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Structure and function of the seed coat of *Theobroma cacao* L. and its possible impact on flavour precursor development during fermentation

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Summary

Seed coats are known to regulate the transfer of substances between the embryo and both the mother plant and the environment. The permeability of the seed coat of the fermenting seeds of *Theobroma cacao* may significantly affect its final flavour quality. Many studies suggest that the flavour quality is genetically determined and uniform within one clone. This implies that the flavour quality can only be inherited exclusively from the mother plant. Consequently, factors affecting flavour quality should be located in the maternal tissues surrounding the seed. As only the fruit pulp and the seed coat are exclusively maternal in origin, whereas the embryo and the endosperm are the progeny of both parents, the flavour quality may be associated with transport characteristics of the seed coat. In this context the influence of the seed coat on the transport of acetic acid, the main product of fermentation is of high relevance.

We investigated the transport characteristics of the seed coat of *T. cacao* with modern light- and fluoroscence microscopic methods. Tracers were used in order to evaluate the potential impact of structures such as barriers and entry sites for the transport of water and solutes. Our morphological, histochemical and microspectrophotometrical data and the interpretation of the tracer distributions demonstrate that certain structures of the seed coat strongly influence the course of transport processes in the mature seed coat of *T. cacao*. These include the inner contact zone of fruit pulp and seed coat, hilum, sclereid layer, hypostase, micropyle and endosperm cuticle. Under natural conditions these structures may prevent desiccation and loss of nutrients of the recalcitrant seed. During fermentation the mentioned structures appear to affect the influx of acetic acid, in analogy to water and solutes. Insufficient or excessive acidification impedes sufficient formation of flavour precursors resulting in a flat or acid taste. Asynchronous and uneven infiltration of acetic acid into the cotyledon cells will lead to inconsistent and suboptimal flavour quality. In conclusion we suggest that flavour quality of the fermented seeds is predominantly due to transport kinetics of water and solutes during the fermentation process rather than a reflection of genetically coded differences in storage proteins.

Introduction

Ripe angiosperm seeds consist of embryo and endosperm or perisperm, which are surrounded by a maternal protection sheath, the seed coat. The protective function of the seed coat in mature seeds is well documented and refers to the protection of the embryo and its storage tissue against loss of water and solutes as well as against physical and biological damage (WERKER, 1997). Due to TEICHMANN and VAN WYK (1994) and CHIN and ROBERTS (1980) the prevention of desiccation is of particular importance in recalcitrant seeds: Although most recalcitrant species occur in moist tropical or aquatic habitats, their embryo is generally well protected against desiccation by the seed coat and/or the pericarp. The morphology or chemical properties of structures within the seed coat that control water transport in recalcitrant seeds have not yet been thoroughly studied. Most previous studies have examined economically important legume seeds in which the dormancy of their dry seeds is based on the impermeability of the seed coat. In recalcitrant seeds however, a completely different adaptation strategy is present, since they must retain relatively high minimum water content in relation to their environment, even in dry periods.

Nevertheless, the function of the seed coat seems to exceed that of a bare protection sheath. It represents a multi-functional structure which co-ordinates the transport of water and nutrients between the vegetative plant parts and the ripening heterotrophic seed (WOLSWINKEL, 1992). Since the vascular bundle of the seed ends in the seed coat before reaching the embryo, there is no direct vascular connection between the mother plant and the embryo or endosperm. Thus all materials that have to be transported from the maternal vascular bundle into the embryo must pass the seed coat via short-distance transport (THORNE, 1985). The seed coat might even directly affect the development of the embryo. The rate of import of water and assimilates in maturing wheat seeds is controlled by transport processes within the seed coat (WANG and FISHER, 1994) and is crucial for the growth rate of embryo and endosperm (JENNER, 1985). In *T. cacao* maternal tissues could play a similar role during seed development. In addition to developmental-physiological considerations another issue of technical importance arises regarding the control of the transfer of substances by the seed coat in seeds of *T. cacao*: The seed coat of *T. cacao* may play an important role in the formation of cocoa flavour during fermentation. Acetic acid is produced during the fermentation of the adhering fruit pulp, which then enters the seeds. In order to reach the embryo and to become effective in the formation of flavour precursors in the cotyledon tissue, acetic acid needs to pass the seed coat. Acidifying the seed is an indispensable condition for the development of flavour precursors, as numerous studies from Biehl have shown (BIEHL and ADOMAKO, 1983; BIEHL et al., 1985). Flavour comparisons demonstrate that the flavour quality of the seeds is at least partially determined by the genotype of the motherplant (CLAPPERTON, 1994b; FIGUEIRA, 1997). If this hypothesis is correct, maternal factors must be responsible for the differences in the genotypically dependent quality parameters. Since only the fruit pulp and seed coat are exclusively maternal in origin, but not the embryo or the endosperm, the flavour quality might be directly correlated with certain features of the seed coat, e.g. transport characteristics of acetic acid, reducing pulp sugars, alkaloids and polyphenolic compounds. Detailed knowledge of the morphology is an important prerequisite for the understanding of the different transport processes within the seed coat of *T.cacao*. Despite the important economic role of the seeds of *T. cacao* its morphological description is, as yet, unsatisfactory (CORNER, 1976). This applies in particular to the seed coat. It has not yet been investigated whether certain structures within the seed coat of the recalcitrant seed of *T. cacao* could represent a barrier for water in order to protect against desiccation, against physical and biological damage or loss of intracellular substances. Likewise it is not known how the short-distance transport of water and assimilates from the mother plant to the embryo takes place, considering that there are existing barriers. The role of the seed coat in terms of

limitation or promotion of the import of acetic acid and fruit pulp sugars and the export of alkaloids and polyphenolic substances during fermentation has never been shown. This study supplies a detailed description of the morphology of the seed coat of *T. cacao* with special emphasis on potential transport barriers or entrance gates for water and solutes. Potential barriers were examined histochemically and microspectrophotometrically and their practical impact was illustrated experimentally by the application of fluorescent tracers. Thus structural aspects of the seed coat were described in accordance to their functional role in transfer processes.

Material and methods

Plant material

Ripe fruits of *T. cacao* clones NA 33, EET 390 and PBC 123 were collected from the genebank Tawau, Malaysia.

Microscopy

The intact seeds were fixed according to GERLACH (1984) in 4% aqueous formaldehyde solution. Specialised structures of the seed coat such as micropyle, hilum, raphe and chalaza as well as parts of the unspecialised seed coat were selected and cut in segments. Subsequently they were dehydrated in a graded series of ethanol and embedded in LR-White Medium Grade (LONDON RESIN COMPANY). Resin embedded, semi-thin sections of 1-5 µm thickness were cut using an ultramicrotome and stained for light microscopy with toluidine blue (0.05% in aqua dest. according to GUTMANN, 1995) or methylen blue/basic fuchsin (1% in 1% sodium borate according to APARICIO and MARSDEN, 1968). The light-microscopical examinations were carried out with an Olympus BH-2 microscope. The photographs were taken with the software program Analysis 3.1 (Soft Imaging System). The fluorescence microscopy investigations were performed with the fluorescence unit BH-2-rfl (Olympus) and the photographs were taken with a camera of the type Olympus OM-2 on Ectachrome 200 ASA slide film. The following filter combinations were used: UV excitation: band elimination filter U (DM-400 + L-420), excitation filter U (UG-1), blue excitation: band elimination filter B (DM-500 + O-515), excitation filter B (BP-490), additional excitation filter EY-455, green excitation: band elimination filter G (DM-580 + O-590), excitation filter G (BP-545) and additional excitation filter EY-475.

Histochemical examination

Toluidine blue (0.05% in aqua dest. according to GUTMANN, 1995). Lignin and polyphenols show green metachromasia, pectin can be identified by its pink metachromatic reaction.

Bismarck brown/toluidine blue (5ml of a stock solution of 5g bismarck brown Y and 25g phenol crystals dissolved in 500ml aqua dest. was made up to 100ml with aqua dest. Counterstaining was executed with 0.05% toluidine blue in benzoate buffer, pH 4.0 according to GRAHAM and PRIYAVADAN, 1996). Bismark brown is replaced by toluidine blue in all cell structures except the cuticle which stains yellow to golden-brown.

Autofluorescence

During UV excitation with the use of colourless additional barrier filters, lignified or suberized cell walls as well as cell walls with esterized phenol acids show a bright blue or bluish-white fluorescence. Cutin fluoresces white (ROST, 1992).

Fluorochromes

Berberine sulphate/aniline blue (0.1% berberine hemi-sulphate in aqua dest. counterstained with 0.5% aniline blue in aqua dest. according to BRUNDRETT et al., 1988). During UV excitation lignin and the phenolic component of suberin fluoresce bright yellowish-green. Aniline blue quenches the non-specific background fluorescence of berberine sulphate and reduces the intensive fluorescence of lignin and the phenolic domain of suberin (BRUNDRETT et al., 1988). Aniline blue is a strong fluorochrome for callose in sieve plates, pit fields and new cell walls. Excited by UV light, callose fluoresces yellow (O'BRIEN and MC CULLY, 1981).

Acridine orange (0.1% in phosphate buffer, pH 6.2 according to ALCHE and RODRIGUEZ GARCIA, 1997). During blue excitation lignin fluoresces green, pectin red, callose yellowish-orange and cutin orange.

Toluidine blue/aniline blue (0.05% toluidine blue in 0.02M benzoate buffer, pH 4.4 as a counterstain and 0.05% aniline blue in 0.067M phosphate buffer, pH 8.5 according to SMITH and MC CULLY, 1978). Toluidine blue quenches the autofluorescence of lignin and polyphenolic compounds as well as the fluorescence of the non-specific binding of aniline blue to cell wall structures. During UV excitation callose in sieve plates, pit fields and new cell walls exhibit yellow fluorescence (O'BRIEN and MC CULLY, 1981).

Toluidine blue /neutral red (0.05% toluidine blue in 0.1M sodium acetate, pH 4.4 used as a counterstain and 0.1% neutral red in 0.1M potassium phosphate, pH 6.5 according to LULAI and MORGAN (1992). Toluidine blue quenches the autofluorescence of lignin and polyphenolic compounds. Neutral red is a lipidfluorochrome that during UV excitation causes cutin and the lipid domain of suberin to fluoresce yellowish and at the same time quenches the autofluorescence of lignified cell walls.

Subcellular UV-spectroscopic investigation (UMSP)

For a topochemical identification of lignified and suberized cell wall layers as well as other aromatic compounds in the tissue of the seed coat, subcellular UV-spectroscopic investigations were carried out using an UV-microspectrophotometer UMSP (Zeiss) with an integrated scanning mechanism.

The fixation and extraction of tissue samples proceeded as described above for microscopical preparation. The segments were then dehydrated in a graded series of acetone and embedded in Spurr's resin (SPURR, 1969). Semi-thin sections of 1 µm thickness were cut with an ultramicrotome and transferred to absorption-free quartz slides.

