Part 1. The Microstructure of and Compartmentation in the Ripe Vanilla Bean

Insights into potential vanillin and flavor formation.

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Curing of vanilla beans is an age old process involving the four basic steps of blanching, sweating or fermentation, drying, and conditioning. Over the last 10 years or so, scientific understanding of this process has improved as a result of developments in plant biochemistry, particularly its contribution to the curing operation. At the same time, greater knowledge of plant tissue microstructure and compartmentation of enzymes and substrates at the microscopic level has facilitated a greater insight into tissue and cellular organization and disorganization during the ripening and senescence process. In the context of the vanilla bean, this comprehension allows a better understanding of the state of the vanilla pod at the ripe stage, and the effects of natural processes and curing on its transformation.

Part 1 of this paper will consider the vanilla bean tissue microstructure and cellular compartmentation of key enzymes and chemical actives in relation to their potential for vanillin and flavor formation in the ripe bean. Part 2, to follow at a later date, will examine the effects of the natural senescence process and the induced curing stages as these relate to tissue microstructure breakdown and flavor development.

Plant Microstructure and Compartmentation

Plant product microstructure is the characteristics of plant cell and tissue assemblies at the microscopic level as defined by the structures generated by the macro chemical components, namely proteins, lipids and the complex carbohydrates. A key structural feature of plant systems is the cell wall, composed of the four basic building blocks of cellulose, hemicellulose, lignin and pectin. Cellulose fibers reinforce a matrix of hemicellulose and either lignin or pectin. Built on this basic wall architecture is the cellular structure of the plant tissue ranging from the largely honeycomb like cells of wood to the closed liquid filled parenchyma cells typified by potato tubers. The combination of these basic building blocks combined in the different cellular arrangements gives a very wide spectrum of mechanical properties of Young's moduli from 0.3 MPa to 30 GPa.[1]

Within the supporting cell wall structure is the selectively permeable lipid bilayer cell membrane. This membrane encompasses the cytoplasmic contents in which are found the nucleus and other organelles, including the mitochondria and chloroplasts. Mature plant cells often additionally exhibit a large membrane-bound central vacuole which may occupy in excess of 90% of the cell volume. These vacuoles are the repository of dissolved sugars and their derivatives as well as mineral ions and other solutes.

Plant cellular organelles, cell structures and tissue assemblies show great variation depending on function. The mature vanilla pod is a green elongated structure weighing around 12–18 g and of length and cross sectional dimensions of ca.15 cm and 1.5 cm, respectively.

The mature vanilla bean, although rounded, approaches an equilateral triangular shape in the transverse section. It exhibits a number of interesting structural features. The outermost epicarp is composed of layers of polygonal cells whose thick walls provide much of the mechanical strength of the bean; their outermost regions transforming into a protective cuticle. The mesocarp is composed of highly vacuolated parenchyma cells which may reach dimensions of 300 µm. In the outer radii of the mesocarp layer are 18 relatively uniformly spaced vascular bundles containing phloem and xylem vessels. The endocarp and mesocarp cells contain chlorophyll and are, up to the advent of senescence, photosynthetically active. The central cavity of the pod, occupying approximately 1/5 of its cross sectional area, is surrounded by an endocarp layer one to two cells deep. Dominating this central cavity are the seeds. These are black in appearance and about 200 µm in their largest oblong dimension. These structures do not contain vanillin or its glucoside precursors. Each of the three sides of the pod bears two placental lamellae bending into the central cavity. These lamellae are composed of four to five layers of parenchyma cells. At the three corners of the internal cavity are a group of elongated cells, the papillae or trichomes. Frozen samples of transverse sections of ripe vanilla are characterized by a distinct whitening of the trichome and placental zones (see **F-1** and **F-2**).

Tapia et al. investigated the microstructure of mature green vanilla beans by microscopy and digital image analysis.[2] They identified and quantified, as a percentage of the space occupied, five main pod compartments, namely epicarp through mesocarp to endocarp (66.55%), placentae (16.09%), seeds (14.42%), trichomes (2.90%) and locular region (0.04%).

Due principally to the work of Odoux and Brillouet, and Brillouet et al., there is now a greater understanding of the distribution and localization of key enzymes and phenol conjugates within these microstructural compartments of the ripe vanilla bean.[3,4]

These workers demonstrated both longitudinal and radial distribution of key enzymes and chemical components in the

ripe vanilla bean. Longitudinal distribution was determined for peroxidase and β-glucosidase, as well as total vanillin (a sum predominantly of glucovanillin and vanillin). A vanilla bean was for the purpose of this analysis divided into 16 equal-sized segments numbered from 1–16, starting from the stem to the blossom end of the pod. For simplicity, the data was collected in fractions based on slices 1–6 (fraction A), 7–11 (fraction B) and 12–16 (fraction C). The summed data for peroxidase, β-glucosidase and total phenols—mainly glucovanillin—in these three fractions are shown in **T-1**.

