Potential interference of fungal endophytes in Vanilla planifolia on vanilla flavor compounds biosynthesis

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Chapter 2: Vanilla flavor production methods: A review.

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ABSTRACT

The biosynthesis of vanillin and other vanilla flavor compounds is still not completely understood. A better understanding of the pathways and how they are regulated may lead to better control of the production of the flavor and thus increase the commercial value of the pods. Non-synthetic vanilla flavor consists of over 250 components, with vanillin being the major one in terms of amount in the pod. Synthetic pure vanillin lacks complex flavor notes, and thus cannot replace vanilla in high quality products. Although customers prefer non-synthetic flavors, non-synthetic vanilla extract is approximately 200 times more expensive than its synthetic counterpart. To produce non-synthetic vanillin, fungi are being used for the biotransformation of vanillin precursors into vanillin. All plants contain several fungal endophytes which do not cause any symptoms of diseases; some endophytes are plant-specific and are thought to play a role in the interaction of the plant with its environment. Certain fungal endophytes have been reported to produce secondary metabolites previously thought to be produced by the plant; such fungi, particularly those isolated from pods, may play a role in the biosynthesis of vanillin and vanilla flavor compounds in the plant. This could be in the form of complete pathways, or parts of the vanillin pathway, interacting with the plant's vanilla flavor compounds biosynthesis machinery. Furthermore, studies on a possible role for endophytes may help to elucidate aspects of the vanillin biosynthetic pathway that still are under debate. Terroir effects on flavor, as observed for vanilla pods, may be due to the presence of microorganisms in the rhizosphere or in the plant itself. Based on this analysis, it is proposed that vanilla plant endophytes are studied and investigated for the presence of possible vanillin and vanilla flavor biosynthetic reactions. As a first step, fungal endophytes have been isolated from vanilla pods from Reunion Island and found to be involved with the development of the vanilla flavor.

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2.1 BACKGROUND

The orchid genus *Vanilla* (Family: *Orchidaceae*) consists of 110 species, three of which are cultivated for their flavor-related commercial value (*Vanilla planifolia* Jacks. ex Andrews, *Vanilla tahitensis* J.W. Moore, and *Vanilla pompona* Schiede, Family: *Orchidaceae*) (Soto-Arenas and Cribb, 2010). Vanilla originated in Central America where it was also used as a flavoring agent; the Spanish brought it to Europe around 1520 and vanilla production on Reunion Island started around 1819 (**Table 1**).

Table 1 – Important events in Vanilla flavor history.

Year	**Events**
Ca. 1520	- Vanilla plants in Europe from Mexico. - Absence of insect pollinator in Europe, no pods obtained.
1819	- Vanilla plants in Reunion Island. - Artificial pollination developed in Reunion Island, pod commercialization follows world-wide.
1858	- Nicholas-Theodore Gobley isolates the vanilla flavor metabolite (vanillin) from vanilla pods.
After 1920s	- Synthetic vanillin synthesized from lignin, followed by commercialization. - Environmental issues with synthetic vanillin synthesis process leading to non-synthetic vanillin synthesis.
1970	- Synthetic ferulic acid biotransformation by *Pseudomonas acidovorans* (den Dooren de Jong) Wen (bacteria) to non-synthetic vanillin.
1998, 2002	- Agricultural byproduct (19,900 mg/l ferulic acid) biotransformation by *Amycolatopsis* sp. Rabenhorst (actinomycete) to non-synthetic vanillin (11,500 mg/L).
2002, 2007	-Synthetic ferulic acid biotransformation by *Aspergillus niger* Tiegh. (fungus) and *Pycnoporus cinnabarinus* (Jacq.) P. Karst. (fungus) to non-synthetic vanillin (300–2800 mg/L, depends on fungal strains, addition of adsorbent resins[a]). - Isotope ratio $\delta^{13}C_{PDB}$ shows non-synthetic and synthetic vanillin differ.
2009	- Non-synthetic vanillin *de novo* synthesis (45-65 mg/L) from glucose by *Schizosaccharomyces pombe* Lindner (yeast) and *Saccharomyces cerevisiae* Meyen (yeast). - Vanillin synthesis only after a novel biosynthetic pathway was genetically engineered in both yeasts.
2011	- World vanilla flavor demand exceeds vanilla pod supply. - To meet demand, substitutes of vanilla extract developed by flavor companies.
2014	- Vanillin from green vanilla pod fermentation by microorganisms. - Complex sensory profile without off-notes. - US patent application for process submitted by Givaudan flavor company.
2015	- Non-synthetic *de novo* vanillin synthesis from glucose by Escherichia coli (bacteria). - Only possible after genetic engineering of E. coli towards a novel biosynthetic pathway. - Vanillin yield from experiments with other genetically modified organisms: 97.20 mg/l from l-tyrosine, 19.30 mg/l from glucose, 13.30 mg/l from xylose, 24.70 mg/l from glycerol.
2017	- Low cost vanilla produced by Firmenich.

Adapted from: Firmenich, 2017; Givaudan, 2016 Ni et al., 2015; Gleason-Allured, 2014; Kaur and Chakraborty, 2013; Hansen et al., 2009; Zheng et al., 2007; Rabenhorst and Hopp, 2002; Lesage-Meessen et al., 2002; Thibault et al., 1998; Rasoanaivo, 1998; Toms and Wood, 1970.

[a] Adsorb produced vanillin away from microorganisms improving total vanillin production.

Apart from environmental concerns, chemical synthesis of vanillin, the major vanilla flavor metabolite, is expensive given the cost of ferulic acid (the precursor for a one-step reaction) whereas agricultural waste is a cheap source of ferulic acid and can be used directly for microbial vanillin synthesis (Chakraborty et al., 2017; Kaur and Chakraborty, 2013). Additionally, there is a lack of substrate specificity in the reactions due to competing by-product formation in the chemical synthesis of vanillin, reducing the yield of the product (Chakraborty et al., 2017; Kaur and Chakraborty, 2013) and making the process less efficient. Producing vanillin through tissue culture of vanilla plants is also not cost effective given the slow growth rate of the plant and the absence of active vanillin biosynthesis. Using microorganisms to produce vanillin is thus a favored alternative. Fungi reportedly have the highest vanillin yield compared to other microorganisms (**Table 1**) and vanillin yield may well be associated with the ability of microorganisms, in general, to tolerate vanillin toxicity; at toxic concentrations, vanillin would be converted into the less toxic products vanillyl alcohol and vanillic acid, which reduces vanillin yield (Kaur and Chakraborty, 2013). Glycosylation of vanillin is another method of reducing vanillin toxicity to microorganisms and increasing vanillin yield. This was engineered by incorporating genes involved in glycosylation in vanillin-producing microorganisms like yeast (Kaur and Chakraborty, 2013). Moreover, vanillin exists in a glycosylated form (glucovanillin) in green vanilla pods, where fungal endophytes responsible for vanillin production may reside. To date, there are no reports of *de novo* vanillin biosynthesis in microorganisms. However, this has been achieved by genetically modifying microorganisms to engineer the lacking biosynthetic steps for *de novo* vanillin biosynthesis (Hansen et al., 2009; Ni et al., 2015). Nevertheless, there are several issues associated with *de novo* vanillin synthesis in genetically engineered organisms. For instance, production of contaminating by-products like isovanillin was observed in the modified *Escherichia coli* (Migula) Castellani and Chalmers (ATCC®8739™) strain (Kaur and Chakraborty, 2013). Additionally, for the latter, the production of vanillic acid as an intermediate reduced the vanillin yield. In the case of engineered yeast, vanillin reduction to vanillyl alcohol was inhibited through gene knockout of the host alcohol dehydrogenase ADH6 enzyme (Hansen et al., 2009). However, an increase in vanillin toxicity in the culture was noted.