The semiquantitative lignin detection and the identification of accessory, aromatic substances were carried out through point measurements with a spot size of 1 µm during sequentially changed wavelengths in the range of 240 to 400nm. Comparison of the fluorescence intensity with a standard preparation was performed for the semiquantitative determination of a specific, aromatic substance within the examined tissue. In addition, the absorption spectra enabled the identification of further accessory, aromatic substances that were absorbing within the UV range (KOCH and KLEIST, 2001).

The distribution of these substances was determined by means of a surface scan that permits the detection of the semiquantitative distribution of an UV absorbing compound within cells or tissue components. The high resolution of this method permits the construction of a subcellular distribution pattern. The sections were

scanned at the defined wavelength of 278nm according to the absorption maximum of angiosperm lignin in digitised fields with a local resolution of 0.25 μm^2. The absorption spectra and the scanned image profile were evaluated with the spectrographic analysis programs „LAMWIN“ and „Apamos“ (Zeiss).

Tracer investigation

For the tracer investigation freshly harvested and opened fruits from the greenhouse of the Biocenter Klein Flottbek were used. Clone indications were not known for these plants.

Ammonium iron (II) sulphate according to BALLARD (1976)
A solution of iron salts stains the area of initial water entry into the seeds in black. Thus the first detectable black region on the surface of the seed coat of seeds imbibing dilute iron salt solutions precisely indicates the first point of water entry (BALLARD et al., 1973).
Half of the seeds of one fruit were released carefully from the adhering fruit pulp with the use of sawdust. Subsequently, 10 seeds each, with and without adhering fruit pulp, were put in a 0.003M aqueous solution of ammonium iron (II) sulphate at ambient temperature until a first outwardly visible black colouring of the seed coat appeared. Then the seeds underwent binocular examination.

Lucifer yellow CH according to STEWARD (1978)
Lucifer yellow CH (LYCH) is a suitable apoplastic tracer due to its lipid insolubility (distribution coefficient toluol:buffer $7 \cdot 10^{-11}$:1) and its high dissociation constant at physiological pH ($pK_a \leq 0.7$). The low distribution coefficient and the high dissociation constant at physiological pH cause impermeability of biological membranes to LYCH. Thus this tracer marks the apoplastistic transport route in the tissue along hydrophilic paths. Additionally LYCH has high fluorescence intensity and is stable between a pH of 1 to 10.
A further advantage is its ability to be bound by aldehyde fixative to the tissue and to retain its fluorescence after dehydration and embedding (STEWARD, 1981). According to CANNY (1889) and STRUGGER (1939; in CANNY, 1986a) the following sites show an accumulation of apoplastic tracers:

1. At apoplastic barriers, where water is removed, i.e. taken up into the symplast, the tracer can not follow due to membrane filtration. In the absence of water only a moderate secondary diffusion of the tracer occurs (CANNY, 1989).
2. When the pathway of water and tracer are identical, i.e. take course in the apoplast, the tracer marks the pathway with the lowest resistance and the highest flow volume of water (STRUGGER, 1939; in CANNY, 1986a). Thick cell walls allow a higher flow volume than thin walls.

Six seeds with adhering fruit pulp of freshly harvested, mature fruits were inserted into a 3% LYCH solution in aqua dest. at ambient temperature, so that the seeds were completely covered. After 30min, 5h and 34h two seeds each were taken out of the solution.
A further 16 seeds were transferred into narrow glasses filled, up to a height of 1cm, with the LYCH solution. Half of these seeds were placed with the broader end containing micropyle and hilum into the colouring solution, the other half were placed with the chalaza into the solution. After 30s, 2min, 5min and 10min two seeds of each orientation were taken out of the solution. After infiltration of the tracer solution the seeds were fixed in 4% formaldehyde in 0.1M sodium phosphate buffer at pH 7.4 at ambient temperature for 24 hours and washed in buffer. The tissue samples were cut, dehydrated in a graded series of ethanol and embedded in LR White. During blue excitation LYCH exhibits yellowish-green fluorescence.

Sulphorhodamine G according to CANNY and MC CULLY (1986b)
Sulphorhodamine G (SR) is water-soluble and has a pKa of 4.9 (CANNY, 1988). At physiological pH SR is dissociated and functions as an apoplastic tracer (CANNY, 1986a). The uptake of SR into cells is pH dependent. If the pH is lowered, SR becomes undissociated. It then crosses the plasma membrane, is taken up and accumulates in the symplast. This occurs at pH 6.5 or below (CANNY, 1988). Between a pH of 6.5 and 3.5 SR acts as an apoplastic and symplastic tracer. If water is taken up into the symplast, e.g. due to apoplastic barriers, the tracer follows. The accumulation of the tracer within cells may indicate either the presence of apoplastic barriers for water or local acidifying e.g. by proton pumps. An apoplastic accumulation of the tracer marks those cell walls having high flow volume for water and solutes. The pathway of SR in acid solution serves as a model for acetic acid penetrating into the seed coat of *T. cacao* during fermentation. Acetic acid is likewise polar and has a similar pKa of 4.74 although a smaller molecular weight.
0.1% solutions of SR in aqua dest. adjusted with acetic acid to pH 6.5, 5.5, 4.5 and 3.5 were prepared. The solutions with pH 3.5, 4.5 and 5.5 were heated up to 45°C and maintained at this temperature, in order to simulate the conditions during fermentation. The solution at pH 6.5 was held at ambient temperature. Subsequently the seeds were completely covered in the solutions for 30min and stirred constantly. In order to preserve the distribution of SR *in vivo* in the tissue and prevent a secondary diffusion, a freeze substitution with following, anhydrous dehydration in acetone and embedding in Spurr's resin according to CANNY and MC CULLY (1986b) was performed. The embedded segments were stored in sealed plastic containers with desiccant. Semithin sections were cut and transferred to slides without the use of water and immediately examined in fluorescence-free immersion oil.
During green excitation SR shows red fluorescence.

Results and discussion

Habitus of the seed

The seeds of *T. cacao* derive from a bitegmic, anatropous ovule (CORNER, 1976). They are 1.5 to 3cm long and 1-1.5cm wide and have a flat, ovoid form with a strongly curved side and a somewhat less strongly curved side. The seed is surrounded by the fruit pulp, which adheres firmly to the seed coat (Fig. 5A). The hilum, recognisable as a horseshoe-shaped scar, is free of fruit pulp and is located at the broader end of the seed. Below the hilum, the micropyle is visible as circular hump (Fig. 5B). At the strongly curved side the main vascular bundle of the raphe runs from the hilum to the chalaza, which is located at the pointed end of the seed. The main vascular bundle of the raphe ends here in a plexus forming approximately 15 postchalazal branches that run back almost all the way up to the micropyle (Fig. 1). In the centre of the chalaza the hypostase is discernible as a small, brown mark.

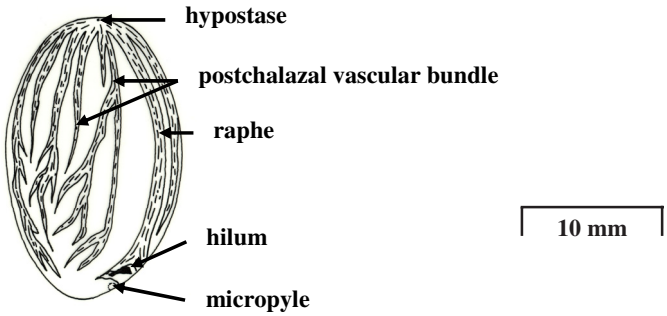


Fig. 1: Habitus of the seed of *T. cacao* (modified according to CHIN and ROBERTS, 1980).

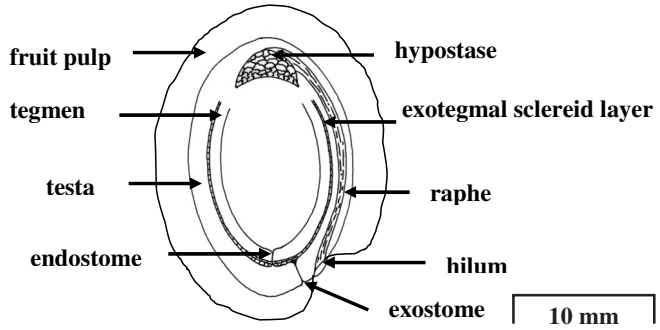


Fig. 2: Schematical longitudial section of the seed of *T. cacao.*

Structure of the seed coat

The seed of *T. cacao* is bitegmic with an outer testa and an inner tegmen. In order to avoid the ambiguity that arises when the integuments of the ovule and the seed are called by the same name, CORNER (1976) referes to the product of the outer integument in the seed as testa and that of the inner integument as tegmen. For the present study we applied CORNER's terminology (1967), as above. The seed coat is multilayered and forms a relatively complex structure. The testa is thicker than the tegmen. The results of our morphological, histochemical and microspectrophotometrical investigations and the interpretation of the tracer distributions demonstrated that there are certain structures within the seed coat of *T. cacao*, which

- inhibit or promote the import and the distribution of water and solutes
- have an ecological function in desiccation protection of the recalcitrant seed
- exert an influence on the process of fermentation.

These include the hilum, the inner contact zone of fruit pulp and seed coat, sclereid layer, hypostase, micropyle and endosperm cuticle.

The hilum: First entry site of aqueous solutions into the seed coat

The hilum is composed of the scar formed by abscission of the seed from the funiculus with the severed vascular bundle in its centre. The hilum of *T. cacao* is free of outer testal epidermis, adhering fruit pulp and hypodermal mucilage (Fig. 5A-C). The injured vascular bundle remains unprotected and represents a potential entry site. After a brief period of immersion in the ammonium iron (II) sulphate rich solution the intact seeds and the seeds that were freed of fruit pulp showed a black colouration in the region of the hilum and the area of the injured vascular bundle (Fig. 5D). The black colouration continued along the main vascular bundle.

During the application of LYCH to the broader end of the seed that contains the hilum and micropyle, a visible fluorescence appeared in the xylem walls of the raphe (Fig. 8F) as well as in the testal parenchyma up to the sclereid layer after merely 30s. Increasing the exposure time lead to an amplification of the fluorescence. The fluorescence in the cell walls of the xylem is most likely caused by water uptake into the vascular lumen, while the tracer is accumulated in the cell walls. The accumulation proceeds evenly along the cell walls of the testal parenchyma indicating that the onward transport in the testal parenchyma runs apoplastically.

With the apoplastic and symplastic tracer SR, a strong uptake into the symplast of bundle sheaths like cells around the vascular bundles takes place. Within these cells a precipitation of SR in the form of red, intracellular deposits is demonstrated, which, according to CANNY (1986a) signifies a particularly strong accumulation of the tracer. These cells may play a role in the temporary uptake of solutes in association with a decrease in the pH due to the high activity of proton pumps.

