Part 2. The Role of Plant Microstructure, Compartmentation and Senescence in Vanilla Curing

Senescence and curing of the ripe vanilla bean.

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The pioneering work by Peter Lillford on food microstructure provided a basis for understanding the major structural elements of food materials that define their textural properties at the microscopic level. Extension of this thinking provided the platform for predicting the pathways for the breakdown of food structures in the environment of the oral cavity.[1] The departitioning of tissue and cellular compartments has a profound effect on the textural properties of food matrices, and influences the formation and release of aroma and taste-active inclusions.[2]

This microstructure approach can be applied to natural tissue materials undergoing major structural changes such as occur in plants during senescence. Natural senescence on the vine or curing of vanilla beans signals major cellular and tissue changes. Major biochemical events occur as a consequence of these processes, namely hydrolytic release of vanillin and related phenols from their β-D-glucosides, and the concomitant "browning" of the tissue.

Part 1 of this review examined the microstructure of the ripe vanilla bean and the compartmentation of the key enzymes and chemical actives that contribute to flavor and color formation during natural post-ripening and curing.[3]

The purpose of this review, Part 2 in this series, is to consider the effect of the senescence process and the decompartmentation that occurs as a consequence of microstructure disassembly of the vanilla pod. These relate to major flavor and color changes that occur during the natural ripening and the induced curing process.

The Release of Vanillin and Browning

The liberation of vanillin from its major precursor, glucovanillin, in the ripe bean is catalyzed by the endogenous enzyme β-glucosidase. Both the enzyme and its substrate exhibit a concentration gradient decreasing from the blossom end of the ripe vanilla bean to the stem junction of the vanilla pod. In addition, both β-glucosidase and glucovanillin are predominantly resident in the placental laminae of the bean and, to a lesser extent, in the trichome and mesocarp zones.[4,5]

There is additional evidence that supports separate compartmentation of the glucosidase enzyme and its substrates within individual placental laminae cells—in the cytoplasm and the vacuole, respectively.[6]

The work of Dignum et al. has clarified the activity of β-glucosidase and other enzymes during the different stages of vanilla curing.[7] All of the enzymes monitored were most active in the green bean. β-Glucosidase found in the ripe bean could not be detected after 24 hours of autoclaving, i.e. the first stage of sweating in which beans, hot from blanching, are placed in airtight containers overnight. This observation highlights the instability of this enzyme and the importance of achieving rapid destructuring of cellular compartments required for bringing substrate and enzyme together.[8] Under the above curing conditions, peroxidase was more stable, with activity decreasing from the ripe stage to about 20% of initial level after 29 days of curing.

Routes that achieve rapid decompartmentation and facilitate reaction between an active β-glucosidase and the phenol glucosides will determine the efficiency of phenols release. Traditional curing operations achieve, in general, less than 50% conversion of the phenol glucosides to the free phenols.[9] Thus, under traditional curing regimes the full potential of vanillin and related glucosidically bound phenols are not fully realized.

One of the first visual signs following the onset of the natural senescence process or of curing is the browning of the tissue. In the vanilla fruit on the vine, this process starts at the blossom terminus of the bean and progresses as a front in the direction of the pod stem end. Interestingly, during curing the browning occurs more uniformly following initial hot water blanching treatment and the subsequent fermentation stage. These variations in the development of browning indicate differences in the directional stimulus between natural and induced senescence. It is probable that the browning process is due to the oxidation of phenols to dimeric and polymeric compounds, of which a number have been characterized in cured vanilla beans.[10] Green beans are virtually free of the dimeric products identified in the above study, confirming that they are curing-associated. The identities of the phenol substrates involved in this oxidation process

Read Part 1 of this article, "The Microstructure of and Compartmentation in the Ripe Vanilla Bean," on Pages 22–27 of the July 2013 issue of *P&F*; *www.PerfumerFlavorist.com/magazine.*

in the vanilla bean have not been fully defined through vanillin. A number of other phenols, including guaiacol, and ferulic, caffeic and chlorogenic acids, probably play a direct or indirect substrate role. These reactions are likely to be free radical in nature and occur via the participation of enzymes, but may also occur via non-enzymatic routes. Oxidation of phenol by aqueous hydrogen peroxide results in the formation of catechol: hydroquinone mixtures whose ratios depend on reaction conditions.[11]