2.2 COMPONENTS OF THE VANILLA FLAVOR

The commercial value of vanilla is in the flavor of the cured pods. Vanillin is the major compound influencing the vanilla flavor, contributing 1–2% w/w of the cured pod (Sinha et al., 2008); however, a series of additional minor compounds from the pods also contribute and are responsible for the differences between the vanilla flavor and pure chemical vanillin. The aroma of cured vanilla beans includes descriptors such as sweet, vanillin, floral, prune/raisin, spicy, woody, and tobacco-like (Ranadive, 2011). Vanillin has organoleptic properties that have been defined as one dimensional, whereas the vanilla flavor is not one-dimensional; however, vanilla flavor has also been described as subtle (Dunphy and Bala, 2012a, b) and is highly complex (Gleason-Allured, 2011), as a consequence of the latter, vanilla pods, the source of vanilla flavor, are considered to be a spice (Cameron, 2011). The non-synthetic vanilla flavor is dependent on changes in the growth conditions of the plant (Dunphy and Bala, 2012a, b). According to Alwahti (2003), a key feature of the vanilla trade is quality, i.e., the correct mixing of flavor compounds to obtain the unique complex vanilla flavor from pods. The organoleptic quality of the flavor is due to the qualitative and quantitative metabolite composition of the pods. More than 250 compounds are now known to influence vanilla flavor and the quality of the cured vanilla pods and the extract thereof is dependent on the relative abundance of these compounds, including vanillin. Consequently, it is important to understand how the concentrations of the vanilla flavor components influence sensory detection. For instance, vanillyl alcohol is detected in the cured pods by olfactory-GC analysis as intensely as vanillin, despite being present at a considerably lower concentration (1000 times less) (Hoffman and Zapf, 2011). Vanillin itself is present in all pods, signifying that it does not contribute significantly (less than 12%) to vanilla flavor variability across cured pods (Hoffman and Zapf, 2011).

Some putative precursors of vanillin also contribute to the aroma and flavor of vanilla, including *p*-hydroxybenzaldehyde. Organoleptic property: sweet (Sigma-Aldrich, 2014), vanillic acid – Organoleptic properties: chocolate, creamy, grape, nutty, wine-like (Sigma-Aldrich, 2014), *p*-hydroxybenzoic acid - Organoleptic property: phenolic (Sigma-Aldrich, 2014), and vanillyl alcohol - Organoleptic properties: mild, sweet, balsamic, and vanilla-like odor (Burdock, 2010). Other components influencing flavor include volatiles such as monoterpenes, sesquiterpenes, ethers, arenes, phenolics, and lactones (Zhang and Mueller, 2012; Toth et al., 2011). However, some of these are volatiles that disappear if exposed to high temperatures, causing vanilla flavor to change upon baking (Kennedy, 2015). Volatiles are more concerned with olfactory receptors, whereas non-volatile components are more concerned with taste receptors; consequently both have different effects on flavor and aroma.

2.3 THE NEED FOR ALTERNATIVE SOURCES OF THE VANILLA FLAVOR

Vanilla is one of the most labor-intensive crops worldwide (Dignum et al., 2001, 2002). As a result, vanilla pods are expensive and the price fluctuates according to political and climatic conditions. This price instability is one reason for the shift to artificial vanilla flavor or pure vanillin by the food and beverage industry. Additionally, non-synthetic vanilla flavor extraction from pods is an also expensive process, with non-synthetic vanilla extract costing up to 200 times that of a manmade vanillin substitute; this has led to the availability of fake vanilla extracts in the market (Royal Society of Chemistry, 2016), making extensive quality control necessary.

Demand for cured vanilla pods, over 16,000 metric tons in 2015 (Givaudan, 2016), exceeds supply by as much as ten to one. With the high prices and the inability of non-synthetic vanilla production to meet the global demand for the vanilla flavor, focus has turned to pure vanillin. The market tendency has now shifted from synthetic vanillin to non-synthetic vanillin produced by environmentally-friendly processes. Consequently, over 95% of the vanilla flavoring globally is derived from pure vanillin. Consumers have become accustomed to the pure vanillin 'vanilla' flavor that it is often preferred to non-synthetic vanilla (Royal Society of Chemistry, 2016). However, biotechnological production of vanillin is not to replace non-synthetic vanilla extract but to replace synthetic vanillin with a non-synthetic flavor at an affordable price (Labuda, 2011). The vanilla flavor from pods will instead be used for high quality products such as expensive chocolates and ice creams, which account for approximately 75% of the total vanilla plant flavor used. Other major uses include Cola-type beverages and baked foods (Royal Society of Chemistry, 2016).

2.4 BIOTRANSFORMATION OR *DE NOVO* BIOSYNTHESIS OF VANILLIN

Non-synthetic flavor compounds obtained by biotechnological processes are gaining importance in the food industry due to increasing consumer demand for non-synthetic food additives (Dionisio et al., 2012). Biotechnological production of aroma compounds can occur through *de novo* biosynthesis or biotransformation. *De novo* biosynthesis is the production of complex substances from simple molecules through complex metabolic pathways, while biotransformation involves single reactions that yield products structurally similar or identical to the desired molecule (Dionisio et al., 2012). Because of the sourcing problems of vanilla extract, efforts are ongoing to screen microorganisms for vanillin production by complete *de novo* biosynthesis or biotransformation of vanillin precursors (Hansen et al., 2009). This has resulted in a non-synthetic process of the bioconversion of ferulic acid into vanillin using bacteria and fungi (Chen et al., 2016; Motedayen and Maznah, 2013).

2.5 ENDOPHYTES AS POTENTIAL SOURCES OF SECONDARY METABOLITES IN PLANTS

In recent years, it has become clear that many secondary metabolites in plants are not produced by the plant itself, but are (at least in part) produced by microorganisms such as the endophytes present in the rhizosphere or living in the plant itself (Gimenez et al., 2007). These microorganisms, including bacteria and fungi, normally reside in plant tissues without causing damage or visible disease symptoms (Souza et al., 2011). What differentiates pathogens from endophytes in this definition is that pathogens cause disease symptoms. However, some aspects of the interactions of an endophyte with its host are a matter of dispute such as whether a pathogenic microorganism colonizing a plant can be considered an endophyte if it loses its virulence (Hardoim et al., 2015). Although the symbiotic relationships between endophytes and host plants remain to be fully elucidated, endophytes likely play a role in plant survival within ecosystems. For example, endophytes can alter the physiology and affect the development of plants, especially under environmental stress conditions such as drought (Bayat et al., 2009), herbivory (de Sassi et al., 2006; Rudgers et al., 2009; Saari et al., 2010), and pathogenic invasion (Álvarez-Loayza et al., 2011; Arnold et al., 2003; Herre et al., 2007). Additionally, the existence of these endophytes in the host plant may be closely related with the metabolome of the host plant (Bálint et al., 2013). Some metabolites with useful pharmacological properties, such as taxol (Stierle et al., 1993; Strobel et al., 1997) and ergoline alkaloids (Kucht et al., 2004; Schardl et al., 2013; Steiner et al., 2008, 2011), were initially referred to as plant metabolites but are now thought to be produced by endophytes, at least in part. This suggests that certain flavors and fragrances derived from plants may be produced by endophytic microorganisms, implying that they may be suitable for studying the *de novo* biosynthesis of novel flavors and fragrances as well as for biotransformation of various precursors. Moreover, such microorganisms may be involved in the terroir effects observed in crop plants, including vanilla.

2.6 VANILLA FLAVOR

2.6.1 Vanillin from *Vanilla* plants

Vanilla flavor metabolites, including vanillin and its precursors, are absent from the leaves of vanilla plants and are found specifically in mature vanilla pods. During the ripening process, the vanillin level gradually increases in the form of its glucoside, reaching a maximum at harvest (8 to 9 months post-pollination). During the curing process, the vanillin glucoside is hydrolyzed by glucosidase activity and, by the end of the curing process, most of the glucoside has been converted into vanillin (Dignum et al., 2001, 2002). Although vanilla pods contain the highest recorded amount of vanillin (**Table 2**), the vanillin biosynthesis pathway is not limited to vanilla plants.

Table 2 - Non-synthetic occurrence of vanillin in plants.

Common name	**Scientific name**	**Botanical family**	**Plant part**	**Vanillin concentration (% dry weight)**
Unicorn plant	*Proboscidea louisianica* (Mill.) Thell.	Martyniaceae	Roots, pod	0.01
Clove	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Myrtaceae	Dry flower buds	Trace
Narcissus	*Narcissus tazetta* L.	Amaryllidaceae	Roots, basal plate	0.01–0.60
Hyacinth	*Hyacinthus orientalis* L.	Asparagaceae	Roots, basal plate	0.20–0.50
Potato	*Solanum tuberosum* L.	Solanaceae	Tuber skin	0.01
Vanilla	*Vanilla planifolia* Jacks. ex Andrews[a]	Orchidaceae	Cured pod	1.00–8.00, although 2.00–3.00 are typically reported[a]
Vanilla	*Vanilla tahitensis* J.W. Moore	Orchidaceae	Cured pod	0.50–2.00
Vanilla	*Vanilla pompona* Schiede	Orchidaceae	Cured pod	0.01–2.00

Adapted from: Havkin-Frenkel and Belanger, 2016.

[a] Highest among the three vanilla species with flavored pods.