All results from the tracer experiments indicate that the hilum represents the site of initial water entry into the seed coat. This takes place through the injured vascular bundle after merely 30s. Water and solutes may be transported further on along the main vascular bundle whereas the short distance transport up to the sclereid layer runs apoplastically.

The fruit pulp: Delay of penetration of aqueous solutions by retention due to swelling and osmotic water binding

The firmly adhering fruit pulp surrounds the entire seed coat except for the hilum (Fig. 5A and B). The metachromatic pink colouration with toluidine blue shows the presence of acid polysaccharides, most likely pectin (O'BRIAN, 1964).

With ammonium iron (II) sulphate the first black colouration of the seeds released from fruit pulp occurred after 5 minutes (Fig. 5D) and in intact seeds with attaching fruit pulp after only 30 minutes. During the application of LYCH, the cell walls of the fruit pulp accumulated the tracer heavily. A particularly strong accumulation of the tracer took place in the cell walls of the inner fruit pulp epidermis within the periplasmatic region, which is located between the plasma membrane and the cell wall (Fig. 5E).

With the apoplastic and symplastic tracer SR the highest fluorescence intensity occurred in the cell walls and the cell lumina of the fruit pulp epidermis adjacent to the seed coat (syn. inner fruit pulp epidermis). In the area between cell wall and cytoplasm of the fruit pulp epidermis cells a particularly high concentration of SR leads to its precipitation which can be visualized as red deposits (CANNY and MC CULLY, 1986b). A somewhat smaller fluorescence intensity was demonstrated, in descending order, in the cell walls and cell lumina of the inner fruit pulp epidermis and in the cell walls and cell lumina of the fruit pulp.

Due to the strong swelling capacity of the mucilaginous fruit pulp and the propensity of free pulp sugars to accumulate water via osmosis, there was retention of considerable amounts of both tracers in the cell walls of the fruit pulp, in particular in its inner epidermis. This applies particularly to the periplasmatic area between plasma membrane and cell wall, a mucilage rich area where mucilage is accumulated after its production (WERKER, 1997). Thus the fruit pulp and in particular its inner epidermis retards the penetration of aqueous solutions into the seed coat. This process of apoplastic water

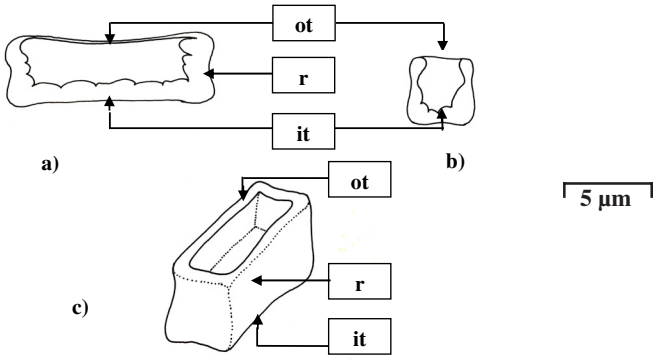


Fig. 3: Schematic construction of an exotegmal sclereid of the sclereid layer in the seed coat of *Theobroma cacao.* **a)** Longitudial section; **b)** Cross section; **c)** Three dimensional construction.
ot: unthickened outer tangential wall; **r:** thickened radial wall; **it**: thickened, inner tangential wall

retention leads to a delay of water transport into the seed coat and may help to protect the desiccation-sensitive seed of *T. cacao* against evaporation.

The sclereid layer: a hygroscopic cell layer with storage and distribution function for water and solutes

The outer epidermis of the tegmen is composed of small, cuboid or shortly oblong sclereids with thickened, strongly pitted radial and inner tangential walls (Fig. 5F-H).

The greenish-blue colouring with toluidine blue showed the presence of small quantities of lignin in all thickened cell walls. During UV microspectrophotometrical investigations the absorption spectra of the wall thickenings of the radial and tangential walls of the sclereid layer correspond to the typical spectrum of angiosperm lignin. The lignin content of the sclereid layer averages approximately one third of that of the lignified secondary walls of a xylem cell of the seed coat of *T. cacao* (Fig. 4).

As shown by the application of LYCH, the sclereid layer does not represent an apoplastic barrier for the tracer solution, since the tracer penetrates unhindered into – and then diffuses laterally within the cell walls of the sclereid layer, as seen after 30min of application (Fig. 5H). The strong accumulation of the tracer in the cell walls of the sclereid layer with particularly high concentrations in the radial central lamella is due to the large cell wall volume, which enables a high flow volume per time unit. The high fluorescence intensity in the central lamella of the radial walls indicate a particularly high flow rate of the tracer solution within these walls and illustrate the direction of transport (Fig. 5H). In addition, the cell walls of the sclereid layer appear to represent a site of water removal by uptake of water into the symplast. This explains the particularly strong fluorescence intensity in the cell walls of the sclereid layer.

The onward transport of water and solutes from the sclereid layer is assumed to proceed symplastically through plasmodesmata within the inner tangential walls of the sclereid layer up to the inner tegmal epidermis. The hardly existing cell wall fluorescence in the tissue below the sclereid layer suggests such a symplastic pathway.

With the apoplastic and symplastic tracer SR the sclereid layer showed a weak fluorescence in its cell walls and cell lumina. This indicates that SR as well as water is at least partly taken up into the symplast of the sclereid layer and transported circumferentially in it through plasmodesmata. The weak cell wall fluorescence of the sclereid layer and the underlying tegmal parenchyma confirms the predominantly symplastic pathway from the sclereid layer up to the inner tegmal epidermis. However, since no symplastic connection is present between maternal and embryonal tissue, the cells of the inner tegmal epidermis must release water and solutes into the apoplast.

Although lignification of cell walls may change their water conductivity, the water absorbency depends on the particular characteristics and quantity of the remaining cell wall matrix (FREY WYSSLING, 1976). The weak lignification of the cell walls of the sclereid layer permits the presence of large quantities of remaining cell wall matrix with high hydration capacity.

The distribution of both tracers supports the assumption that the sclereid layer temporarily stores and circumferentially redistributes water and solutes around the entire seed surface. The even distribution of tracer within the cell walls of the sclereid layer suggests that water and solutes are rapidly transported in its cell walls and symplastically distributed through the large number of plasmodesma in the radial walls (Fig. 5G and H). The water reservoir in the cells of the sclereid layer may represent a micromorphological strategy for the protection of recalcitrant seeds against desiccation in dry periods as well as a significant functional adaption for a more efficient tranfer of nutrients to the embryo. For very large recalcitrant seeds, such as for the seeds of *T. cacao* it is often difficult to achieve a sufficient supply of nutrients (VON TEICHMANN and VAN WYK, 1994). In young seeds the sclereid layer is assumed to distribute water and solutes to the micropyle and particularly to the chalaza for their uptake into the endosperm or cotyledon tissue. The water retention and rapid distribution within this layer could additionally serve for the accumulation of surplus, during phloem unloading at seed maturation obtained water.

In the literature the sclereid layer of *T. cacao* is refered to as an

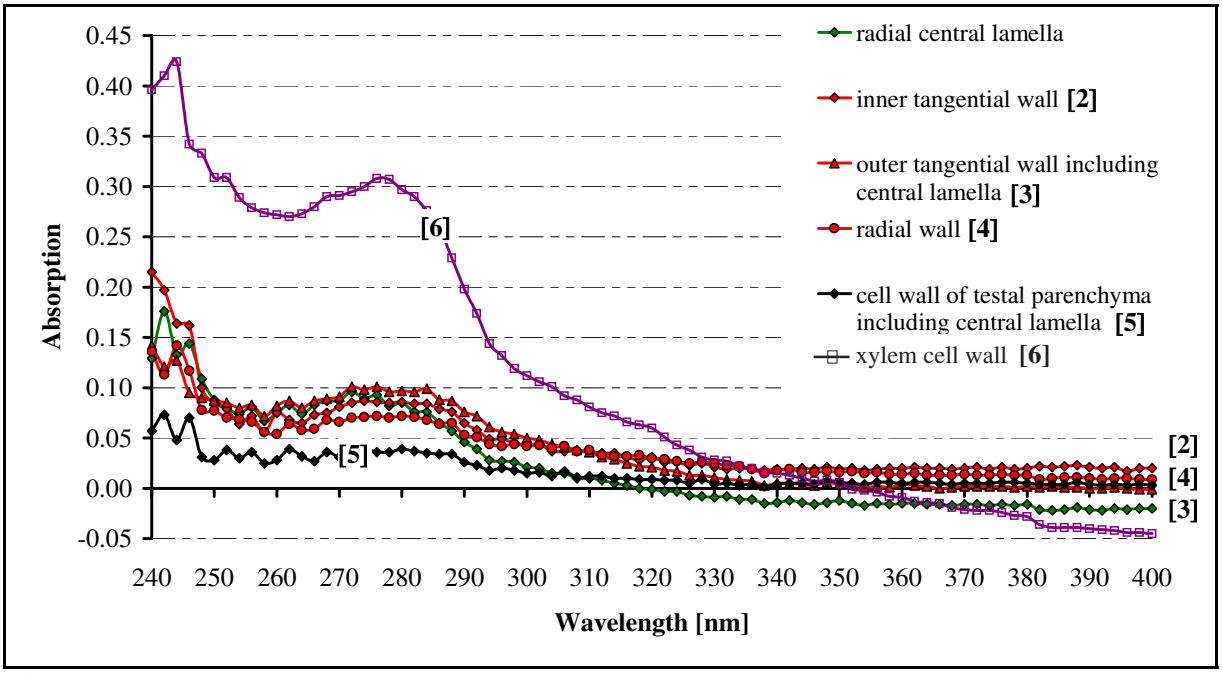


Fig. 4: UV absorption spectra of the cell walls of the sclereid layer compared to that of the xylem cell wall.

exotegmal mechanical hard layer (CORNER, 1976). Within the Sterculiacae a progressive reduction of the hard layer, from a palisade of radially elongated cells to short sclereid cells, occurred in the species *Sterculia* and *Theobroma* (CORNER, 1976). These alterations may represent an ecological adaptation to fruit type or seed spreading mechanism. Contrary to *T. bicolor*, whose exotegmal hard layer is developed as a palisade layer, the fruits of *T. cacao* remain on the mother plant after ripening (YOUNG, 1994) and the protective function of the seed of *T. cacao* is taken over by the firm, fibrous pericarp. The function of the sclereid layer of *T. cacao* therefore may have evolved from mere mechanically reinforcement, such as it is the case in *T. bicolor*, to a layer that provides important water retention and distribution, as indicated in the present study.