Enzyme catalyzed oxidation of phenols occurs in the presence of peroxidase(s) and generally have a requirement for hydrogen peroxide as the hydrogen acceptor. On the other hand, polyphenol oxidase, especially catalase or laccase, utilize molecular oxygen in this role. Ortho-diphenols in the presence of peroxidase and hydrogen peroxide in the peroxidatic mode produce reactive o-quinones, which can react further to produce "brown" polymers. Peroxidase can also operate in the oxidatic mode against selected substrates and have the ability to reduce molecular oxygen to reactive oxygen species such as the superoxide anion. The polyphenol oxidases include catechol oxidase, which oxidizes o-diphenols to o-quinones, and laccase, which oxidizes o-diphenols to o-quinones and can oxidize p-diphenols to p-quinones. These quinones are very reactive and can participate further to generate brown polymers.[12]

A polyphenol oxidase has been isolated from ripe vanilla pods.[13] Substrates for the purified enzyme, molecular weight ca. 34 kD as a monomeric form, were the o-diphenols 4-methyl catechol or catechol. This study demonstrated that the enzyme utilizes the oxidation of mono-and diphenols to catalyze the cooxidation of various cellular substrates, probably including vanillin. Other cosubstrates present in the bean could include tyrosine, and caffeic and chlorogenic acids. Optimum pH for the enzyme was 3.0 for 4-methylcatechol and 3.4 for catechol as substrates. Optimum temperature was 37°C, but with a broad range from 20°–50°C. The enzyme was thermostable at 65°C, and retained up to 90% of its activity after heating for 20 minutes. This high activity at low pH may indicate a vacuolar site for this enzyme. The most potent enzyme inhibitor was 4-hexylresorcinol, followed by ascorbic acid.

The distribution of polyphenol oxidase within the vanilla tissue compartments is currently undefined, unlike the more thoroughly researched peroxidase enzyme. The oxidation of differently substituted phenols, including vanillin, by peroxidase/hydrogen peroxide, is long established. In the case of vanillin, this results in the formation dehydrodivanillin.[14,15] The products of vanillin/polyphenol oxidase/molecular oxygen have not been determined, though they may arise by cooxidation with the o-quinone oxidation products derived from catechol, as indicated above. For browning to occur, the enzymes, substrates and cosubstrates generally have to be present in the same tissues and cell compartments. In healthy, non-senescent cells, enzymes, substrates and cosubstrates are mostly localized in different sub-cellular compartments or in different but adjacent cell types. Therefore, oxidation reactions of this type tend to occur after senescence or other environmental or mechanical stresses have disrupted cell or tissue structures with consequent decompartmentation. Vacuoles are common sites for accumulation of glycosylated phenols and flavanols.[12,16,17]

The sequence of events during the early stages of curing would appear to involve a major destructuring of cellular and tissue compartments, thus facilitating the hydrolytic release of vanillin by β-glucosidase action on the oxidatively stable glucovanillin. Once formed, the free phenols are susceptible to oxidation. The resultant browning is probably due to dimerization/polymerization of vanillin and other suitably functionalized phenols present in the untreated tissues or released during fermentation. As a result, the final level of vanillin and related phenols in cured vanilla beans is positively dependent on the extent of conversion of glucovanillin to vanillin and in the negative aspect to the extent of loss of vanillin by oxidative and other undefined processes.

The Senescence Process

The natural senescence process has three phases:

- An initiation or signal transduction phase resulting in the inactivation and activation of selected genes
- A reorganization phase involving controlled redifferentiation of cell structures, remobilizing of essential materials and a switch in dominance from autotrophic to heterotrophic metabolism
- A terminal phase characterized by the release of free radicals and the irreversible loss of cell integrity and viability[18]