Vanillin biosynthesis, and especially which precursors are involved, is still a matter of debate. Various studies have adopted different approaches and models (vanilla cell culture, vanilla plants, and pods), but the vanillin biosynthesis pathway has not been completely elucidated in vanilla (Korthout and Verpoorte, 2007). Biosynthetic steps found in cell cultures of vanilla are not necessarily the same as in the pods. Dixon (2011) suggested that the simple structure of vanillin is amenable to multiple possible biosynthetic pathways given the promiscuity of enzymes in the plant phenolic metabolic pathways; however, there is currently no experimental evidence to prove this hypothesis although it could explain the different findings reported. **Figure 1** depicts a summary of a putative biosynthetic network, starting from phenylalanine and tyrosine and leading to vanillin.

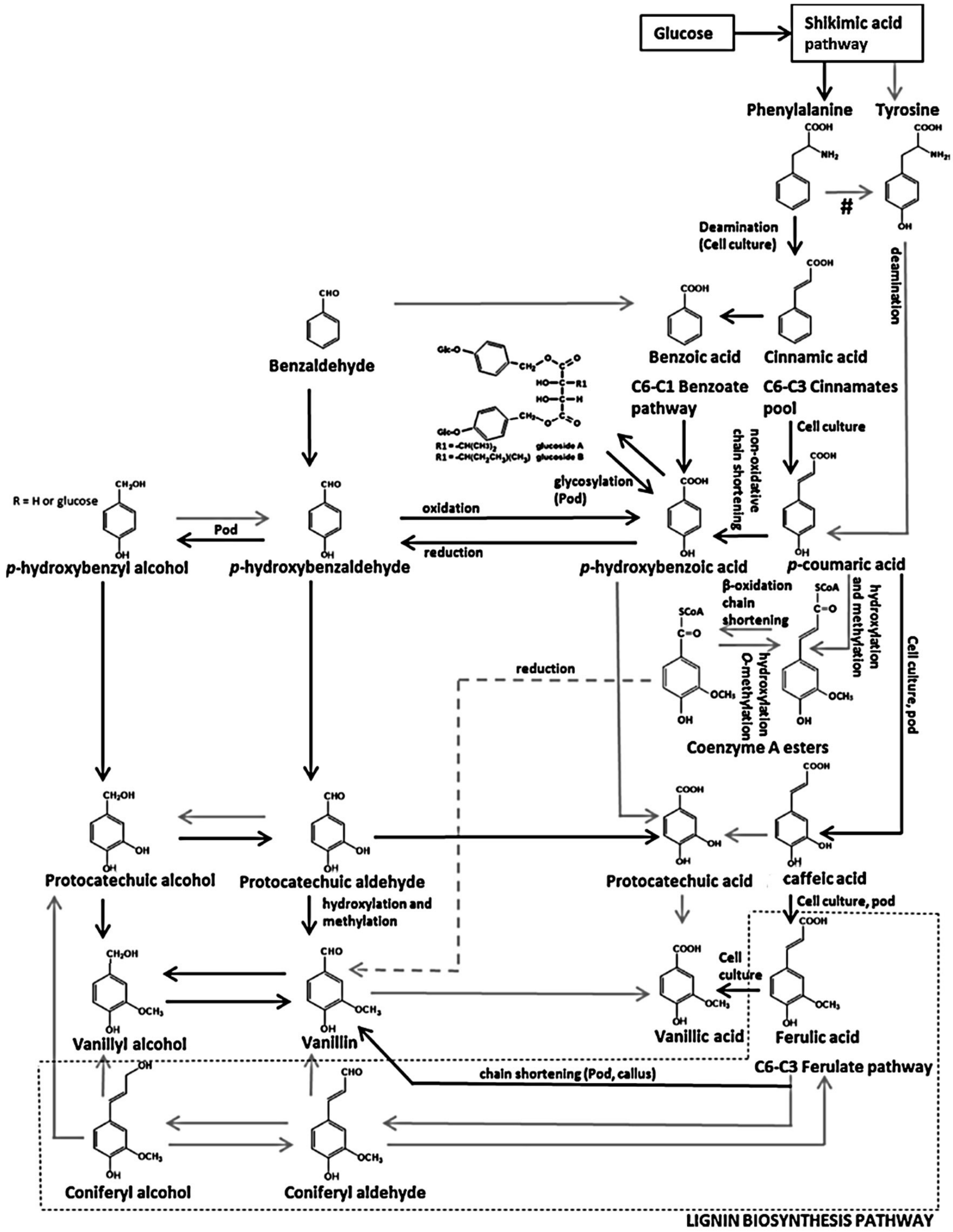


Figure 1. Confirmed vanillin biosynthesis pathways in the plant *Vanilla planifolia* (Bold, Dark lines). The pathways were adapted from (Kundu, 2017; Yang et al., 2017; Gallage et al., 2014; Dixon, 2011; Korthout and Verpoorte, 2007; Havkin-Frenkel and Belanger, 2007; Funk and Brodelius, 1990). The hydroxylation reaction marked with # has not been found in plants and fungi, as phenylalanine and tyrosine are synthesized separately from different precursors (Dewick, 2002). The system (cell culture, pod, callus) in which the step was shown experimentally to occur is indicated.

Vanillin is not an end-product of a pathway, rather it is a possible intermediate for various other products like vanillyl alcohol and vanillic acid.

There is consensus that vanillin originates from the shikimic acid pathway through phenylalanine and tyrosine synthesis (Havkin-Frenkel and Belanger, 2007). However, two major hypotheses exist for the conversion of phenylpropanoid precursors into vanillin. The ferulate pathway involves the hydroxylation and methylation of C6C3, precursor p-caffeic acid, to give ferulic acid or coniferyl alcohol (Havkin-Frenkel and Belanger, 2007); ferulic acid then undergoes chain shortening to yield vanillin. In contrast, the benzoate pathway has the chain shortening event of phenylpropanoids as the first step (Havkin-Frenkel and Belanger, 2007), followed by hydroxylation and methylation of the aromatic ring to yield vanillin. Another very different possibility, not shown in **Figure 1**, is that *p*-hydroxybenzoic acid is derived directly from shikimate (Havkin-Frenkel and Belanger, 2007), bypassing phenylpropanoid production and the degradation of the latter to benzoate pathway intermediates. The phenylpropanoid pathways require phenyl alanine (PAL) or tyrosine ammonia lyase (TAL) activity as a first step, leading to cinnamic acid derivatives. The formation of vanillin via phydroxybenzaldehyde is theoretically feasible; indeed, some intermediates of this pathway have been detected in vanilla pods, e.g., phydroxybenzoic acid, *p*-hydroxybenzaldehyde, protocatechuic aldehyde (Ranadive, 2011), vanillyl alcohol, and *p*-coumaric acid (Hoffman and Zapf, 2011). However, fed radiolabeled *p*-hydroxybenzaldehyde was not incorporated into vanillin or its glycoside (Gallage et al., 2014) which, nonetheless, may be due to highly reactive aldehydes not reaching the site of vanillin biosynthesis. The same enzyme used by Gallage et al. (2014) to prove the ferulate pathway (conversion of ferulic acid to vanillin) was reported to also convert *p*-coumaric acid into *p*-hydroxybenzaldehyde (Havkin-Frenkel et al., 2003, US patent application). These findings all agree with the hypothesis of Dixon (2011) that the plant enzymes may have broad substrate specificity, e.g., one enzyme that can convert all cinnamic acid derivatives into the corresponding aldehyde. Similarly, the β-oxidation or the reduction of the aldehyde may be catalyzed by enzymes that do not distinguish between the different aromatic ring substituents of the substrate. Ferulic acid is the most abundant cinnamic acid derivative found in plants (Mathew and Abraha, 2004), occurring in its free form or covalently linked to lignin and other biopolymers in the cell wall (Lesage-Meessen et al., 1999). Ferulic acid, which has the same substitution pattern on the aromatic ring as vanillin, represents an easy and abundant source for the production of vanillin by biotransformation or synthesis.

