia.
022	An investigation of physiological parameters underlying yield variation in cocoa.
034	Premier cycle de selection recuments du cacaoyer en Cote d'Ivoire et choix....cycle.
040	*Theobroma cacao* L : Genome map and QTLs for *Phytophthora palmivora* resistance.
041	Cocoa genetic diversity assessment using RAPD.
046	Geographical distribution of *CERATOCYTIS FIMBRIATA* on cacao.....,Brazil.
049	Effect of planting density on yield and incidence of Witches' Broom disease.....trees.
051	Anatomical and physiological characteristics of Theobroma spec, seeds..... processsing.
052	The genetic and physiological basis of incompatibility in cocoa.
053	Growth and yield of cocoa grown under area in relation to plant.....architecture.
054	Biological Control of frosty pod (*Moniliophthora roreri*) and.....Peru.
057	Some water relations aspects of Theobroma cacao clones.
063	Tecnicas de Entertia na substituicao da copa de cacaueiros na Bahia, Brazil.
072	The cocoa cluster in the South of Bahia, Brazil.
073	Search for resistance Phytophthora pod rot disease of cocoa; .....variability.
074	Evaluation of the formulated mixture of glyphosate and terbuthylazine in the.....plantation.
075	Comparative weed control studies in young cocoa plantation with formulated ..... Treatment.
082	Sustainable cocoa production in Nigeria: Farmers perception of technology ..... Decision.
083	Nutritional value of urea-treated cocoa husk in pullet growers mash.
095	Efforts to breed for improved cocoa varieties for Ghanaian farmers.
096	Rentabilite economique de la recoite sanitaire utilisee dans le controle des maladies.....Equateur.
097	Creation d'une collection de cacao "Nacional" equitorien, (S.N.A.) sur la .....Organoleptiques.
098	Rentabilite comparee des techniques de rehabilitation par recepage et de renovation ..... Equateur.
100	Evolucion de la production de una plantacion de cacao criollo "Guasare" y su.....Venezuela.

University of Hamburg

Biological Faculty
Institute of Applied Botany

Anatomical and Physiological Characteristics of *Theobroma spec.* Seeds and their Relevance to Processing

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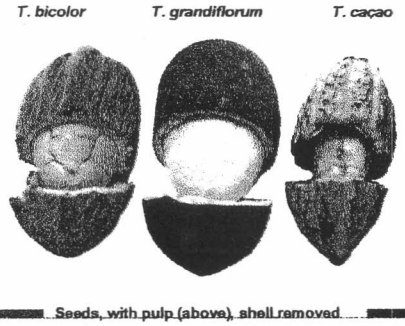
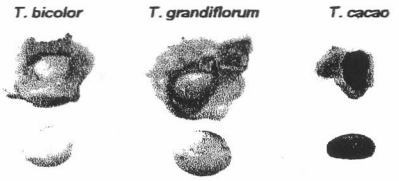
The sampled Species

Fruits



Seeds, with pulp (above), shell removed



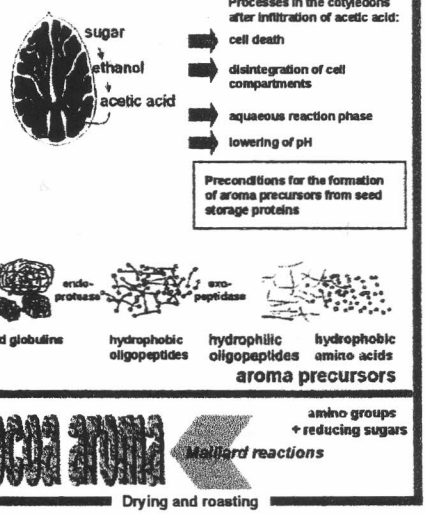
Objective

The objective of our studies was to find out whether the seeds of *Theobroma grandiflorum* (Willd. ex Spreng) Schum. and *Theobroma bicolor* H. B. K. have the potential to develop a good chocolate-like aroma and thus can be used to produce a storable and valuable ware. With this intention, we studied physiological and anatomical features relevant to flavour development of the seeds of the two *Theobroma* species and compared them with the features of *Theobroma cacao* L..