The hypostase: Transport tissue in ripening seeds, apoplastic and symplastic barrier in mature seeds

The main flow of nutritions into the maturing seed proceeds via the chalaza through the main vascular bundle of the raphe to the embryo and endosperm (BOESEWINKEL and BOUMAN, 1984). A symplastic continuity of the maternal tissue exists at the chalaza, as in this area no differentiation into integuments, inner cuticle and sclereid layer is present. The tissue underneath the main vascular bundle is developed as collenchyma with wall thickenings between cells and intercellulars for mechanical reinforcement (Fig. 8A). Below the collenchyma a semi-circular hypostase is present as a multilayered tissue of small, compactly arranged cells without intercellulars. The hypostase can be subdivided into three sections (Fig. 8A and B):

1. The upper section consists of collenchyma cells with markedly thickened, weakly lignified cell walls that contain large amounts of hydrophilic wall matrix and corresponding lumina that are filled with phenolic compounds.
2. The middle section contains cells with heavily lignified, suberized and lightly thickened cell walls, the lumina of which are filled homogeneously with phenolic compounds.
3. The lower section consists of small cells with folded, heavily lignified, suberized and unthickened cell walls. The arrangement of these cells resembles the close fit of pieces in a puzzle that fit without gaps into one another, resulting in a compact tissue (Fig. 8B). All cell lumina are filled homogeneously with phenolic compounds. The inner cells that are directly apposed to the endosperm consist of transfer cells with finger-like wall projections that amplify the plasma membrane surface (Fig. 8D). The presence of these cells clearly specialised to enhance solute transfer shows that in young seeds, the hypostase provides the function of transfer of substances between embryonal and maternal tissue (WERKER, 1997), where no symplastic connection exists.

In the upper section the green-blue colouring with toluidine blue showed the presence of small quantities of lignin in the thickened cell walls of the collenchyma. The results of the microspectrophotometrical examination confirm this finding of light lignification (Fig. 6). The red colouring of the cell walls with methylen blue/basic fuchsin demonstrated the presence of high amounts of hydrophilic wall matrix. The violet colouring of the cell walls of the middle section, using the same colouring process, suggests that these cell walls posses hardly any hydrophilic wall matrix (Fig. 8B).

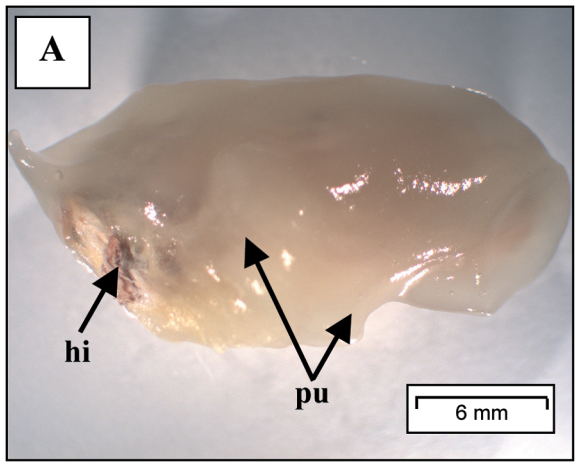
In the middle and lower section a heavy lignification of the cell walls of the hypostase was established with toluidine blue in form of green-blue colouring. The results of our microspectrophotometrical investigation emphasize the extent of these lignifications. In the secondary walls of the middle and lower section of the hypostase we measured extremely high absorption values which corresponded to approximately the ninefold quantity of lignin of the cell walls of the sclereid layer and three times the amount of that of the cell walls of the xylem (Fig. 6). The cell walls of these sections exhibit the bright bluish-white autofluorescence typical for lignin (Fig. 8E) (ROST, 1992).

With berberine sulphate/aniline blue the inner wall region adjacent to the plasma membrane in the middle and lower sections of the hypostase fluoresces whitish; the outer region of the secondary wall shows the yellowish green fluorescence typical for lignin. On the basis of their different emission colours it was suggested that, in addition to lignin, the phenolic component of suberin is present. Toluidine blue/neutral red revealed a thick, bright yellowish-white lipophilic layer between cytoplasm and cell wall, corresponding to the area where the whitish fluorescence detected with berberine sulphate/aniline blue was present (Fig. 8C and D). We used toluidine blue/aniline blue to exclude that this was due to the presence of callose. The whitish fluorescent layer that was visualized with berberine sulphate/aniline blue is interpreted to correspond to the phenolic component of the lipophilic substance, which was established with toluidine blue/neutral red (Fig. 8C). Thus this supports that the lignified cell walls of the middle and lower sections of the hypostase are equipped with a suberin lamella. The results of the acridine orange colouring confirm exactly the outcome of the berberine sulphate/aniline blue method.

Examination of the cell walls of the middle and lower sections of the hypostase with microspectophotometry showed an additional shoulder in the wavelength range between 310 and 320nm, in additon to the spectrum that is typical for lignin (Fig. 6). This suggests the impregnation of the lignified cell walls with suberin. The formation of carbonyl groups or conjugated double bonds between lignin and suberin leads to highly condensed, aromatic compounds that absorb at higher wavelengths. Thus absorption spectra that have a characteristic shoulder in the range of about 330nm occur. NOLDT et al. (2001) described this phenomenon for delignified suberin from *Quercus suber*. Due to the dissolution of 0.25µm, only superimposed spectra of lignin and suberin could be measured. The absorption

Fig. 5: **A:** Seed surrounded by strongly adhering remains of fruit pulp. The region of the hilum is free of pulp.
B: Seed with adhering fruit pulp showing hilum and micropyle.
C: Longitudinal section of the hilum with main vascular bundle. The frame shows the ending of the outer epidermis of the seed coat.
D: Seed coat with black colouration in the region of the hilum and the area of the injured vascular bundle after 5min of immersion in ammonium iron (II) sulphate solution.
E: Longitudinal section of fruit pulp and testa after 30min of application of LYCH, blue excitation. The cell walls of the inner fruit pulp epidermis within the periplasmatic region accumulated the tracer heavily.
F: Cross section of the seed coat with cuboid sclereids with thickened radial and inner tangential walls, toluidine blue.
G: Longitudinal section of the seed coat with strongly pitted oblong sclereids, toluidine blue.
H: Longitudinal section of the sclereid layer after 30min of exposure to the apoplastic tracer lucifer yellow CH, blue excitation. This Fig. shows the strong accumulation of the tracer in the cell walls of the sclereid layer with particularly high concentrations in the radial central lamellae.

A: artefacts produced by dissecting needles; **ab:** area of abscision with the severed main vascular bundle; **e:** endosperm; **ep:** inner fruit pulp epidermis; **hi:** hilum; **iet:** inner testal epidermis; **oet:** outer testal epidermis; **mi:** micropyle; **ph:** phloem; **pl:** plasmodesmata; **pte:** testal parenchyma; **pu:** fruit pulp; **pvb:** postchalazal vascular bundle; **ph:** phloem; **ra:** main vascular bundle of the raphe; **scl:** sclereid layer; **te:** testa; **tg:** tegmen; **x:** xylem.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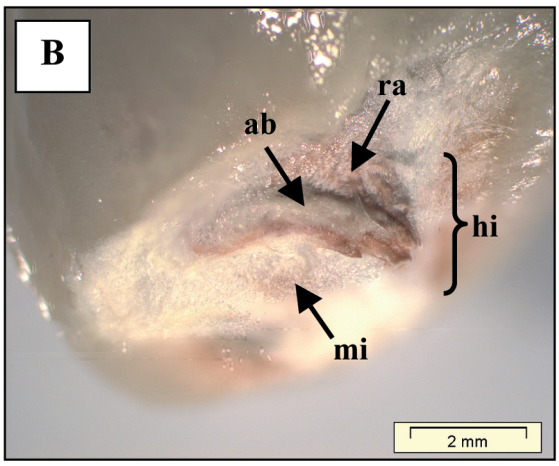
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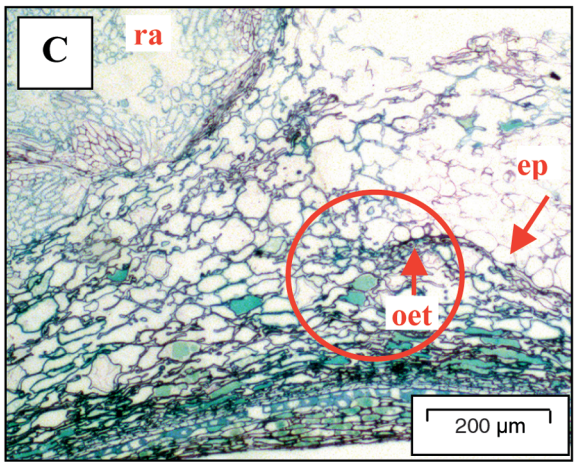
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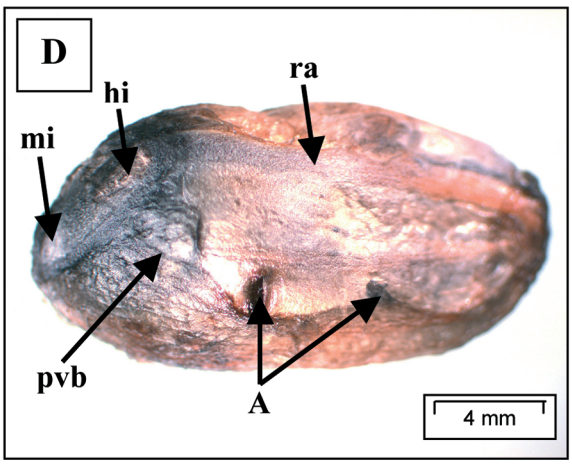
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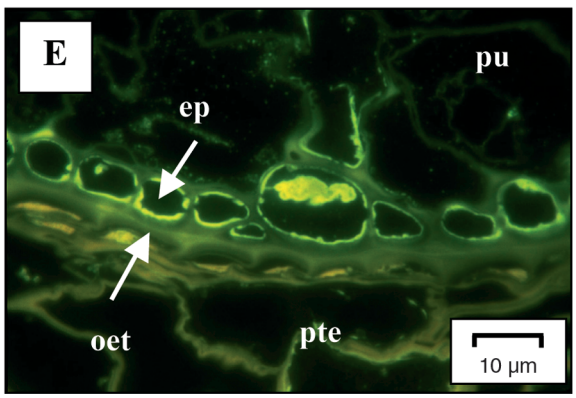
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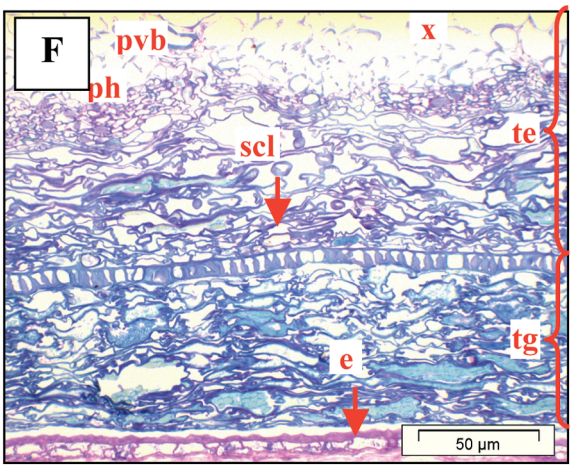
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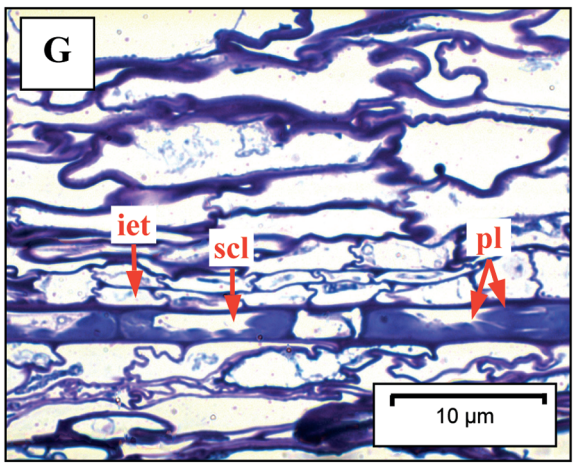
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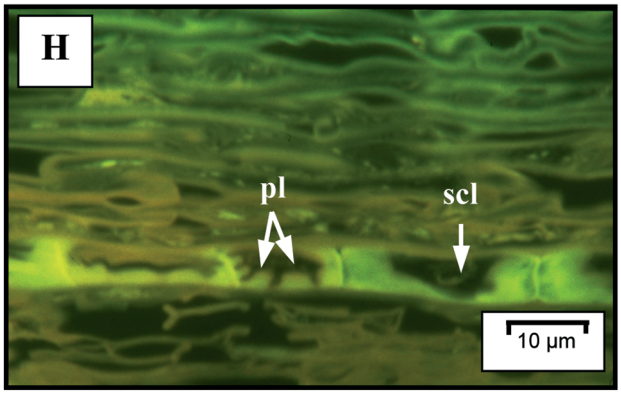
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spectra of the secondary wall of the middle and lower cell sections that we measured showed such an overlay of spectra with a maximum and minimum typical for angiosperm lignin, an abrupt descent of the curve between 282 and 298nm as well as an additional shoulder with a maximum around 330nm (Fig. 6).