2.6.2 Vanilla, vanillin, and microorganisms

Roling et al. (2001) assessed the microbial ecology in vanilla pods from Indonesia at different stages of postharvest processing. Microbial communities showed the biggest change after the scalding of pods in hot water (65–70 °C for 2 min), resulting in a decrease in microbial diversity and the regrowth of fungi. Additionally, the non-pathogenic root mycorrhizal fungi *Ceratobasidium* spp. D.P. Rogers, *Thanatephorus* spp. Donk, and *Tulasnella* spp. J. Schröt. were found to be associated with *V. planifolia* (Porras-Alfaro and Bayman, 2007). Morphological identification, followed by elongation factor gene sequence analysis, showed that several *Fusarium* spp. are present in vanilla plants in Indonesia (Pinaria et al., 2010). Dunphy and Bala (2012a, b) studied the involvement of microorganisms during pod curing with a focus on bacteria and actinomycetes. The bacterial endophytes *Bacillus vanillea* XY18 (= CGMCC 8629=NCCB 100507) and *Bacillus subtilis* (Ehrenberg) Cohn ATCC ® 23857™ isolated from *V. planifolia* pods were shown to increase vanillin content in cured pods compared with the controls (Gu et al., 2015). Endophytes were also identified from roots, stems, and leaves of Colombian *Vanilla* spp. (Gamboa-Gaitán and Otero-Ospina, 2016). Given the consumer demand for non-synthetic products, the current trend is towards the production of vanillin using microorganisms (instead of chemical synthesis), which allows for a non-synthetic label (Lesage-Meessen et al., 1999). Fungal metabolic biosynthesis of compounds with an aromatic ring, including vanillin and its precursors, was studied mainly in the 1940s to 1990s but has since received less attention (Mäkelä et al., 2015). Most vanilla-related studies have focused on the biotransformation of ferulic acid and cinnamic acid and some fungal microorganisms have been found that transform ferulic acid into commercially valuable aromas, including vanillin (Lesage-Meessen et al., 1999; Korthout and Verpoorte, 2007; Mäkelä et al., 2015) (**Figure 2**).

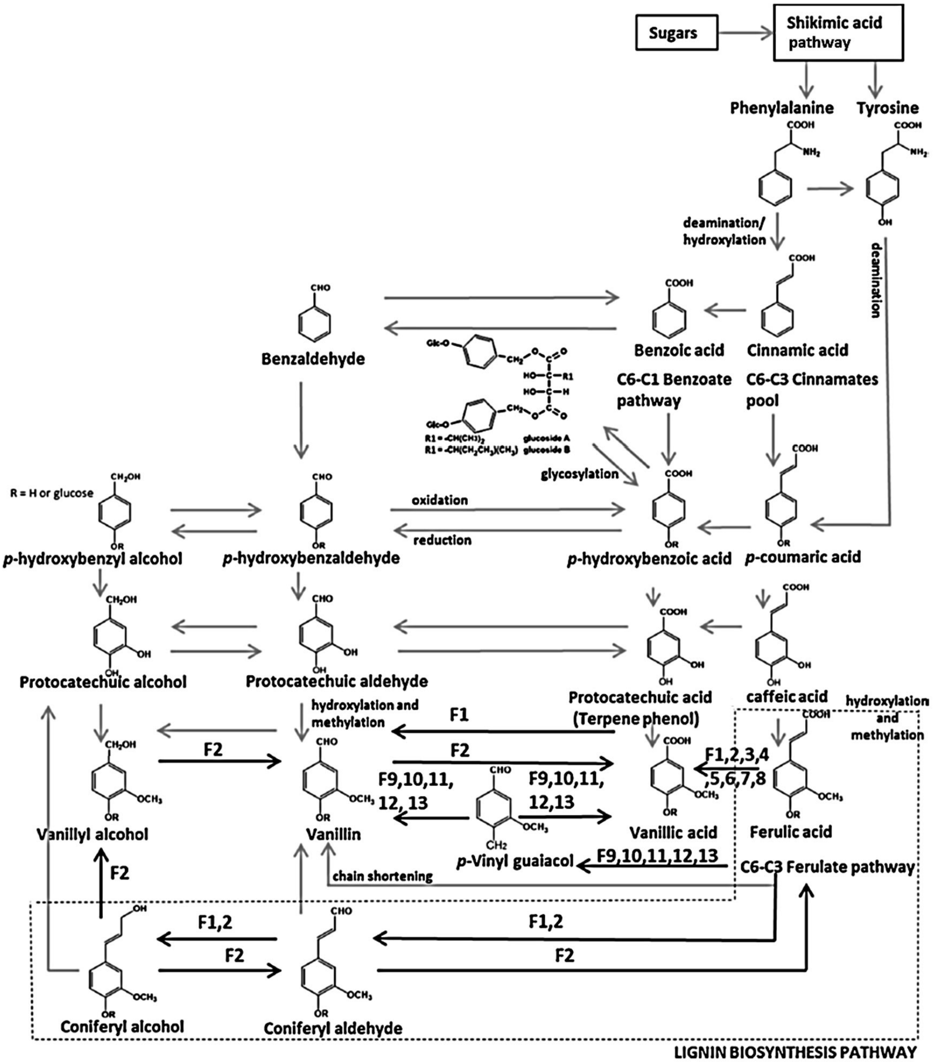


Figure 2. Confirmed vanillin biosynthesis pathways in fungi (Bold, Dark lines). Fungus F1: *Pycnoporus cinnabarinus*, F2: *Trametes* sp., F3: *Aspergillus niger*, F4: *Botrytis* sp., F5: *Cephalosporium* sp., F6: *Penicillium* sp., F7: *Trichoderma* sp., F8: *Verticillium* sp., F9: *Schizophyllum commune*, F10: *Paecilomyces variotii*, F11: *Fusarium solani*, F12: *Sporotrichum thermophile*, F13: *Debaromyces hansenii*. The pathways were adapted from Mäkelä et al. (2015). R=H.

Therefore, it would be of interest to find a possible role for vanilla pod fungal endophytes in the *de novo* biosynthetic and biotransformational reactions of vanilla flavor metabolites. Knowing if fungi affect the vanilla flavor may lead to methods to control the growth and development of such fungi in vanilla plants during postharvest processing of the pods.

2.6.3 Volatile Vanilla flavor metabolites

As discussed in the introduction, the value of cured vanilla pods is not only determined by the vanillin content (Reineccius and Reineccius, 1998). Attention has focused mainly on vanillin and its related analogues (see above) compared to other vanilla flavor metabolites, partly due to vanillin being the first vanilla flavor metabolite to be isolated and commercialized (**Table 1**). The volatiles deserve attention as olfactory receptors are important for vanilla flavor perception. Odor-active compounds can be detected using GC-olfactory detection; however, it is not easy to analyze the effect of the various individual compounds on the complex non-synthetic mixtures in the final flavor.

Initially, artificial vanilla flavor consisted only of vanillin and research did not focus on issues such as detection limit thresholds and organoleptic properties of the other vanilla flavor metabolites. For instance, vanillin is detected as a flavor at a threshold of 0.68 mg/l (Leffingwell and Associates, 2017), whereas guaiacol (another vanilla flavor component) has a substantially lower flavor detection threshold of 0.01 mg/l (approximately 50 times lower); the level of guaiacol in pods is approximately 20 times lower than of vanillin (Takahashi et al., 2013).

Several studies have focused on identifying volatiles from pods that may contribute to the vanilla flavor. A comprehensive list of volatiles from vanilla pods was reported by Toth et al. (2011), but only some of these volatiles are relevant to the vanilla flavor. Gu et al. (2015) reported 42 volatiles from vanilla pods, while Zhang and Mueller (2012) reported 78 odor-active compounds from *V. planifolia*; almost all the volatiles (94%) reported by Gu et al. (2015) were also reported by Zhang and Mueller (2012).