The Origin of Cocoa Aroma

Fermentation

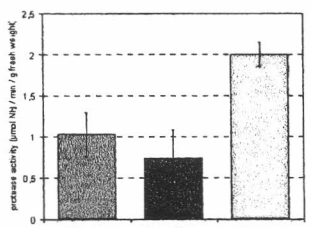
Cocoa seed with adhering pulp



Seed Protease Activities

Aspartic endoproteases

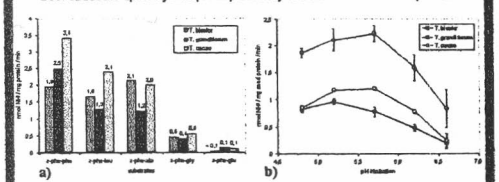
T. cacao cotyledon tissues exhibit the highest endoprotease activities.



Pepstatin-A sensitive activity of aspartic endoproteases incubated at pH 3.5 with 4 mg/ml BSA

Seryl Carboxypeptidases

The highest activities were measured in *T. cacao* seeds.
Both substrate speficity and pH dependency are similar in all three species.

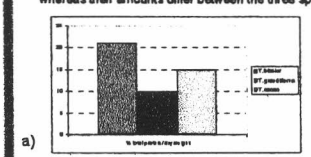


a) PMSF-sensitive activities of seryl carboxypeptidases incubated at pH 5.6 with different z-dipeptides
b) degradation of seed protein by seryl carboxypeptidases at different pH values

Seed Compounds

Protein Contents and Quality

The seed proteins show a high similarity in quality whereas their amounts differ between the three species.



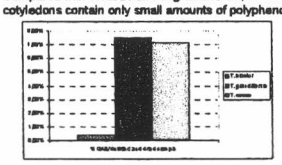
protein	species	mw [kDa]	% of total protein
globulin 1	*T. grandiflorum*	48 kDa	24
	T. cacao	47 kDa	9.5
	T. bicolor	46 kDa	32.9
globulin 2	*T. grandiflorum*	31 kDa	13.4
	T. cacao	30 kDa	12.5
	T. bicolor	30 kDa	16.8
albumin 1	*T. grandiflorum*	20 kDa	20.4
	T. cacao	21 kDa	19.6
	T. bicolor	20 kDa	5

b)

a) Total protein contents estimated by Bradford protein assay
b) Analysis of seed proteins by SDS gel electrophoresis

Total Phenolics

Compared with *T.cacao* and *T. grandiflorum*, *T. bicolor* cotyledons contain only small amounts of polyphenols.

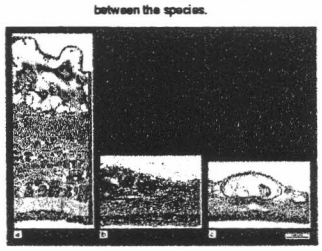


Total phenolics estimated with Folin-Ciocalteu reagent and calculated as gallic acid equivalents (GAE)

Seed Tissues

Seed Shells

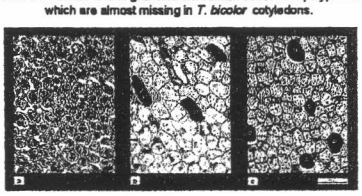
The thickness of the seed shells and the palisade layers varies strongly between the species.



Seed shells of *T. bicolor* (a), *T. grandiflorum* (b) and *T. cacao* (c).
■: outer integument
: inner integument
▦: palisade resp. sclereide layer (outer epidermis of the inner integument)

Cotyledon Tissues

Cotyledons of *T. cacao* and *T. grandiflorum* contain about 10% polyphenol cells, which are almost missing in *T. bicolor* cotyledons.