These different phases are influenced by senescence accelerators and inhibitors, including hormones, and environmental and developmental factors. Fruit senescence is characterized by dramatic changes in major organelles, particularly the plastids of mesophyll and parenchyma cells. These ultrastructure changes are accompanied by altered biochemical pathways, the most visual and common of which is chlorophyll degradation. Interestingly, this pathway in higher plants has a molecular oxygen-dependent step catalyzed by a monooxygenase. The enzyme that affects the opening of the chlorophyll macrocyclic ring structure by conversion of pheophorbide A to a linear tetrapyrrole was only found in senescent plants.[19] During senescence the complex, multibranched phenylpropanoid pathway for the formation of secondary metabolites such as phenolics, tannins, flavonoids and lignin is activated, leading to accumulation and changes in their patterns of production. An example of this process is observed in the ripe vanilla bean. During the first hour of fermentation both vanillin and the N-heterocyclic compound indole appear and begin to accumulate. Unlike vanillin, which continues to increase in level over the subsequent 24–36 hours, indole maximizes after about three hours of fermentation and disappears by the seventh hour.[20] Indole is an intermediate in the final stages of the primary phenylpropanoid metabolic pathway, starting from indole-3-glycerophosphate and catalyzing by tryptophan synthase to the amino acid tryptophan. Mechanical damage of tissues, including elicitation by herbivores feeding on plant tissue, activates a transient secondary metabolic pathway, via indole-3-glycerophosphate lyase, liberating free indole.[21] An additional consequence of the onset of senescence is a limitation of carbon resources brought about by declining photosynthesis. This initiates a switch from autotrophic to heterotrophic metabolism, which is manifested as significant modifications to respiratory and oxidative processes.[18]

The onset of senescence can be induced by many different factors both biotic and abiotic. Environmental stresses such as elevated or sub-ambient temperatures, drought, poor light, pathogen attack or mechanical damage can all result in the premature activation of the process.[22]

Senescence is a complex, highly regulated developmental stage in the life of a plant and is a component part of the process of programmed cell death. Consequences of senescence include loss of cellular integrity, especially membrane disruption, associated accelerated oxidative deterioration involving free radicals primarily derived from molecular oxygen and the related breakdown of antioxidant defense mechanisms. Extensive degradation of membrane-associated unsaturated lipids is initiated as a consequence of the oxidative burst. This latter process involves a rapid, transient production of excessive levels of reactive oxygen species (ROS) and is one of the earliest observable aspects of a plant's defense strategy.[23]

This accelerated generation of ROS is one of the commonest responses to both abiotic and biotic environmental stress. These chemical species are a characteristic feature of the senescing cell. Molecular oxygen is converted to ROS by univalent reduction (transfer of an electron) or by energy transfer. The common ROS produced in plants include superoxide (O_2•–), perhydroxy radical (HOO•), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), alkoxy radical (RO•), peroxy radical (ROO•), singlet oxygen ($1O_2$) and alkyl hydroperoxides (ROOH).[24]

ROS are generally produced as unmanaged byproducts of normal aerobic metabolism, involving largely the chloroplast membrane-linked electron transport processes, redox cascades and metabolism, whose normal production are perturbed under the influence of unfavorable environmental conditions. In all aerobic organisms under normal conditions, the concentration of ROS is tightly controlled by ROS-scavenging pathways. However, an inbalance in generation and metabolism of ROS leads to a variety of physiological challenges by disrupting redox homeostasis of the cell, which is collectively known as "oxidative stress." Plants possess, under normal status, an efficient antioxidant defense system that protects the cell from oxidative damage caused by oxidative stress.[25]

ROS are versatile molecular species and radicals that are at the center of a sophisticated network of signaling pathways of plants and act as core regulator of cell physiology and cellular responses to the environment.[23] Lipid peroxidation and the subsequent generated products thereof are characteristically associated with stress and senescence.[26]

Hydrogen peroxide and the superoxide anion are ROS generated in normal cellular reactions in chloroplasts, peroxisomes and glyoxisomes. Under homeostatic cellular conditions, any excess of ROS produced are inactivated by catalases and superoxide dismutase coupled to the ascorbate-glutathione cycle.[27]

During senescence the redox balance of the defense system is compromised, probably resulting in an altered, enhanced, profile of ROS. Excess and unregulated levels of these ROS via propagation of free radical cascades can result in cell death in the final stage of senescence.