The cell lumina of the hypostase are homogeneously filled with phenolic compounds, which could be shown due to their green colouring with toluidine blue. The results of the microspectrophotometry established that two different material classes were present. One of them did not show aromatic properties; the other could be defined as a polyphenolic compound with extremely high absorption values at 278nm (Fig. 7).

The surface scanning profile at 278nm shows both: the local distribution of large quantities of lignin and the ubiquitous occurrence of lignin or other polyphenolic compounds, including the phenolic component of suberin in all cell walls and cell lumina of the middle and lower section of the hypostase. The high absorption values demonstrated the magnitude of the lignification and impregnation of the cell walls and established the inclusion of extremely large quantities of polyphenolic compounds in the cell lumina (Fig. 8G).

The application of the apoplastic tracer LYCH lead to a marked accumulation of the tracer in the hydrophilic cell walls above the middle section of the hypostase (Fig. 8F). The lignified, suberized cell walls in the upper layer of the middle section of the hypostase represented a semipermeable, apoplastic barrier for the tracer solution. There was no fluorescence in the cell walls of the middle and lower section of the hypostase due to the selective exclusion of the tracer. Heavy lignification of the cell walls of the middle section prevented the transport of tracer into these cells because a strong lignification reduces the permeability of the cell walls for solutes until it corresponds to that of the plasma membrane (CLARKSON, 1974; in COCHRANE, 1983). Following the removal of water the tracer in the lightly lignified cell walls concentrates in the upper section of the hypostase, forming a „sump“ (Fig. 8F) (CANNY, 1989). Due to the suberin lamella, solutes that have been taken up into the symplast can not leave the symplast again. During the application of LYCH to the chalaza none of the selected application times produced any fluorescence in the middle and lower section of the hypostase.

The entire area of the hypostase showed negligible fluorescence with the apoplastic and symplastic tracer SR. The tracer becomes lipophil at lower pH and is then taken up into the symplast. This explains the particularly low concentration of SR in the cell walls. On the other hand polyphenolic compounds in the cell lumina bind to proteins and prevent all symplastic transport in mature seeds (COCHRANE, 1983) which may be responsible for the absent, intracellular fluorescence.

Our investigations and the results thereof establish that in mature seeds of *T. cacao* the hypostase functions as a barrier tissue against the loss of water and nutrients from embryo and endosperm. Being located between the end of the main vascular supply and the embryo, the hypostase of the mature seed of *T. cacao* runs across the main pathway of water and nutrients and functions as a seal of the chalazal opening. This apoplastic barrier completely blocks off any apoplastic backflow of solutes from the endosperm and may represent a protection strategy of the not desiccation-tolerant embryo of *T. cacao* against the loss of water.

The barrier is build of:

- heavily lignified cell walls with significantly reduced permeability for water (Fig. 6) (COCHRANE, 1983).
- a layer of suberin between the plasma membrane and the lignified cell wall (Fig. 8C and D).
- the inclusion of extremely large quantities of polyphenolic compounds in the cell lumina (Fig. 7 and 8G).

The overall distribution of extremely large quantities of lignin in the cell walls of the middle and lower section of the hypostase reduces the permeability of the cell walls for water and solutes considerably (CLARKSON, 1974; in COCHRANE, 1983). However, the heavy

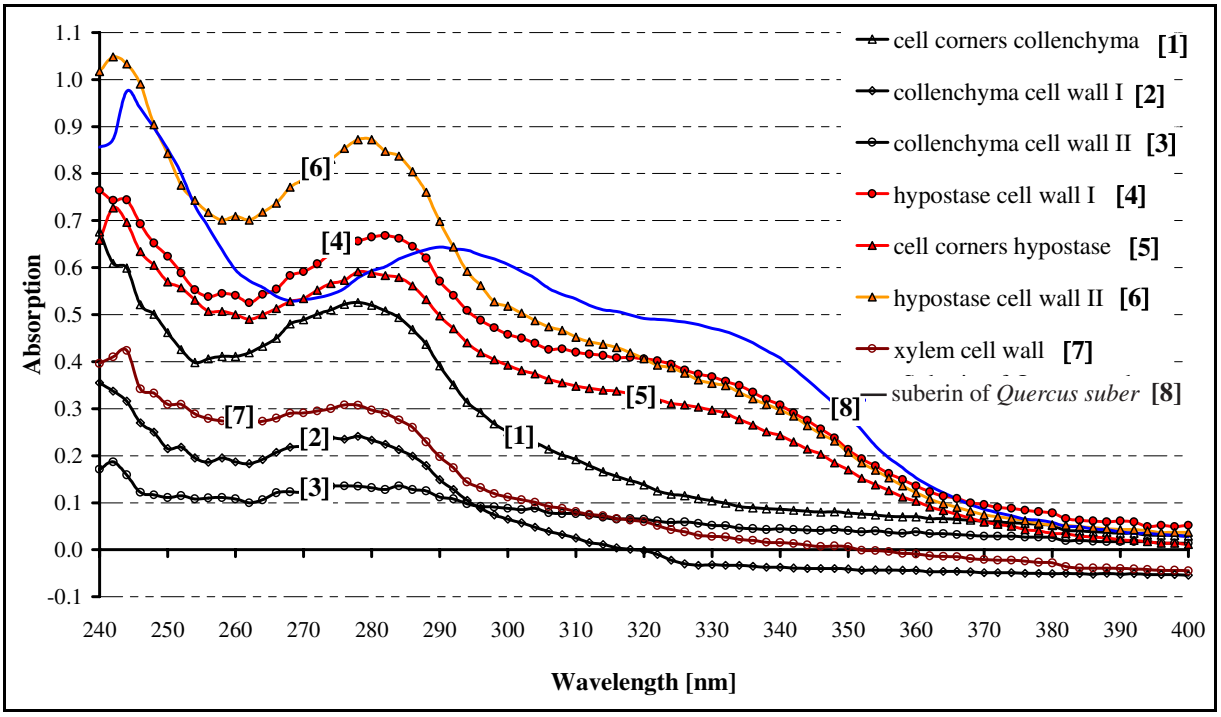


Fig. 6: UV absorption spectra of the cell walls of the hypostase compared to that of a xylem cell wall and the reference spectra of delignified suberin from *Quercus suber.*

lignification of the cell walls only occurs in mature seeds, which remain connected to the mother plant through the funiculus in *T. cacao*. The mature seed is physiologically separated from the mother plant by the hypostase, which functions as a barrier, so that a return flow of substances into the sink area of the mother plant is prevented. The function of the suberin lamella consists in the separation of symplast and apoplast in regions where a strong flux of water and assimilate need to occur in close proximity and limited space (CANNY and MC CULLY, 1986b). Solutes can not exit the symplast of suberized cells once uptake has taken place. Water and assimilates may still reach the seed via symplastic transport while water proceeds within the apoplast of the chalazal tissue in the opposite direction (COCHRANE, 1983). This may have profound implications for young seeds with less heavily lignified cell walls of the hypostase.

The ubiquitous occurrence of extremely large quantities of polyphenolic compounds in all cell lumina of the middle and lower sections of the hypostase contributes to its function as a barrier tissue. The degree of permeability of seed coats for water is directly correlated with their content of polyphenolic compounds (MARBACH and MAYER, 1974) and the oxidation in the seed coat is thought to be responsible for its water impermeability (WERKER, 1997). If, in mature seeds, the polyphenol-enclosing membranes of cells of the hypostase break down, the polyphenolic compounds thus released precipitate proteins and terminate all symplastic transport (COCHRANE, 1983). The hypostase then fulfils its function as symplastic barrier and terminates any further embryo growth (SOFIELD et al., 1977; in COCHRANE, 1983).