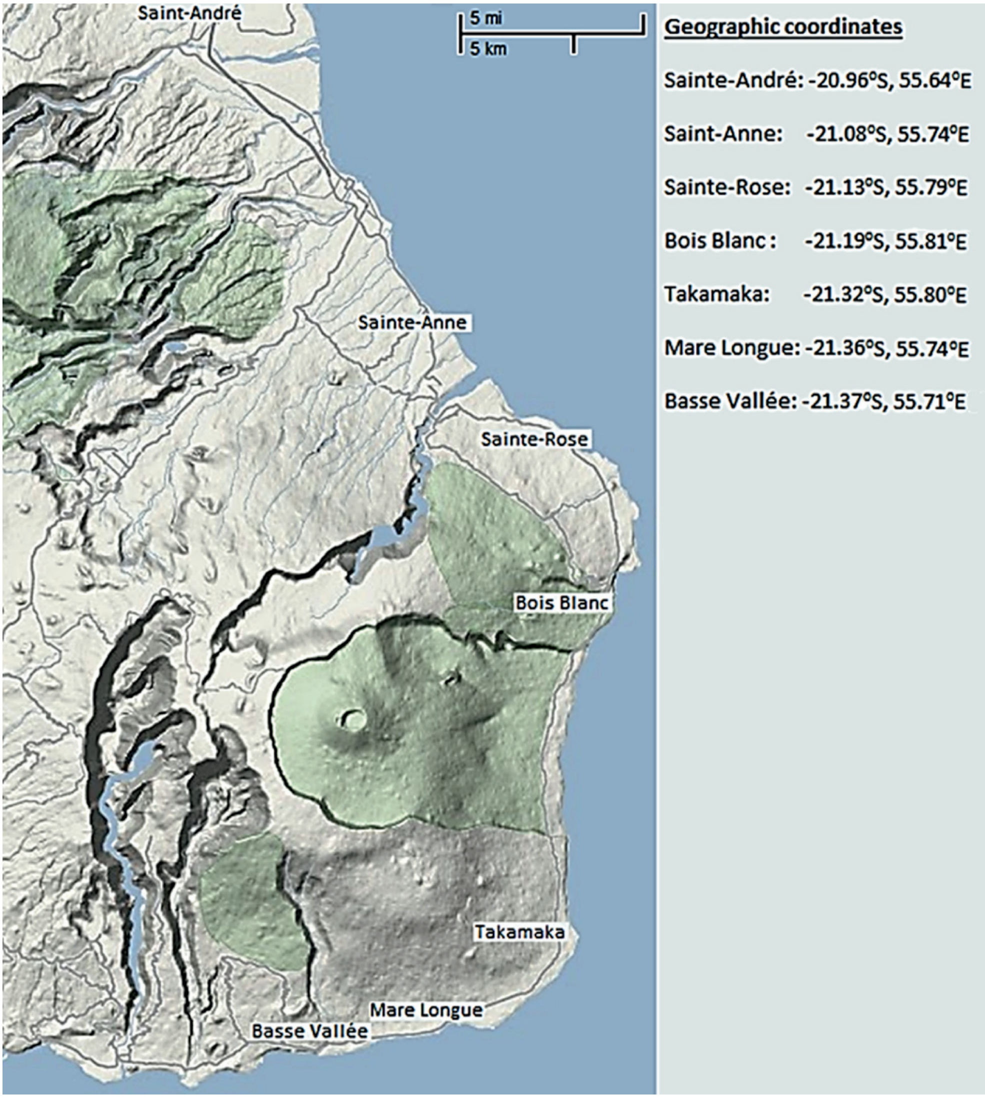
The list of volatiles from the above-mentioned studies includes monoterpenes, sesquiterpenes, ethers, arenes, phenols, and lactones. As an example of volatiles in this list, sesquiterpenes contribute to the vanilla flavor in the pods with a woody, organoleptic tone important for good quality vanilla (Buccellato, 2011). The organoleptic notes associated with these volatiles can be found in catalogues, e.g., from Sigma-Aldrich (2014) (see above).

To date, no reports have been published on a possible role for vanilla-derived endophytes in the production of volatile vanilla flavor notes. Endophyte participation could be an interesting research direction as some fungi are known to synthesize often odor-active volatiles such as terpenoids (Grice et al., 2009), which have potential as flavoring agents (Mäkelä et al., 2015). Vanilla endophytes may similarly be capable of producing some volatile vanilla aroma compounds.

2.7 ENDOPHYTES

2.7.1 Geographical effects on the endophyte community

The community of fungal endophyte species inhabiting a host plant species varies across sites where the host plant is cultivated (David and Seabloom, 2016). The same may apply to vanilla fungal endophytes on Reunion Island (**Figure 3**).



Geographic coordinates

Sainte-André:	-20.96°S, 55.64°E
Saint-Anne:	-21.08°S, 55.74°E
Sainte-Rose:	-21.13°S, 55.79°E
Bois Blanc :	-21.19°S, 55.81°E
Takamaka:	-21.32°S, 55.80°E
Mare Longue:	-21.36°S, 55.74°E
Basse Vallée:	-21.37°S, 55.71°E

Figure 3. East and South Eastern regions in Reunion Island where vanilla cultivation is performed. Fungal endophytes of vanilla were isolated from the same regions to obtain representative samples in Reunion Island.

The variation in aroma and flavor in the same vanilla species from different regions of the world is somewhat distinctive (Ranadive, 2011) (**Table 3**).

Table 3 - A comparison of the organoleptic descriptors of *Vanilla planifolia* pods from different regions of the world.

Region of pod origin	**Vanilla type**	**Organoleptic descriptor**
Madagascar, Reunion Island, Comoro Islands, Seychelles, Mauritius	Bourbon vanilla (*Vanilla planifolia*)	Sweet, creamy, rich, full bodied, tobacco-like, woody, deep balsamic, sweet spicy back notes.
Mexico	Mexican vanilla (*Vanilla planifolia*)	Sharp, slightly pungent, sweet, spicy, lacks body compared with Bourbon vanilla.
Indonesian Islands	Indonesian vanilla (*Vanilla planifolia*)	Less sweet and creamy than Bourbon vanilla. Lacks bouquet. Strong woody, slightly smoky, freshly sharpened pencil note.
India	Indian vanilla (*Vanilla planifolia*)	Full bodied, less sweet, and creamier than Bourbon vanilla. Lacks balsamic notes. Slightly pungent sour notes.

Adapted from: Ranadive (2011)

If indeed vanilla flavor-producing endophytes are found in *V. planifolia*, comparison of endophytes from this plant cultivated in different regions would be of interest with regard to the terroir effect.

Variation in *V. planifolia* pod flavor has been observed across regions of vanilla cultivation on Reunion Island, despite all the plants being cloned and thus genetically identical and the same curing procedure being used for all pods (Bertrand Côme - La Vanilleraie, pers. comm.) (**Figure 3**).

There may be a terroir effect of the cured pod product, similar to that observed for wine. Results from Zarraonaindia et al. (2015) and from Bokulich et al. (2014) showed regional differences in grape microbiota. Zarraonaindia et al. (2015) also showed that besides soil, physicochemical properties, particularly the soil microbiome, determine the metabolome of the vine and this in turn affects the flavor characteristics of wine. The authors speculated that a specific wine terroir could be reproduced at any location by inoculating certain soil microbes onto harvested grapes. A similar microbial participation in the terroir effect of vanilla may exist and, at least in the curing phase, effects of different microbiomes could be important in this regard. It would thus be of interest to study the fungal endophyte distribution in vanilla pods and other organs like the leaves across Reunion Island, and compare this with the metabolome of the pods to chemically characterize the terroir effect. Moreover, the biochemical potential of the endophytes to produce any of the vanilla flavor compounds and related precursor(s) should also be investigated.

2.7.2 Plant-Endophyte interactions

Research on endophytes has grown in recent years (Varma and Gange, 2014). To date, every study for the presence of endophytes in different plant species resulted in the isolation of at least one species of endophytic fungi, while many plants were found to contain hundreds, or even thousands, of species (Faeth and Fagan, 2002). The interaction between endophyte and host plant species may be specific and rather complex.

One of the natural plant responses to microbial infections is the production of phytoalexins, but endophytes do not elicit this defense response in the host plant. However, the interaction of an endophyte with the corresponding host plant may induce the constitutive production of metabolites by the endophyte or the biotransformation of plant metabolites.

These secondary metabolites may play a role in constitutive plant defenses. In the case of infection with other microorganisms, the response becomes even more complex, with the plant producing phytoalexins and possibly inducing endophyte secondary metabolism, with subsequent conversion of the compounds produced by the plant. Because plants contain many endophyte species, the fungi plant interactions are biochemically extremely complex (Pusztahelyi et al., 2015). Such interactions likely influence the vanilla flavor and could explain the terroir effects.

2.7.3 Vanilla endophytes

Several *Bacillus* spp. (*B. amyloliquefaciens* Borris (ex Fukumoto 1943) Priest, *B. subtilis*, *B. vanillea*, *B. licheniformis* (Weigmann) Chester ATCC ® 21415™, *B. smithii* Nakamura et al. ATCC ® 35670™ are reported bacterial endophytes isolated from vanilla material (Gu et al., 2015; White et al., 2014; Roling et al., 2001). However, only *B. subtilis* and *B. vanillea* increased vanillin amounts in cured pods (Gu et al., 2015). Whereas the previously mentioned bacterial endophytes of vanilla were isolated mainly from pods after curing treatment (e.g., after scalding), fungal endophytes were isolated mainly from green pods before curing. Fungal endophytes were isolated from green vanilla pods and leaves from seven different regions in Reunion Island, as shown in **Figure 3** (Khoyratty et al., 2015), confirming fungal endophyte presence in the pods and leaves collected from all seven regions. Thirteen fungal species were from pods, i.e., 57% of all 23 isolated endophyte species. Although there were overlaps, pod fungal endophyte species generally differed (twelve species) from those from the leaf and this may be correlated with the pod having the vanilla flavor while the leaf lacks this. The pod-derived endophytes were grown on media consisting of vanilla green pod material, the organ from which they were isolated.