Ethane is an important trigger initiating senescence in climacteric. Ethane is produced endogenously from the amino acid methionine via the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid.[28] In many fruits, such as tomato, there is a developmentally regulated burst of respiration and ethene production, followed by related ripening activities such as color change and flavor development. Chemical inhibitors

of ethene synthesis in plants have been shown to hinder or delay the ripening process. Supra- or suboptimal environmental factors can also trigger senescence, e.g. drought, day length change or extremes of temperature.

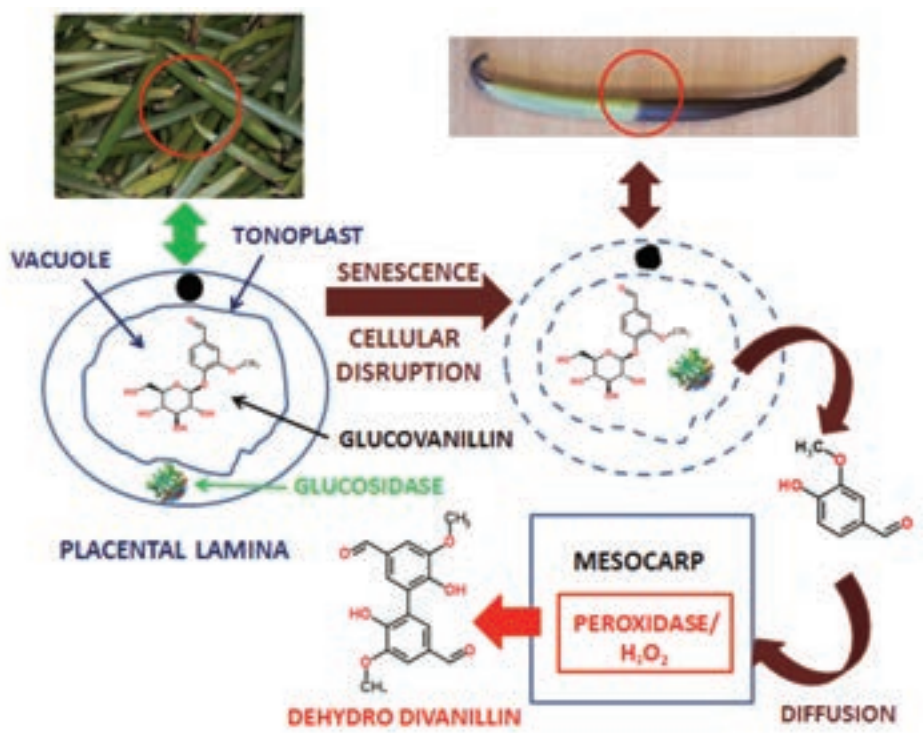
The vanilla bean is a climacteric fruit, and as such will respond to endogenous ethene production by initiation of the respiratory burst and induction of senescence.[29]

Senescence and Curing

A ripe green vanilla bean on the vine and left to stand, will undergo a number of visual and textural manifestations of senescence, namely loss of turgidity and increase in tissue flexibility, degradation of chlorophyll followed by browning, and then blackening of the tissue. This process on the vine commences at the flower end of the pod and gradually spreads toward the proximal or stem end.

The moving front could be initiated by one or more hormone triggers and perpetuated by the senescing cells.

F-1. Schematic representation of localized decompartmentation in the placental laminae cells during natural senescence and traditional curing



Based on previous knowledge, the senescence process in the vanilla pods probably displays a number of key phases:

- Extensive cellular and tissue destruction
- Formation of excessive levels of reactive oxygen species as part of the inbalance between formation of ROS and the failing efficiency of the antioxidant defense mechanism
- Activation of oxidoreductases, including peroxidase(s) and, probably, polyphenoloxidase(s)
- Induction of hydrolases, of which β-glucosidase is crucial to the hydrolysis of glucovanillin
- Further oxidation of the liberated phenols to dimeric and complex polymeric structures mediated by the phenol oxidases
- Potential oxidation of unsaturated lipids, including membrane-associated galactolipids and phospholipids
- Chlorophyll degradation
- Activation of the phenylpropanoid pathway of secondary metabolism