There were parallels in the longitudinal distribution of β-glucosidase and the total phenols. Peroxidase activity, although showing elevated activity at the blossom end of the pod, exhibited a more uniform distribution along the rest of the bean length. "Browning" starts at the blossom and moves toward the stem end of the bean, as evidenced by the concentration toward the blossom end of the fruit of glucovanillin, its hydrolytic enzyme β-glucosidase, and peroxidase, an enzyme capable of phenol oxidative dimerization and browning.

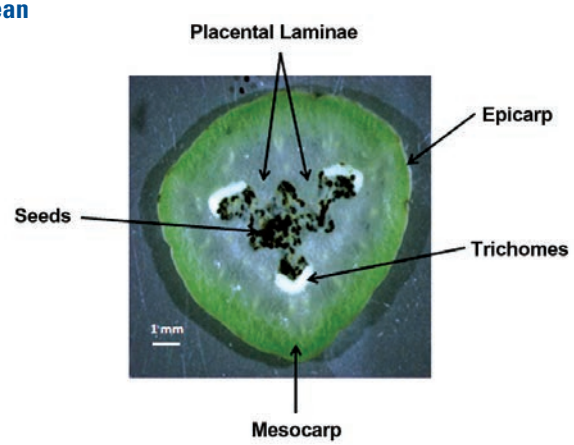
Alternatively, this observation may be the consequence of the process of senescence, a facet of programmed cell death, which results in the destruction of the cellular organization of the pod and, as one of its consequences, the activation of hydrolytic and oxidoreductase enzymes and de-compartmentation in cells and tissues. Such processes may be initiated at one apex of the pod and spread in a semi-autocatalytic progressive way following initiation by the hormone ethylene and other plant hormones.[5]

Radial distribution of the same key enzymes and the phenol conjugate in the mesocarp, the papillae and the trichome compartments were examined in slice #14. This segment was considered to be an average representation of the bean microstructure as well as its enzyme and chemical actives distribution. The disposition of the homologous γ-pyrone lipid family and the mucopolysaccharides, or glycosaminoglycans, were also evaluated (**T-2**).

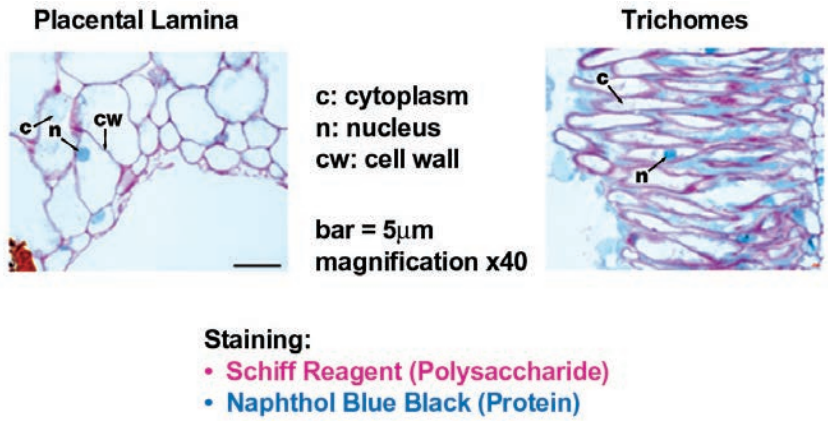
The colocalization of β-glucosidase and glucovanillin in the placental compartment is of interest particularly in the context of liberation of vanillin during the subsequent curing operation. Previous work has provided additional evidence for the presence of both substrate and enzyme in the same tissue. Glucovanillin is reported to be present in the vacuole, while β-glucosidase is predominantly cytoplasmic.[6] Peroxidase appears to be localized almost exclusively in the mesocarp compartment. This enzyme in plants is frequently associated with the xylem structures of the vascular bundles. The strength and physical properties of xylem vessels are fortified by the presence of lignin, which is deposited in the cell walls of these structures. Key monolignols include coniferyl alcohol, which can be oxidized by peroxidase in the presence of hydrogen peroxide to carbon-based radicals that dimerize and further polymerize subsequent to the first oxidation step. Coniferyl alcohol is often found as the glucoside coniferin, which may act as a transport vehicle for monolignols, as an intermediate, or storage form of the primary alcohol.[7, 8] There was no peroxidase activity evident in the trichome fraction.