Through ^{1}HNMR and HPLC analysis, it was shown that three pod fungal endophyte species (*Diaporthe phaseolorum* (Cooke & Ellis) Sacc., *Pestalotiopsis microspora* (Speg.) Bat. & Peres, *Fusarium oxysporum* Schltdl.) increased the levels of vanilla flavor compounds present in the pod-based growth media (vanillin, vanillyl alcohol, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid) (Khoyratty et al., 2015). The pod-derived fungal endophyte species varied according to isolation region, suggestive of a terroir effect (**Table 4**).

Table 4- Endophyte fungal species isolated from *Vanilla planifolia* and the increase in vanilla flavor compounds (^{1}HNMR analysis) after growth on a vanilla pod agar medium for 30 days.

Fungal species	Isolation organ		Isolation region in Reunion Island							Increase in vanilla flavor compounds in green pod medium
	Pod[a]	Leaves	St Andre	St Anne	St Rose	Bois Blanc	Takamaka	Mare Longue	Basse Vallee	
Fusarium proliferatum (Matsush.) Nirenberg ex Gerlach & Nirenberg	Y	N	Y	Y	Y	Y	Y	Y	Y	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
	N	Y	Y	Y	Y	N	Y	Y	N	Acetate
Nigrospora sp1 Zimm.	N	Y	Y	N	N	N	N	N	N	Acetate
Diaporthe phaseolorum (Cooke & Ellis) Sacc.	Y	N	Y	N	N	N	N	N	N	Vanillin Vanillyl alcohol *p*-hydroxybenzaldehyde *p*-hydroxybenzoic acid
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.	Y	N	Y	N	N	N	N	N	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
Xylaria sp. Hill ex Schrank	N	Y	Y	N	N	N	N	N	N	Acetate
Colletotrichum sp2 Corda	N	Y	Y	N	N	N	N	N	N	Acetate
Penicillium citrinum Thom	N	Y	Y	N	N	N	N	N	N	Acetate
Sarcosomataceae spp. Kobayasi	N	Y	N	N	N	N	Y	N	N	Acetate
Guignardia mangiferae A.J. Roy	N	Y	N	N	N	N	N	Y	N	Acetate
Perenniporia nanlingensis B.K. Cui & C.L. Zhao	N	Y	N	N	N	N	N	Y	N	Acetate
Fusarium oxysporum Schltdl.	Y	N	N	Y	N	N	N	N	N	Vanillin Vanillyl alcohol *p*-hydroxybenzaldehyde *p*-hydroxybenzoic acid
Cunninghamella blakesleana Lendn.	Y	N	N	Y	N	N	N	N	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
Botryosphaeria ribis Grossenb. &	Y	N	N	Y	N	N	N	N	N	Glucoside A Glucoside B

Duggar										*p*-hydroxybenzaldehyde glucoside
Nigrospora sp 2 Zimm.	Y	N	N	Y	N	N	N	N	N	Vanillin Acetate
Aspergillus fumigatus Fresen.	Y	N	N	Y	N	N	N	N	N	Glucoside A Acetate
Acremonium implicatum (J.C. Gilman & E.V. Abbott) W. Gams	Y	N	N	N	Y	N	N	N	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
Nemania bipapillata (Berk. & M.A. Curtis) Pouzar	Y	N	N	N	Y	N	N	N	N	Glucoside A Acetate
Pestalotiopsis microspora (Speg.) Bat. & Peres	Y	N	N	N	Y	N	N	N	N	Vanillin Vanillyl alcohol *p*-hydroxybenzaldehyde *p*-hydroxybenzoic acid
Botryosphaeria ribis Grossenb. & Duggar	Y	N	N	N	Y	N	N	N	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
Phomopsis phyllanthicola C.Q. Chang, Z.D. Jiang & P.K. Chi	Y	N	N	N	N	N	N	Y	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside
Mycosphaerella marksii Carnegie & Keane	Y	N	N	N	N	N	N	Y	N	Glucoside A Glucoside B *p*-hydroxybenzaldehyde glucoside

Adapted from: Khoyratty et al., 2015.

[a] Pod-derived endophytes are of particular interest given that only pods contain the vanilla flavor.

Pod fungal endophyte species unique to only one region include two from St. Andre, four from St. Anne, three from St. Rose, and two from Mare Longue, whereas no unique pod fungal endophyte species from Basse Vallee, Takamaka, and Bois Blanc were observed. However, further experiments are required to confirm the terroir effect and find volatile emissions related to the vanilla flavor from pod-derived fungal endophytes.

2.7.4 Vanilla Flavor Synthesis in Endophytes and Non-Endophytes

As reported here, several endophytic and non-endophytic bacteria and fungi were shown to synthesize vanilla flavor metabolites. The highest vanillin yield (11,500 mg/L) reported for a microorganism is from *Amycolatopsis* sp. Rabenhorst (**Table 1**) after feeding of the actinomycete with 19,900 mg/l of ferulic acid (Rabenhorst and Hopp, 2002). However, no similar experiments were conducted on other micro-organisms (in **Table 1**), i.e., none have been fed the same amount of ferulic acid. Given this difference, it is difficult to accurately compare synthesized vanillin yield across microbial endophytes and non-endophytes from these reports. Vanilla endophytes offer an advantage over non-endophytes as a terroir effect (unique vanilla flavor) in a region may be reproduced in pods in any cultivation region by infecting vanilla plants with vanilla endophytes. Additionally, experimental conditions should be found to maximize vanillin synthesis by endophytes.

2.8 CONCLUSIONS

This review illustrates the biosynthetic network for vanillin and related vanilla flavor compounds, parts of which have also been found in various other microorganisms. Indeed, some microorganisms are now known to produce vanillin through precursor biotransformation and, additionally, genetic engineering has resulted in microorganisms capable of producing vanillin *de novo*. Endophytes have also been shown to produce secondary metabolites with economic value that were originally thought to be produced by the plant, and several excellent reviews are available on this topic (Pusztahelyi et al., 2015; Ludwig-Müller, 2015; Hongsheng et al., 2010). Although some studies have been performed on bacteria isolated from vanilla plants, this area requires further investigation. Fungal endophytes and other microorganisms connected with vanilla plants may contribute one or more steps towards vanillin biosynthesis, which could be useful for the production of vanillin, or even the vanilla flavor, during the maturing of the pods or postharvest processing. Moreover, fungal endophytes may play a role in terroir effects which are common for the vanilla flavor. Studies on vanilla fungal endophytes may help to explain some of the controversial findings concerning vanillin biosynthesis and lead to new microorganisms that can produce non-synthetic vanillin and vanilla flavor. As a first step towards this goal, vanilla pod-derived fungal endophytes have been shown to affect the spectra of the vanilla flavor in growth media based on vanilla pods (Khoyratty et al., 2015).

2.9 REFERENCES

Álvarez-Loayza P., White J.F., Torres M.S., Balslev H., Kristiansen T., Svenning J.C., Gil N. (2011) Light Converts Endosymbiotic Fungus to Pathogen, Influencing Seedling Survival and Niche-Space Filling of a Common Tropical Tree, Iriartea deltoidea. PLoS ONE 6:e16386.

Alwahti A.Y. (2003) A taste of *Vanilla*. The TED Case Studies. Number 686. http://www1.american.edu/TED/vanilla.htm (accessed: July 30, 2016)

Arnold A.E., Mejía L.C., Kyllo D., Rojas E.I., Maynard Z., Robbins N., Herre E.A. (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences USA 100:15649-54.

Bálint M., Tiffin P., Hallström B., O'Hara R.B., Olson M.S., Fankhauser J.D., Piepenbring M., Schmitt I. (2013) Host Genotype Shapes the Foliar Fungal Microbiome of Balsam Poplar (*Populus balsamifera*). PLoS ONE 8:e53987.

Bayat F., Mirlohi A., Khodambashi M. (2009) Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. Russian Journal of Plant Physiology 56:510-16.

Bokulicha N.A., Thorngated J.H., Richardsone P.M., Mills D.A. (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proceedings of the National Academy of Sciences USA 111:E139-48.

Buccellato F. (2011) Vanilla in Perfumery and Beverage Flavors. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp.235-40.

Burdock G.A. (2010) Fenaroli's Handbook of Flavor Ingredients. 6th edition. CRC Press. NY P 2001.

Cameron K.M. (2011) Vanilla phylogeny and classification. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp. 243.

Chakraborty, D., Selvam, A., Kaur, B., Wong, J., Karthik, O., 2017. Application of recombinant *Pediococcus acidilactici* BD16 (fcs +/ech +) for bioconversion of agrowaste to vanillin. Applied Microbiology and Biotechnology 101:5615–26.