The sequence of these events is not defined, but the terminal phase of the senescence process involves the release of free radicals, the formation of ROS, and the irreversible loss of cell integrity and viability. These processes result in the loss of membrane, organelle and cellular structure. Vacuolar membrane disruption affects the decompartmentation of glucovanillin and β-glucosidase in the placental laminae, releasing vanillin to a greater or lesser extent. This reaction converts the stable glucovanillin to the oxidatively unstable vanillin. Released vanillin can be transformed directly or via a couple with other phenols by the oxidoreductase enzymes peroxidase/hydrogen peroxide and/or polyphenol oxidase/molecular oxygen. The outcome of these enzyme reactions with the liberated phenols present in vanilla beans, as exemplified by vanillin, is oxidation to dimeric and polymeric compounds of the type described above.

The development of the phenol oxidation reactions can be observed, in transverse sections of the vanilla bean, by the onset and spread of the brown pigmentation from the center of the pod in a radial direction and encompassing the vascular bundles. The first darkening appears in the inner mesocarp region. This darkening extends outward at the early stages of browning into the outer mesocarp. This is in keeping with the known distribution of glucovanillin, β-glucosidase and peroxidase, and the association of the latter enzyme with the xylem elements of the vascular bundles. At the fully brown stage, all areas of the bean from the epicarp to the central zone take on the dark coloration of the fully senesced bean. The inner placental laminae and the trichome layer show no evidence of browning (**F-1**).

Extensive cellular disruption occurs during the senescent decline of the ripe vanilla bean under natural conditions and by curing. Most traditional curing methods exhibit limited cellular destruction compared to that which occurs in the naturally senescing material. In this latter process, the moving senescence front is highlighted by the surface brown/black interface with the green chlorophyll-containing area. After a blanching pretreatment in water at 60°–65°C for 2–3 minutes, a sweating stage at temperatures in the range of 40°–45°C and 24 hours of incubation, the vanilla bean assumes a uniform brown color.

Clearly, the natural and induced senescence processes are different in terms of the focus and direction of the browning reaction. The significance of these differences is not clear, but it is worth noting that in the natural process the tissue damage at

the senescence front is extensive and appears to be programmed from blossom to stem end. The directional process is probably under hormonal control in terms of both initiation and propagation. It is likely that the process, once started, progresses in a semi-autocatalytic way from damaged to normal cell. In the case of the cured bean, the curing treatments, such as hot water immersion, are less damaging to cellular structure than the natural process, and so the impact, as reflected by browning, is more uniform. Blanching and sweating are multidirectional in their impact, and as such probably instigate a uniform senescence response that probably precludes hormonal intervention. In addition, ripe beans, if removed from the vine and left standing at room temperature, will eventually begin to brown in a random, rather than unidirectional, manner. This suggests that the development of senescence of the bean on the vine may be determined at the plant level rather than the pod level.

The localized changes that occur during natural senescence at the moving bean/senescing interface are an efficient process, at least in terms of the conversion of glucovanillin to vanillin, which is of the order of 96%. On this basis, there would be a strong case for allowing senescence to occur on the vine, except that the process is slow, and during its timescale dehiscence often occurs, particularly in *Vanilla planifolia*, with increased risk of microbial incursion. In traditional curing, the process is more rapid, but there is significantly less tissue damage, and the glucovanillin conversion is <50%.[9] Senescence development in leaves is a stimulus-dependent process. If environmental stress is the trigger, then the start of senescence is a random process. In the absence of environmental factors, senescence commences at the leaf tip and margins and progresses to the leaf base. In this way nutrients can be withdrawn from the leaf and transferred to the roots for storage.[a] The same situation probably prevails in the vanilla pod. In the natural process, the senescent front moves from the blossom end to the stem end of the bean. During traditional curing, where heating and mechanical stresses are the trigger, this process is random.

Light micrographs of transverse sections of the ripe bean and the vanilla bean following natural senescence or traditional curing are shown in **F-2**.

In the morphology of the ripe bean there are large placental laminae cells. The cells are highly vacuolated and appear mostly empty. Cell walls are often clearly defined with attached cytoplasmic material. Turgor is apparent, and as a consequence cells are regularly shaped and appear perfectly intact with clearly delineated nuclei and nucleoli.