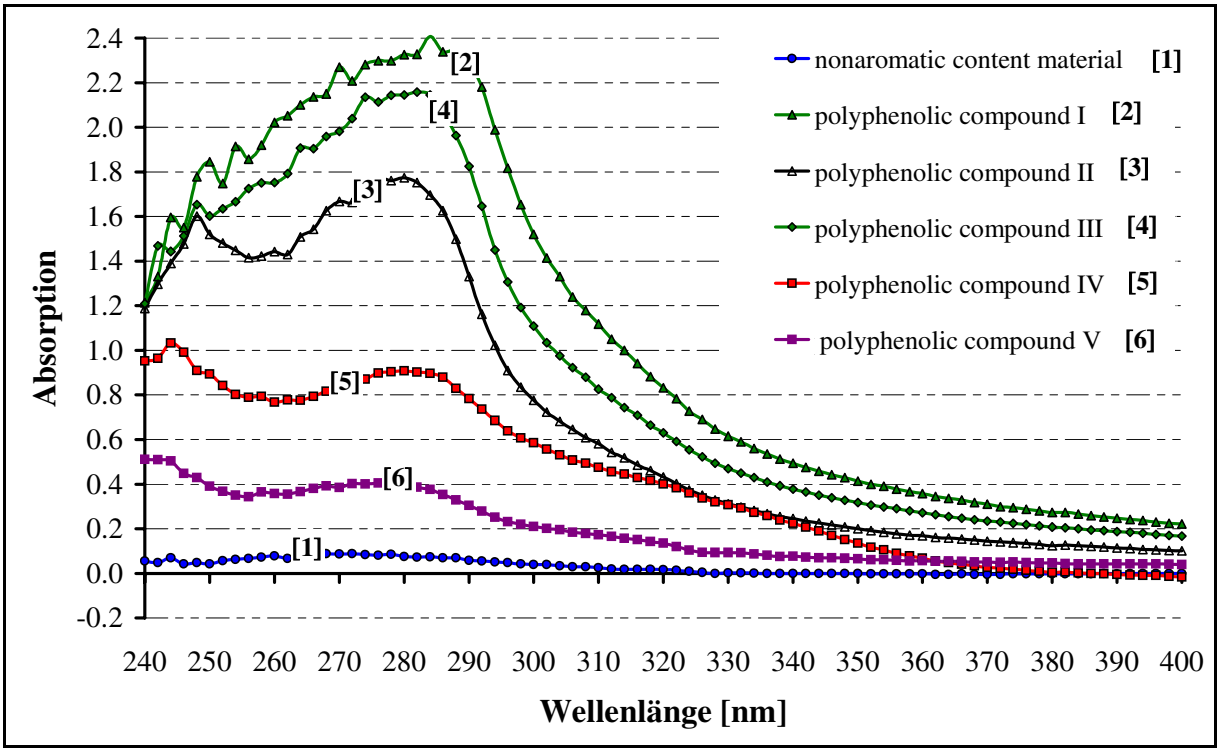


Fig. 7: UV absorption spectra of the cell content materials of the cells of the hypostase.

Fig. 8: **A:** Cross section of the chalaza showing the hypostase, methylene blue/basic fuchsin. The cell walls of the upper section contain large amounts of hydrophilic wall matrix (red) whereas the violet colouring of the cell walls of the middle section suggests that these walls posses hardly any hydrophilic wall matrix.

B: Detail of the hypostase, methylene blue/basic fuchsin. Phenolic compounds stain blue. The lumina of the cells of the middle and lower section of the hypostase are filled with phenolic compounds.

C: Cross section of the upper and middle section of the hypostase showing the heavily lignified, suberized and lightly thickened cell walls of the middle section, neutral red/toluidine blue, UV-excitation.

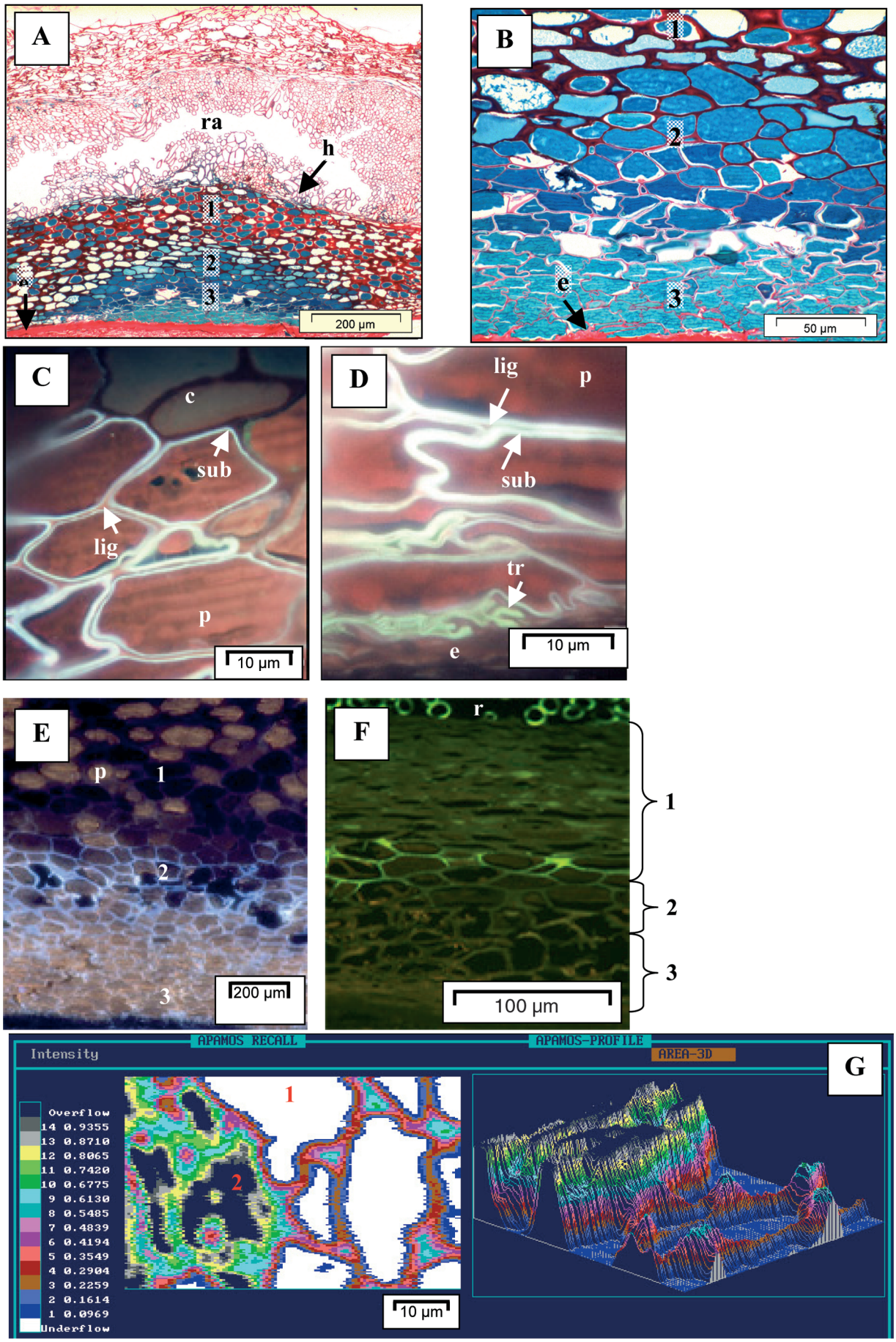
D: Cross section of the lower section of the hypostase showing the heavily lignified, suberized and unthickened cell walls of the lower section. The inner cells directly apposed to the endosperm consist of transfer cells, neutral red/toluidine blue, UV-excitation.

E: Cross section of the hypostase showing the blue autofluorescence of the lignified cell walls of the middle and lower section of the hypostase and the copper-coloured autofluorescence of phenolic compounds in the cell lumina, UV-excitation.

F: Longitudinal section of the chalaza with hypostase after 30min of application of LYCH showing a marked accumulation of the tracer in the hydrophilic cell walls above the middle section of the hypostase, blue excitation.

G: Scanning-micrograph and 3D profile at 278nm of a cross section of collenchyma and hypostase, showing the great extent and ubiquitous distribution of lignin and other phenolic compounds in cell walls and lumina of the hypostase.

e: endosperm; **c:** collenchyma; **lig:** lignified cell walls; **p:** cell lumina filled with polyphenolic compounds; **ra:** main vascular bundle of the raphe; **sub:** suberin lamella; **tr:** transfer cell; **1:** outer section of the hypostase; **2:** middle section of the hypostase; **3:** inner section of the hypostase.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G
APAMOS RECALL
APAMOS-PROFILE
AREA-3D
Intensity
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14 0.9355
13 0.8710
12 0.8065
11 0.7420
10 0.6775
9 0.6130
8 0.5485
7 0.4839
6 0.4194
5 0.3549
4 0.2904
3 0.2259
2 0.1614
1 0.0969
Underflow
1
2
10 µm

Endosperm with cuticle as semipermeable, apoplastic barrier in matures seeds

In mature seeds of *T. cacao* the endosperm consists of one or more cell layers of small cells with lightly thickened outer walls, which adhere to the seed coat (Fig. 5F) and invert into the numerous foldings of the cotyledon tissue. The enlargement of surface area between embryo and endosperm by rumination of the cotyledon tissue may assist in improving the water and nutrient uptake of the embryo (WERKER, 1997). In the area of the micropyle the endosperm consists of many cell layers. At the chalaza the endosperm is tightly compressed and only the cell walls remain visible (Fig. 8A and D).

On histochemical examination the bismack brown/toluidine blue stain revealed a thick cuticle on the outer epidermis of the endosperm with its yellowish-brown colouring (Fig. 10A). This was confirmed by its yellowish fluorescence with toluidine blue/neutral red. In the area of the chalaza an endosperm cuticle could not be detected. Whether an endosperm cuticle is present in the area of the micropyle, such as it is the case in grapefruit seeds (DAVIS and KOLATTUKUDY, 1980), or whether it is indeed absent in that area, such as in barley (COLLINS, 1918), requires further investigation.

With berberine sulphate/aniline blue and toluidine blue/aniline blue punctuate callose inclusions were shown in the cell walls of the endosperm in the region of the chalaza in the form of a bright yellow fluorescence. These are interpreted as enclosures of formerly functional plasmodesmata in this area.

The cutin adcrustation between the endosperm and the seed coat is thought to be responsible for the apoplastic semipermeability within seeds. Such deposits may function as a molecular sieve, permitting water exchange but holding back solutes in the endosperm (KYU OCK YIM, 1998). Semipermeable barriers between endosperm and seed coat are the rule rather than the exception (GOLA, 1905; in KOTOWSKI, 1927). The semipermeability prevents the loss of solutes from the endosperm during backflow of surplus water to the mother plant (BRADFORD, 1994).

With the application of the apoplastic tracer LYCH the endosperm did not show any accumulation of the tracer in its cell walls. Although only a smaller quantity of tracer was available, as considerable amounts were retained in the cell walls of the sclereid layer, it was evident that the intensity of the fluorescence of the endosperm cell walls was significantly less than the fluorescence produced by the cell walls of the adjacent parenchyma cells of the seed coat. Thus the endosperm cuticle acts as a semipermeable, apoplastic barrier, preventing the passage of the apoplastic tracer from the seed coat into the endosperm.