Cotyledon tissues of *T. bicolor* (a), *T. grandiflorum* (b) and *T. cacao* (c).
Cells marked with p: polyphenol cells
Others: storage cells, containing lipids (white, aleuron (purple) and starch (arrows)

Discussion

Our studies show that *T. bicolor* and *T. grandiflorum* seeds generally meet the biochemical requirements for the generation of aroma precursors comparable to those of cocoa seeds. Fermented *T. grandiflorum* and *T. bicolor* seeds are of a light colour - comparable with those of Criollo varieties. Like those, they might be used for the manufacture of light coloured chocolates.
However, simple application of the fermentation practices known from cocoa will not yield sufficient aroma precursors, since structural differences especially of the seed shells may influence the duration in which the acetic acid infiltrates the seeds. Additionally, the distinct contents of phenolic compounds of *T. bicolor* seeds most likely result in a differing flavour development during fermentation.

Appropriate fermentation procedures need to be developed for the production of chocolate-like products of a high and reproducible quality from seeds of *T. bicolor* and *T. grandiflorum*. First studies are being performed to test new fermentation technologies meeting the particular requisites of seeds of *T. grandiflorum*.

For further information please refer to our full paper in the Conference Proceedings or contact us:
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Anatomical and Physiological Characteristics of *Theobroma spec.* Seeds and their Relevance to Processing

1. Introduction

All 22 species of the genus *Theobroma* L. produce berry fruits with large seeds embedded in a pulp. Nevertheless, only *Theobroma cacao* L. became a main tropical crop plant due to its seeds which are processed to raw cocoa, the basic substance of chocolate and related products.
Besides *T. cacao* the seeds of at least 12 other *Theobroma* species are used in the Amazon Basin for the production of home-made chocolate (Cuatrecasas 1964). Among these, *T. grandiflorum* (WILLD. EX SPRENG) SCHUM. and *T. bicolor* H. B. K. are considered to be the most important species.
T. grandiflorum, the cupuaçu tree, is an excellent crop for locally adapted land use systems in the Amazon region. The fruit pulp fetches relatively high market prices while the seeds are not yet commercially used.
T. bicolor is commonly cultivated throughout all humid tropical America. Its pulp is used similar to the one of *T. grandiflorum*, while in some regions the seeds are utilised for the production of chocolate for local markets (CUATRECASAS 1964).

The unique chocolate aroma is formed in the course of fermentation and roasting of the seeds. During fermentation acetic acid is formed by microbial degradation of the fruit pulp. After permeating the seed shell, the acid penetrates the cotyledons where it effects a disintegration of the cellular compartments. A continuous aqueous phase is generated by the subsequent fusion of the cotyledonar liposomes. Within this phase the lowering of the pH activates two types of proteases, which corporately digest the major cocoa storage protein, a vicilin-type globulin. The resulting mixture of amino acids and oligopeptides represents essential aroma precursors.
During drying and roasting the aroma precursors react with reducing sugars to Maillard-products, forming the proper cocoa aroma (BIEHL et *al.* 1982, 1985, 1993, BIEHL and PASSERN 1982, VOIGT et *al.* 1994a, 1994b, 1994c, 1994d, VOIGT and BIEHL 1995, ZIEGLEDER and BIEHL 1988).

Phenols form another group of flavour compounds, causing an astringent and bitter taste. During fermentation, cocoa phenols leak from the vacuoles and tan significant portions of the surrounding proteins (BIEHL et *al.* 1983). The function of the resulting products as flavour components is disputed (ZIEGLEDER and Biehl 1988). However, the tanning prevents large amounts of storage proteins from proteolytic cleavage and thus affects the flavour development.

Besides the physiological aspects the success of fermentation depends on the histological and cytological structure of the seeds. Both the permeability of the

seed coat to acetic acid and the specific characteristics of the cotyledon tissue are to be mentioned in this context (LOPEZ et *al.* 1987).

The objective of our studies was to find out whether the seeds of *T. grandiflorum* and *T. bicolor* have the potential to develop a good chocolate-like aroma and thus can be used to produce a storable and valuable ware. With this intention, we studied physiological and anatomical features relevant to flavour development of the seeds of the two *Theobroma* species and compared them with the characteristics of *T. cacao.*

2. Materials and Methods

2.1 Samples

Ripe fruits of *T. cacao* of the Forastero variety, *T. grandiflorum* and *T. bicolor* were obtained from the experimental field and the surroundings of the German-Brazilian project SHIFT near Manaus, Brazil. The *Theobroma* seeds were analysed at the Institute of Applied Botany, University of Hamburg, Germany.