Following natural senescence, vanilla beans became very dark in color. The placental laminae cells were dramatically reduced in size, deformed and often had partially disintegrated. Vacuolar spaces were drastically reduced, and cell walls were irregular shaped and wrinkled. Cytoplasmic material appeared disintegrated and was no longer attached to the cell walls. Cells seemed to have lost most of their fluid content, and turgor was absent. Nuclei were ill-defined or unrecognizable, while nucleoli were not visible. Overall, the tissue appeared to be at an advanced stage of degradation.

Following traditional curing, cells of the placental laminae retained some of their turgor, but tissue integrity was largely perturbed. Cytoplasmic material was irregularly attached to the cell walls. Nuclei, though distinguishable, were no longer rounded but irregularly shaped. Nucleoli were not observed.

The results above indicate that senescence and, to a lesser extent, traditional curing alters cytoplasmic integrity and probably destroys cellular compartmentation. Only mature cells were turgid and contained distinct and well-preserved nuclei and nucleoli. The extent of cellular disruption probably equates to the accessibility of the β-glucosidase to glucovanillin in the placental laminae.

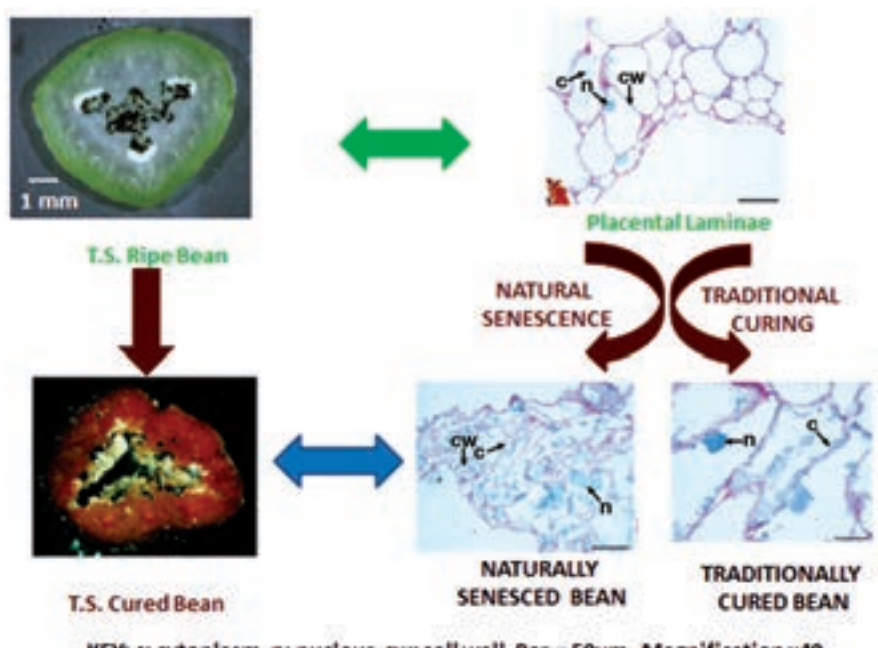
In iris petal cells during senescence, closure of plasmodesmata occurred along with destruction of the tonoplast. The destruction of the tonoplast appears to precede the loss of the plasma membrane integrity.[30]

As indicated, traditional curing operations achieve, in general, less than 50% conversion of the phenol glucosides to the free phenols, and show only partial loss of cellular integrity, but virtually complete loss of β-glucosidase activity. Under natural senescence conditions, however, at the dark zone of the moving front cellular integrity was completely destroyed, glucovanillin to vanillin was ca. 96% complete, while β-glucosidase activity was completely lost. This suggests that total disruption of the cellular microstructure with integrated and available β-glucosidase activity were the major critical factor for glucovanillin hydrolysis.[8]

How then can one achieve the controlled cellular destruction and accompanying

[a] *www.plant-biology.com/Leaf-Senescence.php*

F-2. Morphological changes in vanilla beans following natural senescence or traditional curing