The large vasculature associated with the mesocarp may account for the majority of the peroxidase activity of this fraction. Some association of peroxidase(s) and vanillin, released from the glucoside, may account in part for browning observed in ripe vanilla beans, though it is not clear at this stage as to how

F-1.Transverse section of a frozen sample of a ripe vanilla bean



F-2. Microscopy of the trichomes and placental lamellae region of ripe vanilla beans



T-1. Longitudinal distribution of key enzymes and chemical actives in ripe vanilla beans[1]

Component	Fraction A	Fraction B	Fraction C
β-Glucosidase[2]	22	40	38
Peroxidase[3]	31	27	42
Total phenols[4]	17	43	40

1. Data modified from reference 3
2. nKatals/g fresh weight
3. 10^2 units/g fresh weight
4. g % fresh weight

T-2. Radial distribution of key enzymes and chemical actives in ripe vanilla beans[1]

Zone	β-Glucosidase (nKatal/g f.w.)	Glucovanillin g % f.w.	Peroxidase Units/g f.w.	γ-Pyrone g % f.w.	Mucopolysaccharide % d.w.
Mesocarp	1409 (26.9)[2]	0.03 (0.2)	7124 (97.5)	n.d.[3]	n.d.
Placentae	3534 (69.3)	14.61 (78.7)	186 (2.5)	n.d.	n.d.
Trichome	200 (3.8)	3.93 (21.1)	n.d.	45	32

1. Data modified from references 3 and 4
2. % composition
3. Not detected

much of the browning is due to oxidation of vanillin by peroxidase resident in the mesocarp tissue.

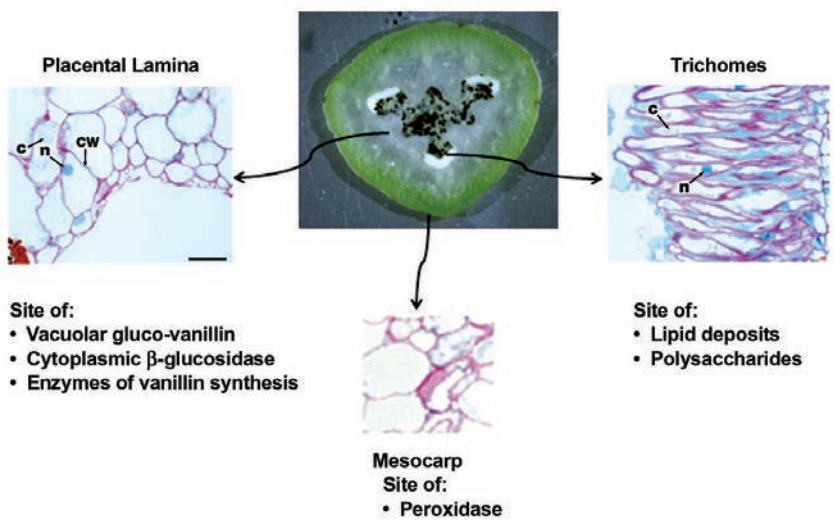
Histological treatment of transverse sections of mature vanilla pods demonstrated high concentrations of oleoresin material concentrated in the trichome compartment. These very dense trichome cells were very rich in lipids and mucopolysaccharides and to a lesser extent protein, as demonstrated by Nile Red, Periodic acid-Schiff reagent[a] and Naphthol Blue Black,[b] respectively.[9,6]

This lipid material was present at ca.45% of the fresh weight of the trichomes and was virtually absent from the mesocarp and placentae fractions. The principal lipid compounds present therein were a homologous series of γ-pyrones of molecular mass range of 376–516. The major homologue present was the γ-pyrone of molecular weight 432 and molecular formula $C^{29}O^{2}H^{52}$. The position of the *cis*-double bond in the omega-9 position in all of the homologues suggest a biogenetic origin of the side chain by C2 extension, using acetyl CoA, of a *cis*-Δ^{9} fatty acid. Previous studies had identified the γ-pyrone family of compounds isolated by pentane extraction of crushed ripe beans from two vanilla bean species.[10] However, this earlier study did not identify the location of these lipids. In addition to the lipid fraction a mucilaginous mucopolysaccharide material, staining with periodic acid Schiff reagent (PAS) was also present in the trichome compartment at about 32% by weight of the dry solids. This material was rich in the uronic acids, arabinose and galactose as the major sugar constituents. These materials were absent from the papillae and mesocarp fractions.[3] The function of the γ-pyrones and the mucopolysaccharides in the vanilla bean is not known.

[a] *www.jhu.edu/iic/PDF_protocols/LM/Glycogen_Staining.pdf*
[b] *www4.mpbio.com/ecom/docs/proddata.nsf/(webtds2)/806717*

The major structural zones in the ripe vanilla pod and their inclusions of importance are shown in **F-3**.