2.2 Microscopical Studies

Entire seeds were cut into slices and fixed in 4 % formaldehyde. Tissue samples of seed coats and cotyledons were dehydrated first in 60 %, then in 100 % ethanol and subsequently embedded in LR White ® Medium Grade. Semithin sections (1-2 µm) were fixed on a slide. After incubating in 12 % sodium hypochloride they were dyed with 0,05 % aq. Toluidine Blue and Lugol's Solution to stain protein bodies, starch grains, polyphenols and cell walls (GUTMANN 1995, modified).
The semithin sections were analysed with an Olympus BH-2 light microscope.

2.3 Quantification of Total Phenolics

After removal of the seed coat the embryos were shock frozen and freeze dried.
Samples of 5 g were ground with 20 ml hexane until the particle size was reduced to less than 0,04 mm. Most of the residual seed fat was extracted by flushing the solids with petrol ether in a Buchner funnel.
The phenolic compounds were extracted by agitating 0.5 g of the fat-free sample on ice once with 80 % aq. acetone and twice with 60 % aq. acetone (50 ml respectively). After centrifugation the supernatants were combined in a flask containing 5 ml of acetic acid. The acetone was removed by rotary evaporation under partial vacuum at 40 °C. The aqueous residue was brought to 200 ml in a volumetric flask. Total phenolics were determined by the Folin-Ciocalteu procedure (SINGLETON and ROSSI 1965, modified).

2.4 Preparation of Acetone Dry Powder (acdp)

The fat of freeze dried and ground seed material was extracted exhaustively with petrol ether in a Twisselman apparatus. The phenolic compounds were removed by agitating twice with 80 % aq. acetone and once with 70 % aq. acetone containing 5 mm sodium ascorbate. After centrifugation the solids containing the proteins and proteases were dried to an anhydrous powder (acetone dry powder, acdp) by extracting twice with 100 % acetone and subsequent removal of the solvent under vacuum.

2.5 Analysis of the Seed Proteins

Total protein content was measured by Biuret (COOPER, 1981).

In order to obtain the albumin fraction, the acdp was extracted in a low salt buffer (0.01 mol/l NaCl, 0.05 mol/l TRIS/HCl pH 7.5, 2 mmol/l Na_2-EDTA, 5 mmol/l sodium ascorbate, 5 µg/ml pepstatin A). The precipitate was extracted in a high salt buffer (0.5 mol/l NaCl, 0.2 mol/l TRIS/HCl pH 8.0, 5 µg/ml pepstatin A) to obtain the globulin fraction.

For SDS gel electrophoresis, portions of 10 mg acdp or fractions were diluted in 1.5 ml SDS sample buffer containing 0.0625 mol/l Tris/HCl, pH 6.8, 2 % SDS, 5 % mercaptoethanol, 10 % glycerine and 0.001 % Bromophenol blue.

The electrophoresis was conducted using BIO-RAD ® 10-20 % polyacrylamide gradient ready gels. The protein bands were stained with Coomassie Brilliant Blue or with silver staining methods.

2.6 Characterization of the Proteases

The aspartic endoprotease was extracted from the acdp according to HANSEN et. *al.* 1998. After centrifugation, aliquots of the supernatant were incubated at 35°C both in presence and absence of 20 µg/ml pepstatin A in McIlvaine buffer, pH 3,5, containing 4 mg/ml BSA as substrate.

The seryl exoprotease was extracted with 20 mmol/l Na_2HPO_4 containing 0,5 mol/l NaCl, 0,5 % Triton X-100 and 20 g/l Pepstatin A (pH 6,8). After centrifugation, aliquots of the supernatant were incubated both in presence and absence of 1 mmol/l phenyl methane sulfonyl fluoride (PMSF) in McIlvaine buffer pH 5,6 containing 5 mmol/l N-benzyl-oxycarbonyl dipeptides as substrates.

The proteolytic reactions were stopped by adding a solution of trichloro acetic acid to a final concentration of 4 % (w/v).

The amount of liberated α-NH_2 groups was measured in the supernatants according to SHUTOV et *al.* 1992. The activities were determined as mol α-NH_2 liberated per minute and g fresh weight.