The accumulation of the apoplastic and symplastic tracer SR in cell walls and cells of the endosperm at pH 6.5 or below demonstrate that lowering the pH renders the tracer lipophilic and enables its permeation through the endosperm cuticle into the endosperm (Fig. 10B).

The micropyle: outward closure, storage and inward transport of water and solutes

The micropyle of *T. cacao* is zigzag shaped and consists of an inner endostome and an outer exostome, which are not in line with each other (Fig. 2 and Fig. 10C-F). The cells of the sclereid layer in this area are enlarged and possess thickened, strongly lignified radial and tangential walls. Below the sclereid layer approximately 5 additional cell layers with thickened, lignified radial and tangential walls are developed (Fig. 10C-G).

The exostome in mature seeds is firmly closed by compression of its wavy edges (Fig. 10C and F). The endostome exhibits an immigration of a large number of small, reserve material containing cells, which resemble those of the endosperm (Fig. 10D). These are interpreted to be the remains of an endosperm haustoria, the function of which lies in the improvement of nutrient absorption in developing seeds by imparting a more direct contact between endosperm and vascular supply. The endosperm haustoria represents another achievement to ensure a sufficient nutrient supply in large seeds. In mature seeds the endosperm haustoria seems to serve in locking the endostome opening (NETOLITZKY, 1926). If, with the abscission of the seeds, the connection to the vascular supply of the funiculus is interrupted, the remaining haustoria cells may function in the conduction of water and nutrients from the reservoir of the sclereid layer and its underlying cell layers to the embryo or endosperm and thus reduce the loss of water in recalcitrant seeds.

The use of toluidine blue produces a green-blue colouring of the thickened cell walls of the sclereid layer and underlying cell layers within this area, which signifies the lignification of these areas. With UV excitation these thickened cell walls show the bright bluish-white autofluorescence typical for lignin (Fig. 10E) (ROST, 1992). The presence of lignin in the thickened cell walls of the sclereid layer and the underlying cell layers was supported by the application of berberine sulphate/aniline blue, resulting in a yellow-green fluorescence. Acridine orange confirms these results. The outer tangential walls of the sclereid layer were sealed additionally by lipophilic adcrustacion as shown by toluidine blue /neutral red and with acridine orange by its orange fluorescence. This could be interpreted to represent the remains of the cuticle between both integuments and may serve to seal off the sclereid layer as storage tissue, preventing loss of water or solutes through the endostomal opening.

The microspectrophotometrical investigations confirmed the occurrence of large quantities of angiosperm lignin in the thickened cell walls of the sclereid layer and those of the underlying cell layers within this area (Fig. 9). The sclereid layer of this region exhibits approximately a fivefold higher lignification, when being compared to the sclereid layer at other areas of the seed coat. The greatest lignification occurs in the tangential walls of the sclereid layer. The lignification of the radial secondary walls of the sclereid layer and the thickened secondary walls of the underlying cell layers correspond approximately to that of the xylem walls, while the outer tangential walls of the sclereid layer display almost twice as much lignin (Fig. 9). The surface scan, which was accomplished at a wavelength of 278nm, demonstrates the distribution pattern of lignin even more clearly (Fig. 10G). In the outer tangential walls of the sclereid layer very high quantities of lignin were detected whereas in the cell layers below the sclereid layer lower absorption values were measured.

Below the sclereid layer and underlying cell layers with thickened cell walls toluidine blue revealed a strong accumulation of polyphenolic compounds in the tegmal cells, the lumina of which are completely filled with these compounds (Fig. 10D).

In the cell lumina within the area of the micropyle, there was an accumulation of material which we identified as lignin-like material due to the typical absorption spectra of angiosperm lignin on microspectrophotometry. There was a very strong absorption maximum. Furthermore there was previously not identified aromatic cell content material detected that had an absorption maxima at 269nm. These cells resemble those of the hypostase and likewise may form a barrier with sealing function in this area.

The exostome did not show any significant accumulation of the tracer LYCH and therefore does not represent a preferential entry site for the tracer solution (Fig. 10F). High concentrations of LYCH had accumulated in the thickened, strongly pitted cell walls of the sclereid layer and adjacent cell layers below (Fig. 10F). The wall thickenings of the sclereid layer show a somewhat stronger fluorescence intensity than those of the underlying cell layers. Similar to other parts of the seed coat, the sclereid layer is characterised by a high flow volume of the tracer solution. The cell walls of the sclereid layer and those of the underlying cell layers may represent also in this area a location

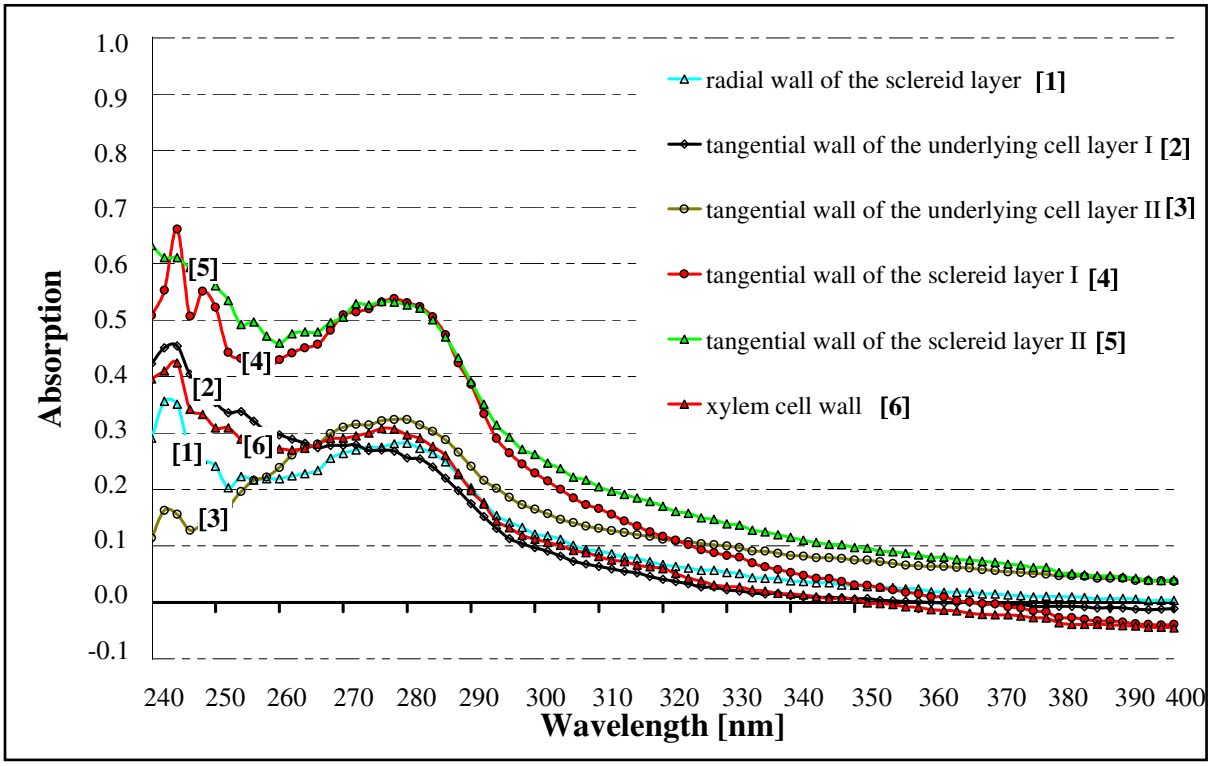


Fig. 9: UV absorption of the cell walls of the sclereid layer and underlying cell layers of the region of the micropyle compared to that of a xylem cell wall.

from which water is removed via uptake into the symplast. The sclereid layer and the underlying layers within this area form an extensive reservoir for water and probably also solutes. This reservoir is protected outwardly from loss of these substances by a lipophilic adcrustation on the outer tangential walls of the sclereid layer in the area of the endostome opening.

With the apoplastic and symplastic tracer SR the thickened cell walls of the enlarged cells of the sclereid layer as well as those of the underlying cell layers showed a weak intracellular fluorescence, which suggests the presence of small tracer quantities in the symplast of these cells.

Overall the tracer experiments demonstrate that the exostome does not constitute a significant entry site for water and solutes into the seed coat. The enlarged cells of the sclereid layer and the adjacent layers situated below, as well as the sclereid layer at other areas of the seed coat, accumulated high concentrations of the apoplastic tracer LYCH in their strongly pitted wall thickenings (Fig. 10F). The sclereid layer at this area, with the assistance of the endosperm haustoria (Fig. 10D), may provide an increased reservoir of water and solutes and may promote a more efficient transport of these substances to the embryo.

Hypothetical way of acetic acid through the seed coat during fermentation

The ammonium iron (II) sulphates test showed that the entire surface of the seed coat is highly permeable for aqueous solutions, although this permeability is still less than that in the area of the hilum (Fig. 5D). Fruit pulp, outer testal epidermis with cuticle and hypodermal mucilage, which are absent within the area of the hilum (Fig. 5A-C), represent diffusion-retarding, but not diffusion-preventing structures. This is confirmed by the LYCH application, where the tracer solution reached its final distribution after just 30min what clearly demonstrates that the apoplastic pathway through the seed coat represents a pathway with little resistance. Fruit pulp, outer testal

Fig. 10: A: Longitudinal section of tegmen and endosperm showing the endosperm cuticle.

B: Tangential section of the endosperm after 30min of application of sulforhodamine at pH 3.5 and 45°C, blue excitation. The strong accumulation of SR in cell walls and cells of the endosperm at pH 6.5 or below demonstrate that lowering the pH renders the tracer lipophilic and enables its permeation through the endosperm cuticle into the endosperm.

C: Longitudinal section of the exostome.

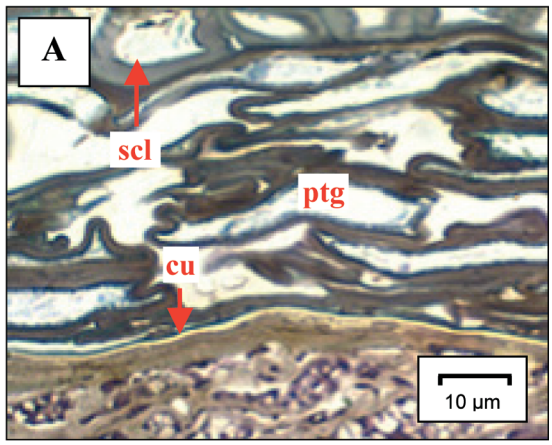
D: Longitudinal section of the micropyle showing the endosperm haustoria, toluidine blue.

E: Longitudinal section of the micropyle showing the autofluorescence of the endostome.

F: Longitudinal section of the micropyle with exostome after 5h of incubation of LYCH, blue excitation. High concentrations of LYCH had accumulated in the thickened, strongly pitted cell walls of the sclereid layer and adjacent cell layers below.