Chen, P., Yan, L., Wu, Z., Li, S., Bai, Z., Yan, X., Wang, N., Liang, N., Li, H. (2016) A microbial transformation using Bacillus subtilis B7-S to produce natural vanillin from ferulic acid. *Scientific Reports* 6. https://doi.org/10.1038/srep20400.

David A.S., Seabloom E.W. (2016) Plant Host Species and Geographic Distance Affect the Structure of Aboveground Fungal Symbiont Communities, and Environmental Filtering Affects Belowground Communities in a Coastal Dune Ecosystem. Microbial Ecology 71:912–26.

De Sassi C., Müller C.B., Krauss J. (2006) Fungal plant endosymbionts alter life history and reproductive success of aphid predators. Proceedings of the Royal Society. Biological Sciences 273:1301-6.

Dewick P.M. (2002) The shikimate pathway: aromatic amino acids and phenylpropanoids. In: Dewick P.M. (ed.). Medicinal natural products. John wiley and sons Ltd, West Sussex, pp.121-66.

Dignum M., Kerler J., Verpoorte R. (2001) Vanilla production: technological, chemical and biosynthetic aspects. Food Research International 17:199-219.

Dignum M.J.W., Kerler J., Verpoorte R. (2002) Vanilla curing under laboratory conditions. Food Chemistry 79:165-71.

Dionisio A.P., Molina G., Carvalho D.S., Pastore G.M. (2012) Natural flavourings from biotechnology for foods and beverages. In: Baines D., Seal R. Natural Food Additives, Ingredients and Flavourings. Woodhead Publishing Limited. Cambridge, pp.231-59.

Dixon R.A. (2011) Vanillin Biosynthesis – Not as Simple as it Seems? In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp. 292–8.

Dunphy P., Bala K. (2012a) The role of Microorganisms in Vanilla Curing. Part 1: evidence for microbial involvement. Perfumer and Flavorist 37:24–9.

Dunphy P., Bala K. (2012b) The role of Microorganisms in Vanilla Curing. Part 2: Microbial transformation of phenols and other compounds. Perfumer and Flavorist 37:22–7.

Faeth S.H., Fagan W.F. (2002) Fungal Endophytes: Common Host Plant Symbionts but Uncommon Mutualists. Integrative Comparative Biology 42:360-8.

Firmenich (2017) Firmenich enriches *Vanilla* palette with sustainable and cost-effective natural tonalities. http://www.firmenich.com/en_INT/company/news/FIRMENICH-ENRICHES-VANILLA-PALETTE.html (accessed: September 09, 2017)

Funk C., Brodelius P.E. (1990) Phenylpropanoid Metabolism in Suspension Cultures of *Vanilla planifolia* Andr. Plant Physiology 94:95-101.

Gallage N.J., Hansen E.H., Kannangara R., Olsen C.E., Motawia M.S., Jørgensen K., Holme I., Hebelstrup K., Grisoni M., Møller B.L. (2014) Vanillin formation from ferulic acid in *Vanilla planifolia* is catalysed by a single enzyme. Nature Communications 5:4037.

Gamboa-Gaitán, M.A., Otero-Ospina, J.T., 2016. Colombian vanilla and its microbiota. III. Diversity and structure of the endophytic community. Acta Botanica Hungary 58:241–56.

Gimenez C., Cabrera R., Reina M., Gonzalez-Coloma A. (2007) Fungal Endophytes and their Role in Plant Protection. Current Organic Chemistry 11:707-20.

Givaudan (2016) Anything but Plain Vanilla. Givaudan Taste Essentials Vanilla. https://www.givaudan.com/flavours/world-flavours/tasteessentials/vanilla (accessed July 29, 2016)

Gleason-Allured J. (2011) Vanilla: Anything but Plain. Perfumer and Flavorist. http://www.perfumerflavorist.com/flavor/application/vanilla/132347233.html (accessed: July 30, 2016)

Grice K., Lu H., Atahan P., Asif M., Hallmann C., Greenwood P., Maslen E., Tulipani S., Williford K., Dodson J. (2009) New insights into the origin of perylene in geological samples. Geochimica et Cosmochimica Acta 73:6531-43.

Gu F., Chen Y., Fang Y., Wu G., Tan L. (2015) Contribution of Bacillus isolates to the flavor profiles of vanilla beans assessed through aroma analysis and chemometrics. Molecules 20:18422-36.

Hansen E.H., Møller B.L., Kock G.R., Bünner C.M., Kristensen C., Jensen O.R., Okkels F.T., Olsen C.E., Motawia M.S., Hansen J. (2009) De Novo Biosynthesis of Vanillin in Fission Yeast (*Schizosaccharomyces pombe*) and Baker's Yeast (*Saccharomyces cerevisiae*). Applied and Environmental Microbiology 75:2765-74.

Hardoim P.R., Overbeek L.S.V., Berg G., Pirttila A.M., Compant S., Campisano A., Doring M., Sessitsch A. (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiology and Molecular Biology Reviews 79:293-320.

Havkin-frenkel D., Podstolski A., Dixon R.A. (2003) Vanillin Biosynthetic Pathway Enzyme from *Vanilla planifolia*. US Patent US20030070188 A1 (US Patent Office: United States, 2003).

Havkin-Frenkel D., Belanger F.C. (2007) Application of metabolic engineering to vanillin biosynthetic pathways in *Vanilla planifolia*. In: Verpoorte R., Alfermann A.W., Johnson T.S. (eds.). Applications of plant metabolic engineering. Springer, Dordrecht, pp.175-196.

Havkin-Frenkel D., Belanger F.C. (2016) Biotechnological production of vanillin. In: Havkin-Frenkel D., Dudai N. (Eds) Biotechnology in flavor production. 2nd Ed. John Wiley & Sons, Ltd. West Sussex, pp.168.

Herre E.A., Mejía L.C., Kyllo D.A., Rojas E., Maynard Z., Butler A., Van Bael S.A. (2007) Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88:550-8.

Hoffman P.G., Zapf C.M. (2011) Flavor, Quality, and Authentication. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp.162-79.

Hongsheng Y., Zhang L., Li L., Zheng C., Guo L., Li W., Sun P., Qin L. (2010) Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiological Research 165:437-49.

Kaur B., Chakraborty D. (2013) Biotechnological and Molecular Approaches for Vanillin Production: a Review. Applied Biochemistry and Biotechnology 169:1353–72.

Kennedy C.R. (2015) The Flavor Rundown: Natural vs. Artificial Flavors: What's in a Flavor? *Harvard University*. http://sitn.hms.harvard.edu/flash/2015/the-flavor-rundown-natural-vs-artificial-flavors/ (accessed July 29, 2016)

Khoyratty, S., Dupont, J., Lacoste, S., Palama, T.L., Choi, Y.H., Kim, H.K., Payet, B., Grisoni, M., Fouillaud, M., Verpoorte, R., Kodja, H., 2015. Fungal endophytes of Vanilla planifolia across Réunion Island: isolation, distribution and biotransformation. BMC Plant Biology 15:142.

Korthout H., Verpoorte R. (2007) Vanilla. In: Berger RG. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. Springer Science + Business Media. Leipzig, pp.203-17.

Kucht S., Groß J., Hussein Y., Grothe T., Keller U., Basar S., König W., Steiner U., Leistner E. (2004) Elimination of ergoline alkaloids following treatment of *Ipomoea asarifolia* (Convolvulaceae) with fungicides. Planta 219: 619-25.

Kundu A. (2017) Vanillin biosynthetic pathways in plants. Planta 245:1069-78.

Labuda I. (2011) Biotechnology of Vanillin: Vanillin from Microbial Sources. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of *Vanilla* Science and Technology. Wiley-Blackwell, Oxford, pp. 301-27.

Leffingwell & Associates (2017) Odor & Flavor Detection Thresholds in Water (In Parts per Billion). http://www.leffingwell.com/odorthre.htm (accessed: 5 March 2017)

Lesage-Meessen L., Stentelaire C., Lomascolo A., Couteau D., Asther M., Moukha S., Record E., Sigoillot J., Asther M. (1999) Fungal transformation of ferulic acid from sugar beet pulp to natural vanillin. Journal of the Science of Food and Agriculture 79:487–90.

Lesage-Meessen L., Lomascolo A., Bonnin E., Thibault J.F., Buleon A., Roller M., Asther M., Record E., Ceccaldi B.C., Asther M. (2002) A biotechnological process involving filamentous fungi to produce natural crystalline vanillin from maize bran. Applied Biochemistry and Biotechnology. 103:141-53.

Ludwig-Müller J. (2015) Plants and endophytes: equal partners in secondary metabolite production? Biotechnology letters 37. 10.1007/s10529-015-1814-4.