2.7 Seed Incubation Experiment

Fermentation-like seed incubation was performed during 5 days with 1 % acetic acid in glass vessels in a water bath at 40 °C.

Every day, three seeds of each species were sampled and sliced. The pH of the intersections was measured by means of a pH electrode with a flat membrane (Mettler Toledo InLab ®).

3. Results

3.1 The Structure of the Seed Shells

The *Theobroma* seed shells species consist of two integuments, of which the outer one is much thicker than the inner. Its predominantly collapsed parenchyma contains vascular bundles and, beneath the outer epidermis, large chambers filled with slime. Significant differences between the three examined *Theobroma* species were found in the thickness of the seed shells that ranges between 385 µm (*T. cacao*) and 1670 µm (*T. bicolor*).

The parenchyma of the inner integument consists of only a few cell layers. Its outer epidermis is composed of a single layer of thick-walled, lignified cells.

The thickness of the outer epidermis of the inner integument also varies significantly between the three species. In the seed coat of *T. cacao* the epidermal cells form a sclereide layer that is about 9 µm thick. The homologue palisade layers of the two other examined species are 30 µm (*T. grandiflorum*) and 130 µm (*T. bicolor*) thick.

3.2 The Structure of the Cotyledons

The cotyledonar tissue of *Theobroma* seeds mainly consists of storage cells which contain about 60 % fat, 20 % proteins and 5-10 % starch in separated compartments.

Besides the storage cells, polyphenol cells amount to a portion of 8-11 % of the cotyledonar cells of *T. cacao* and *T. grandiflorum*. Due to anthocyanins, these cells cause the purple colour of Forastero-type cocoa seeds but they are colourless in cupuaçu seeds. Apart from a higher frequency beneath the vascular bundles, the polyphenol cells are evenly distributed in the cotyledonar tissues of *T. cacao* and *T. grandiflorum* seeds. In some sections they form small groups and rows which mainly appear close to the upper epidermis of the cotyledons.

The seed tissue of *T. bicolor* is virtually free of polyphenol cells, which appear sporadically beneath the vascular bundles.

3.3 Total Phenolics

While the phenolics of *T. cacao* and *T. grandiflorum* cotyledons amounted to 7-7,5 % of freeze-dried and defatted sample, their portion was only 0,5 % in *T. bicolor*. These results correlate with the few polyphenol cells found in *T. bicolor* seed tissue.

3.4 Seed Proteins

The patterns of seed proteins of all three *Theobroma* species are marked by two predominant globulins and at least one predominant albumin. The molecular weights of the two classes of globulins are similar for all three species (46,5 ± 1 kDa and 30,3 ± 2 kDa). Considering the quantity of globulins, the seeds of *T. bicolor* are prominent regarding their high content of the 46 kDa storage protein, while only the seeds of *T. grandiflorum* contain more albumins than globulins. However, in all three species the relative quantity of the 30 kDa globulin vary around 15 % of total protein content.

3.5 Incubation Experiment

Before incubation *T. grandiflorum* seeds showed a pH of 5,7 ± 0,3 whereas a pH of 5,9 ± 0,3 was measured at the intersections of *T. cacao* and *T. bicolor* seeds. During incubation the pH was declining in all of the species. Seed samples drawn after 4 days of incubation indicated pH values of 4,78 ± 0,1 (*T. cacao*), 4,71 ± 0,3 (*T. grandiflorum*) and 5,1 ± 0,3. (*T. bicolor*).

During seed incubation, 70% of the total protein content of T. cacao seed was degraded after two days whereas even after four days a decrease of only 15% resp. 3% was measured in *T. grandiflorum* and *T. bicolor* seed.

3.6 Proteolytic Activities

The seeds of all three examined Theobroma species contain pepstatin-A sensitive endoproteolytic activities, which gives rise to classify the respective enzymes as aspartic endoproteases. All of these activities are considerably high but the one of cocoa is twice as high as of *T. bicolor* and *T. grandiflorum* (fig.). The pH-dependency of the aspartic proteases is similar in all three species with an optimum range at pH 3.4-3.6.