G: Scanning-micrograph and 3D profile at 278nm of a longitudinal section of the micropyle in the region of the endostome. The surface scan demonstrated the occurrence of large quantities of angiosperm lignin in the thickened cell walls of the sclereid layer and those of the underlying cell layers within this area.

cu: endosperm cuticle; **e:** endosperm; **eh:** endosperm haustoria; **es:** endostome; **ex:** exostome; **p:** cell lumina filled with polyphenolic compounds; **pte:** testal parenchyma; **ptg:** tegmal parenchyma; **pu:** fruit pulp; **scl:** sclereid layer; **lig:** lignified cell wall of the tegmen.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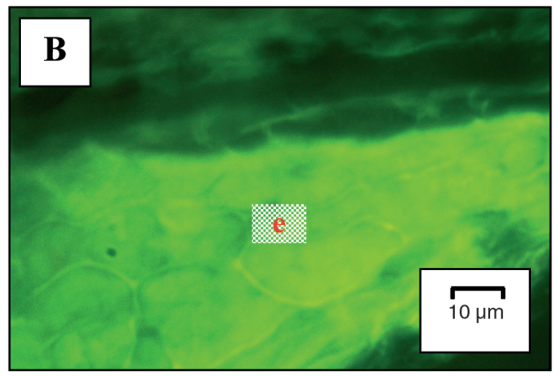
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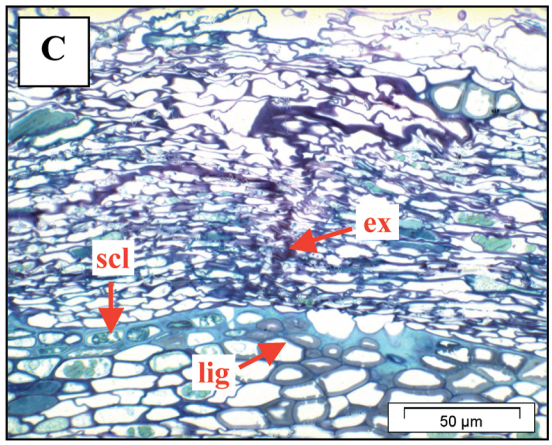
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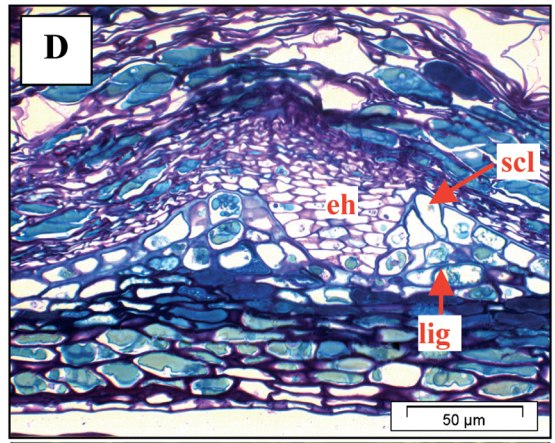
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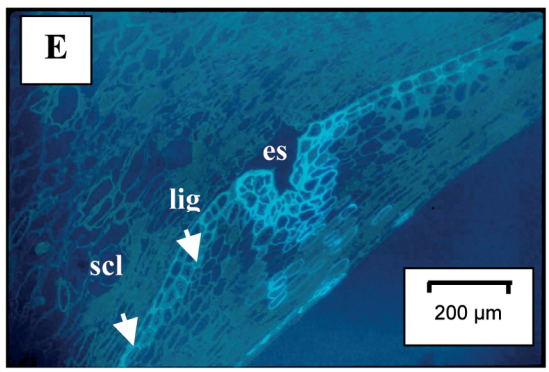
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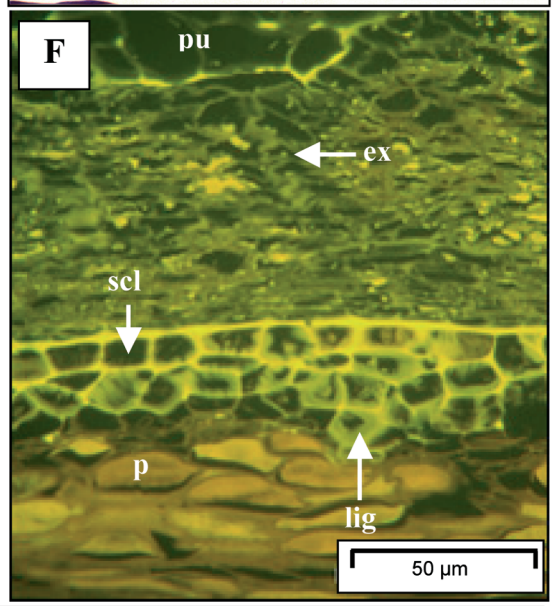
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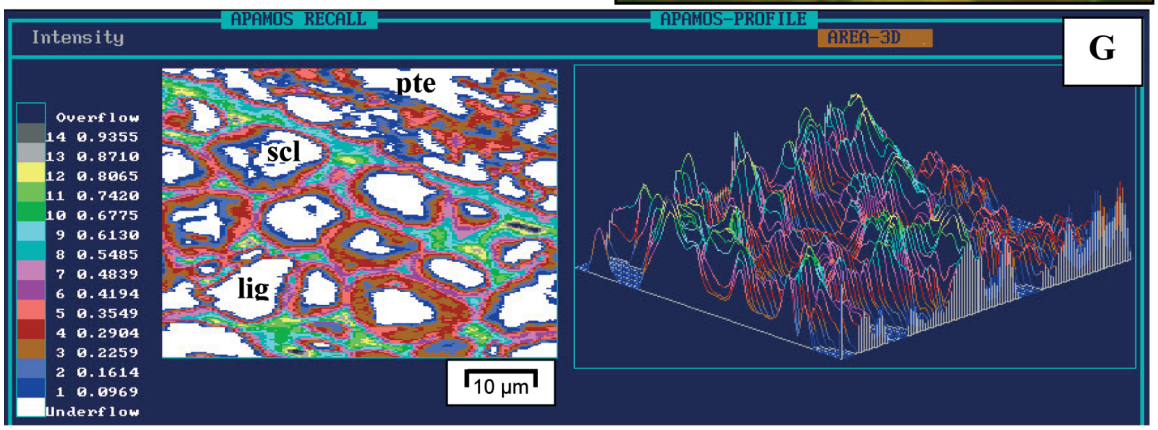
APAMOS RECALL
APAMOS-PROFILE
Intensity
AREA-3D
G
Overflow
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13 0.8710
12 0.8065
11 0.7420
10 0.6775
9 0.6130
8 0.5485
7 0.4839
6 0.4194
5 0.3549
4 0.2904
3 0.2259
2 0.1614
1 0.0969
Underflow
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10 µm

cuticle and sclereid layer do not represent absolute, apoplastic barriers for aqueous solutions.

On its way into the seed coat, acetic acid first encounters the adhering, pectinous fruit pulp. The fruit pulp and the inner pulp epidermis in particular, play a role in the retention of acetic acid during fermentation at least up to their microbial decomposition. The fruit pulp and especially the inner pulp epidermis have a high water absorption capacity due to pectin which enables them to bind the diluted acetic acid solution by swelling as well as the through osmotic water binding of pulp sugars. The acetic acid is retained in cells, cell walls and particularly within the periplasmatic region (Fig. 5E). This results in a decreased, delayed and synchronous uptake of acetic acid into the seed coat, which favours good flavour quality.

The thin cuticle on the outer testal epidermis did not act as a considerable barrier for the uptake of acetic acid into the seed coat. No significant retention of acetic acid was present in the hypodermal, probably pectin-containing mucilage of freshly harvested seeds.

In pre-dried seeds the pectinuous fruit pulp and the hypodermal mucilage may lower the permeability of the seed coat for acetic acid. During desiccation and in the presence of oxygen pectinous mucilage shrinks and goes through chemical changes, which can make the seed coat impermeable (WERKER, 1997). Since the presence of water limits the admission of oxygen, drying of the seeds prior to fermentation could lower the permeability of the seed coat, which could lead to a decrease in acidity and thus to a better fermentation result (PASS, 1996). This effect can be augmented by the oxidation of polyphenolic compounds in the seed coat, which occur in large quantity (WERKER, 1997; MARBACH and MAYER, 1974).

During fermentation the hilum, which is free of outer testal epidermis, adhering fruit pulp and hypodermal mucilage, is thought to constitute the first entry site of acetic acid into the seed coat (Fig. 5A-C). The onward transport proceeds within the injured main vascular bundle of the raphe. Short-distance transport probably runs predominantly apoplastically within the testal parenchyma up to the sclereid layer. The exostome does not represent a preferential entry site for acetic acid into the seed coat of freshly harvested seeds (Fig. 10F).

Once taken up into the seed coat, acetic acid is probably transported apoplastically up to the sclereid layer. It is taken up into the symplast of the sclereid layer and transported circumferentially within this layer around the entire seed surface (Fig. 5H). During fermentation the sclereid layer probably forms a reservoir for acetic acid and imparts a rapid and even redistribution. Thus, the sclereid layer potentially facilitates the even distribution and the simultaneous infiltration of acetic acid into the cotyledon tissue. A prominent sclereid layer with its ability to provide an ample pool with high buffer capacity may constitute a distinguishing feature of good flavour producing varieties.

Since the sclereid layer within the area of the micropyle is increased and extended by several layers (Fig. 10C-G), acetic acid can be intensely accumulated in the sclereid layer and adjacent cell layers below. The remains of the endosperm haustoria (Fig. 10D) may help in directing acetic acid from the reservoir of the sclereid layer and its underlying cell layers to the embryo or endosperm. However, after loss of seed coat integrity during fermentation, the endostome may constitute a predetermined breaking point enabling a high influx of acetic acid into the seed.

Inwards of the sclereid layer the acetic acid may be transported symplastically up to the inner tegmal epidermis.

The hypostase (Fig. 8A and B) represents a barrier, which prevents the penetration of acetic acid into the chalazal opening, impeding an excessive, inhomogeneous infiltration that would lead to an uneven, suboptimal flavour quality. Thus, a pronounced hypostase may be an important prerequisite of good flavour producing varieties.

At the inner tegmal epidermis, the acetic acid will be released from the maternal tissues. The uptake into the endosperm must take place symplastically as the semipermeable, apoplastic endosperm cuticle (Fig. 10A) prevents an apoplastic uptake.

The endosperm cuticle functions as an apoplastic barrier for acetic acid until an acid pH is reached. Acetic acid can be taken up strongly into the endosperm cells when the pH drops to 3.5 during fermentation (Fig. 10B). The endosperm cuticle allows acetic acid to permeate in a synchronised manner into the endosperm resulting in an even, homogenous uptake of acetic acid into all cotyledon cells.

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