Mäkelä M.R., Marinović M., Nousiainen P., Liwanag A.J., Benoit I., Sipilä J., Hatakka A., de Vries R.P., Hildén K.S. (2015) Aromatic metabolism of filamentous fungi in relation to the presence of aromatic compounds in plant biomass. Advances in applied microbiology 91:63-137.

Mathew S., Abraha T.E. (2004) Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Critical Reviews in Biotechnology 24:59-83.

Motedayen, N., Maznah, I., 2013. Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725. African Journal of Biotechnology 12:6618–24.

Ni J., Tao F., Du H., Xu P. (2015) Mimicking a natural pathway for *de novo* biosynthesis: natural vanillin production from accessible carbon sources. Scientific Reports 5:13670. doi:10.1038/srep13670.

Gleason-Allured J. (2014) Givaudan Submits Patent Application for Vanilla Fermentation Process. Perfumer and Flavorist

http://www.perfumerflavorist.com/networking/news/company/Givaudan-Submits-Patent-Application-For-Vanilla-Fermentation-Process-238896611.html (accessed July 29, 2016)

Pinaria, A.G., Liew, E.C.Y., Burgess, L.W. (2010) *Fusarium* species associated with vanilla stem rot in Indonesia. Australasian Plant Pathology 39:176-83.

Porras-Alfaro, A., Bayman, P. (2007) Mycorrhizal fungi of vanilla: Diversity, specificity and effects on seed germination and plant growth. Mycologia 99:510–25.

Pusztahelyi T., Holb I.J., Pócsi I. (2015) Secondary metabolites in fungus-plant interactions. Frontiers in Plant Science 6:573. doi:10.3389/fpls.2015.00573.

Rabenhorst, J., Hopp, R., 2002. Process for the preparation of vanillin and suitable microorganisms. European Union patent EP0761817.

Ranadive A.S. (2011) Quality control of vanilla beans and extracts. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of *Vanilla* Science and Technology. Wiley-Blackwell, Oxford, pp.141-60.

Rasoanaivo, P. (1998) Essential oils of economic value in Madagascar: Present state of knowledge. HerbalGram 43:31–59.

Reineccius G. (1998) Natural Flavoring Materials. In: Reineccius G. (ed.) Source Book of Flavors. 2nd Edn. Chapman & Hall, NY, p.357.

Roling W.F.M., Kerler J., Braster M., Apriyantono A., Stam H., Verseveld H.W.V. (2001) Microorganisms with a taste for *Vanilla*: microbial ecology of traditional Indonesian vanilla curing. Applied Environmental Microbiology 67:1995-2003.

Royal Society of Chemistry (2016) Chemistry in its element – vanillin. http://www.rsc.org/chemistryworld/podcast/CIIEcompounds/transcripts/vanillin.asp (accessed July 29, 2016)

Rudgers J.A., Afkhami M.E., Rúa M.A., Davitt A.J., Hammer S., Huguet V.M. (2009) A fungus among us: broad patterns of endophyte distribution in the grasses. Ecology 90:1531-9.

Saari S., Sundell J., Huitu O., Helander M., Ketoja E., Ylönen H., Saikkonen K. (2010) Fungal-Mediated Multitrophic Interactions - Do Grass Endophytes in Diet Protect Voles from Predators? PLoS ONE 5:e9845.

Schardl C.L., Young C.A., Hesse U., Amyotte S.G., Andreeva K., Calie P.J., Fleetwood D.J., Haws D.C., Moore N., Oeser B., Panaccione D.G., Schweri K.K., Voisey C.R., Farman M.L., Jaromczyk J.W., Roe B.A., O'Sullivan D.M., Scott B., Tudzynski P., An Z., Arnaoudova E.G., Bullock C.T., Charlton N.D., Chen L., Cox M., Dinkins R.D., Florea S., Glenn A.E., Gordon A., Güldener U., Harris D.R., Hollin W., Jaromczyk J., Johnson R.D., Khan A.K., Leistner E., Leuchtmann A., Li C., Liu J., Liu J., Liu M., Mace W., Machado C., Nagabhyru P., Pan J., Schmid J., Sugawara K., Steiner U., Takach J.E., Tanaka E., Webb J.S., Wilson E.V., Wiseman J.L., Yoshida R., Zeng Z. (2013) Plant-Symbiotic Fungi as Chemical Engineers: Multi-Genome Analysis of the Clavicipitaceae Reveals Dynamics of Alkaloid Loci. PLoS Genet 9:e1003323.

Sigma-Aldrich (2014) Ingredients Catalog: Flavors & Fragrances.

Sinha A.K., Sharma U.K., Sharma N. (2008) A comprehensive review on vanilla flavor: extraction, isolation and quantification of vanillin and others constituents. International Journal of Food Science and Nutrition 59:299–326.

Soto-Arenas, M.A., Cribb, P., 2010. A new infrageneric classification and synopsis of the genus *Vanilla* Plum. ex Mil. (Orchidaceae: Vanillinae). *Lankesteriana* 9, 355–98.

Souza J.J.D., Vieira I.J.C., Rodrigues-Filho E., Braz-Filho R. (2011) Terpenoids from Endophytic Fungi. Molecules 16:10604-18.

Steiner U., Hellwig S., Leistner E. (2008) Specificity in the interaction between an epibiotic clavicipitalean fungus and its convolvulaceous host in a fungus/plant symbiotum. Plant Signaling & Behavior 3:704-6.

Steiner U., Leibner S., Schardl C.L., Leuchtmann A., Leistner E. (2011) Periglandula, a new fungal genus within the Clavicipitaceae and its association with Convolvulaceae. Mycologia 103:1133-45.

Stierle A., Strobel G., Stierle D. (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214-6.

Strobel G.A., Hess W.M., Li J.Y., Ford E., Sears J., Sidhu R.S., Summerell B. (1997) Pestalotiopsis guepinii, a Taxol-producing Endophyte of the Wollemi Pine, *Wollemia nobilis*. Australian Journal of Botany 45:1073-82.

Takahashi M., Inai Y., Miyazawa N., Kurobayashi Y., Fujita A. (2013) Key odorants in cured Madagascar vanilla beans (*Vanilla planifolia*) of differing bean quality. Bioscience, Biotechnology and Biochemistry 77:606-11.

Thibault F.J., Asther M., Colonna-Ceccaldi B., Couteau D., Delattre M., Duarte J.C., Craig F., Heldt-Hansen P.H., Kroon P.A., Lesage-Meessen L., Micard V. & C. Renard, M. Tuohy, Van Hulle S., Williamson G. (1998) Fungal Bioconversion of Agricultural By-Products to Vanillin. Food Science and Technology-Lebensmittel-Wissenschaft & Technologie 31:530-6.

Toms A., Wood J.M. (1970) Degradation of trans-ferulic acid by *Pseudomonas acidovorans*. Biochemistry 9:337–43.

Toth S., Lee K.J., Havkin-Frenkel D., Belanger F.C., Hartman T.G. (2011) Volatile Compounds in *Vanilla*. In: Havkin-Frenkel D., Belanger F.C. (eds.). Handbook of Vanilla Science and Technology. Wiley-Blackwell, Oxford, pp.183-218.

Varma, V.C., Gange, A.C. (2014) Advances in Endophytic Research. Springer, India. White, J.F., Torres, M.S., Sullivan, R.F., Jabbour, R.E., Chen, Q., Tadych, M., Irizarry, I., Bergen, M.S., Havkin-Frenkel, D., Belanger, F.C. (2014) Occurrence of *Bacillus amyloliquefaciens* as a systemic endophyte of vanilla orchids. *Microscopy Research and Technique* 77:874–885.

Yang H., Barros-Rios J., Kourteva G., Rao X., Chen F., Shen H., Liu C., Podstolski A., Belanger F., Havkin-Frenkel D., Dixon R.A. (2017) A re-evaluation of the final step of vanillin biosynthesis in the orchid *Vanilla planifolia*. Phytochemistry 139:33-46.

Zarraonaindia I., Owens S.M., Weisenhorn P., West K., Hampton-Marcell J., Lax S., Bokulich N.A., Mills D.A., Martin G., Taghavi S., van der Lelie D., Gilbert J.A. (2015) The soil microbiome influences grapevine-associated microbiota. Microbial Cell 2:171-3.

Zhang S., Mueller C. (2012) Comparative Analysis of Volatiles in Traditionally Cured Bourbon and Ugandan Vanilla Bean (*Vanilla planifolia*) Extracts. Journal of Agriculture and Food Chemistry 60:10433−44.

Zheng L., Zheng P., Sun Z., Bai Y., Wang J., Guo X. (2007) Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus*. Bioresource Technology 98:1115-9.