In seeds of *T. bicolor* and *T. grandiflorum* we found PMSF-sensitive carboxy exopeptidases of an activity equal to the respective enzyme of cocoa seeds (Fig.). Thus, the particular enzymes of all three species are to be classified as seryl type carboxypeptidases. The pH optimum of the enzymes ranges from pH 5.4-5.7.

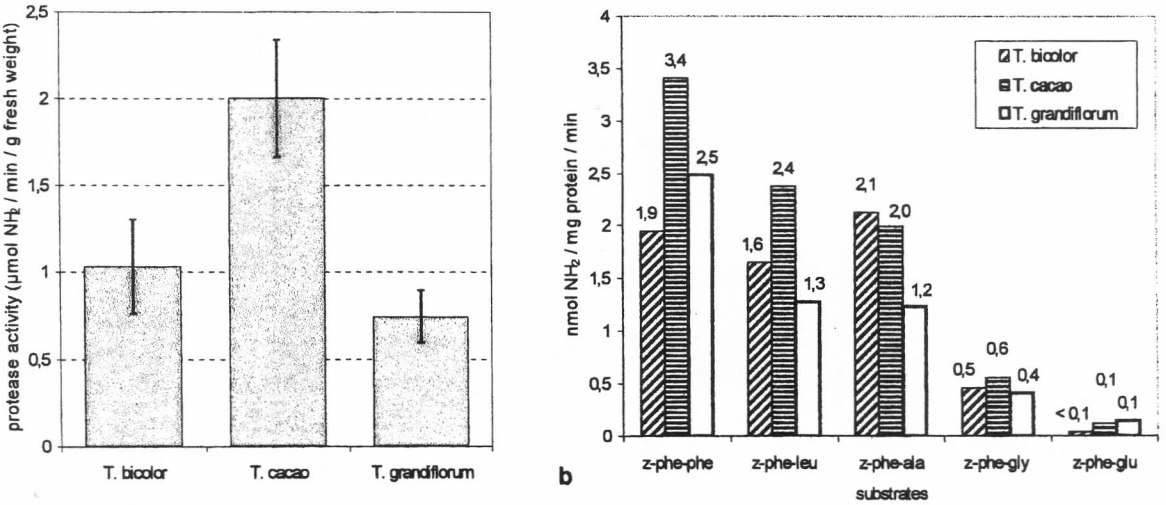

Fig: Proteolytic Activities in Cotyledons of three *Theobroma* Species
a: Pepstatin-A sensitive activity of aspartic endoproteases incubated at pH 3.5 with 4 mg/ml BSA
b: PMSF-sensitive activities of seryl carboxypeptidases incubated at pH 5.6 with different z-dipeptides

4. Discussion

Our studies show that *T. bicolor* and *T. grandiflorum* seeds generally meet the biochemical requirements for the generation of aroma precursors comparable to those of cocoa seeds. The quantitative and qualitative characteristics of the seed globulins and the cotyledonar proteases are similar in all three species. The slight differences regarding the proteolytic activities and the globulin contents are not likely to influence the potential yield of aroma precursors. Furthermore, due to broad range of intraspecific variation of *T. grandiflorum* and *T. bicolor*, there should exist proveniences with higher globulin and protease contents. Fermented cupuaçu and *T. bicolor* seeds are of a light colour and so they might be used for the manufacture of light coloured chocolates.

However, simple application of the fermentation practices known from cocoa will not yield sufficient aroma precursors, since structural differences especially of the seed shells may influence the duration in which the acetic acid infiltrates the seeds. In fact, both the decrease in pH and the generation of flavour precursors are delayed in *T. bicolor* seeds which may be due to the comparatively thick seed shell with its massive palisade layer.

Additionally, the distinct contents of phenolic compounds of *T. bicolor* seeds most likely result in a differing flavour development during fermentation.

Our results show that appropriate fermentation procedures need to be developed for the production of chocolate-like products of a high and reproducible quality from seeds of *T. bicolor* and *T. grandiflorum*. First studies are being performed to test new fermentation technologies meeting the particular requirements of seeds of *T. grandiflorum*.

5. Literature

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