F-3. The major structural zones and their inclusions in the ripe vanilla bean



It is clear from the above studies that the major enzymes involved in phenol formation and liberation from their principal glucoside precursors are present in the vanilla pod in the placentae and trichome compartments. The proximity of glucovanillin and its hydrolyzing enzyme(s) β-glucosidase is well established. Based on this knowledge, it now may offer possibilities to formulate curing interventions to effect maximum efficiency of vanillin liberation from the glucoside.

The work of Perez-Silva et al. indicated that about 13 of the 24 key aroma compounds identified in cured vanilla beans were phenolic and that the mode of their formation was in most cases by their liberation from the parent β-D-glucopyranosides.[11] Joel et al. demonstrated the localization of 4-hydroxy-benzaldehyde synthase (HBS) in the vanilla pod trichomes by immunofluorescence techniques and showed that these cells contained enzymes that are involved in vanillin biosynthesis.[12] Havkin-Frenkel et al. hypothesized the role of papillae in the biosynthesis of glucovanillin and related precursors of phenolics and their subsequent excretion into the medium surrounding the seeds.[13] The exact site of synthesis of vanillin and the related phenols and their glucosides is still undetermined. Indeed, the pathway of formation of vanillin in the maturing vanilla bean is still not fully clarified, though it is still considered to proceed via the phenylpropanoid pathway from phenylalanine.[14] Negishi et al. provided evidence for the intermediate role of ferulic acid, derived from p-coumaric acid, in the formation of vanillin.[15] These authors also hypothesized that vanillin was subsequently converted to the glucoside, presumably by the action of the appropriate glycosyltransferase.[16]

The remaining compounds identified by Perez-Silva et al. were a collection of aliphatic acids, C4 alcohols and ketones, and a family of mono- and diunsaturated linear chain C7 to C12 aldehydes.[11] The potential biogenetic origin of these compounds in the vanilla bean during curing has already been considered, though to date there is little experimental evidence to support their routes of formation. [17]

The pathways of the non-phenolics are probably catabolic and feed from substrate pools of monosaccharides, short- and long-chain fatty acids, peptides and amino acids, and as such would be dependent on the degradative transformation of complex lipids (galactolipids and phospholipids), polysaccharides and proteins.[17] A typical composition of the macro components found in ripe vanilla beans is shown in **T-3**.[18]

The lipids represent an important chemical group in the green bean. As indicated, they probably comprise phospholipid and galactolipid components of the lipid bilayers in photosynthetic tissue and the unsaponifiable homologous γ-pyrones and long-chain β-diketones.[19] The esterified fatty acid, present in the phospholipids and galactolipids, comprise linoleic acid:oleic acid:linolenic acid approximately in the ratio of 20:4.5:1.[20] Mechanical damage to plant tissues results in the activation of lipid ester hydrolases and rapid release of free fatty acids from their esterified forms in complex lipids indicated above. Indeed, this reaction is a prelude to the peroxidation of di-and triunsaturated fatty acids by plant lipoxygenases.[21] The catabolism of macromolecules probably occurs by hydrolytic activity during the natural senescence process or during induced curing.[22]

T-3. Macro component composition of ripe vanilla beans[1]

Fraction	g/100 g d.w.	Component and % of each		
Fiber	45	Lignin 62	Cellulose 27	Hemicellulose 11
Sugars	10	Sucrose 80	Glucose 15	Fructose 5
Lipids[2]	12	Linoleic acid 54	Oleic acid 10	Palmitic acid 10
Protein	3			
Organic acids	5	Citric 50	Malic 30	
Minerals	10	Potassium 28	Calcium 10	Magnesium 2
Glucovanillin	10			

1. Data modified from reference 18
2. Percentage of fatty acids in relationship to saponifiable content

Summary

A number of the key enzymes and chemical actives relevant to the curing of vanilla beans exhibit both longitudinal and radial distribution in the ripe vanilla pod. The inner part of the bean, comprising the placentae and the trichomes contained the majority of the vanillin precursor and the enzyme, β-glucosidase, responsible for its hydrolysis. Of the potential oxidoreductases involved in phenol oxidation, the enzyme peroxidase is almost exclusively localized in the mesocarp zone. The major γ-pyrone lipid family, whose function is not clear, is restricted to the trichome fraction. Residing in this same fraction is a large mass of mucopolysaccharide, again of undefined function.

The dispositions of a number of these important compounds have an influential role on flavor and color development during senescence of the pod or during the industrial curing of the vanilla bean. This topic will be the focus of Part 2 of this paper.

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