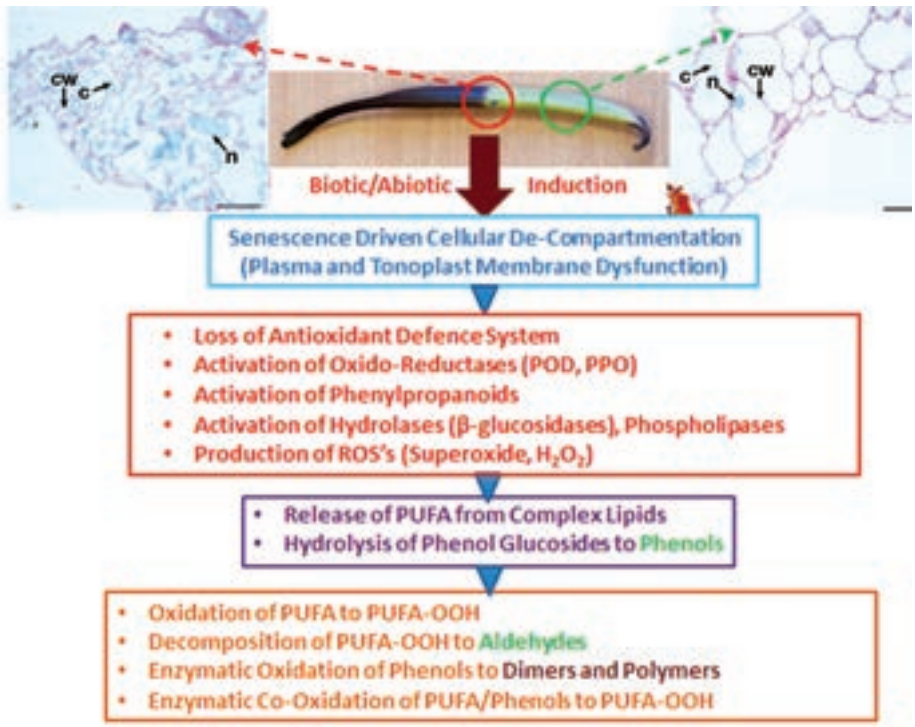


flavor formation/transformation in the curing operation that nature achieves so elegantly?

Summary

Extensive cellular microstructure disassembly is a key feature of the senescence process in the ripe vanilla bean. The extent and direction of destruction is dependent on whether the process is natural or induced by the curing stages. The loss of cellular integrity is much greater at the localized level in the natural process, compared to traditional curing. This is reflected by microscopic examination of the cellular status and reflected in the extent of glucovanillin hydrolysis. A common feature of senescence is the destruction of lipid structures, especially the tonoplast and plasma membranes. In this way the compartmentation of the different cellular zones are compromised (**F-3**).

F-3. Key structural changes, activations and transformations during vanilla bean senescence



The senescence process is initiated by senescence gene activation and hormone release in the natural process, and by the abiotic stress of elevated temperature and mechanical damage during curing. An early consequence is hydrolysis of the complex lipids in the membranes and the formation of ROS, the oxidative burst, which initiate extensive oxidation of membrane lipids. These changes upset order and organization in the cell, resulting in a catastrophic cascade of destruction involving activation of hydrolytic and oxidoreductase enzymes, degradation of chlorophyll and loss of the cellular antioxidant defense system. From a vanilla flavor perspective, the three major developments are the hydrolysis of glucovanillin and other glucosylated phenols, the oxidation of complex lipids, and the separate or linked oxidation of phenols to dimeric and polymeric brown compounds. Since the curing process is protracted, there are opportunities for both enzymatic and non-enzymatic reaction to occur. Among the key enzymes in this context are lipases, β-glucosidase, peroxidase and polyphenol oxidase. The non-enzymatic actives include ROS such as the alkoxy radicals and lipid hydroperoxides.

The key question still remains: What are the flavor differences between natural senescence and curing in the ripe vanilla bean, both with respect to tissue and cellular destruction and flavor formation?

To answer this question, the processes that initiate senescence in the vanilla bean and the factors contributing to the extensive destruction of the pod microstructure must be better understood, as should the importance of the enzymology and chemistry taking place. To achieve this, analysis of the flavor compounds present during natural senescence and their mode of formation should be carried out on either side of the senescent front in the vanilla bean. In this way it may be possible to throw light on the origin of these compounds, as well as the much studied hydrolysis of the resident phenol-β-D- glucosides.

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