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

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Chapter 1: *Vanilla* endophytes. General Introduction and outline of thesis

1.1 BACKGROUND

Vanilla is the most popular among flavors and fragrances in the world (Dunphy and Bala 2012; Rain 2004). Vanilla flavor originates from pods of *Vanilla planifolia* Jacks. ex Andrews, a member of the Orchidaceae family (Ramachandra and Ravishankar 2000). This natural flavor is widely used in many industries such as food, beverages, pharmaceuticals, cosmetics and tobacco (Medina et al. 2009). Vanilla flavor products include cured pods and alcoholic extracts of cured pods. These products are expensive. The supply is low compared to the demand. Vanilla flavor is complex (Toth et al. 2011). It consists of a myriad of organoleptic descriptors like vanillin, balsamic, anisic, floral and others. Vanilla extract contains more than 250 flavor active components. Those components include volatiles and non-volatiles. Vanillin is a major contributor to the flavor. It occurs at 1-2% of the dry weight of pods (Havkin-Frenkel and Belanger, 2016). Unexpectedly, sensory analysis showed little correlation between good Vanilla flavor and vanillin content of the pods (Hoffman and Zapf, 2011). In fact, vanillin explains less than 12% of the organoleptic variability across *Vanilla* pods. Hence, other than vanillin, the volatile and semi-volatile compounds present in Vanilla extracts are responsible for many of the sensory attributes of the pods. Vanillin has an aryl ring system. Other compounds with an aryl ring system, which are volatiles, detected in *Vanilla* pods include acids, alcohols, aldehydes, ethers and ketones. Additionally, monoterpenoids and sesquiterpenoids were detected in non-derivatized extracts of Bourbon and Ugandan dark pods through GC-MS analysis (Zhang and Mueller 2012).

Vanillin can be produced by *de novo* synthesis or by biotransformation of natural precursors. *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillyl alcohol. Furthermore, ferulic acid can all be converted to vanillin by biotransformation (Hoffman and Zapf 2011, Labuda 2011). Ferulic acid tends to be the preferred precursor for vanillin formation by microorganisms (Labuda 2011).

(Bio)Synthetic pure vanillin is used in low cost products due to its much lower price than natural *Vanilla* material. However, for high quality products, natural *Vanilla* is preferred, because of the much richer complex flavor than pure vanillin. Plant-originated Vanilla extract has a delicate, yet rich and mellow aroma after-taste (Reineccius 1994). On the other hand, pure vanillin has a heavy, grassy odor and a less pleasant after-taste than plant Vanilla flavor.

Before the 1980's, the major advantage of *V. planifolia* flavor over pure vanillin was its more elegant fragrance due to the presence of additional flavor compounds. In the early 1980's the demand in international markets for natural products gained more importance, as they were regarded as safe and less toxic. Besides, the introduction of food safety laws, regulations and procedures intended to protect consumers' health and reduce the cost of health care contributed to the preference for natural Vanilla flavor over synthetic vanillin (Medina et al. 2009).

The world market demands more natural Vanilla extract instead of its replacement pure (synthetic) vanillin, ethyl vanillin and coumarin. Additionally, coumarin has been associated with cancer (Medina et al. 2009). As the supply of *V. planifolia* flavor remains too low for the demand, the price is very high. Moreover, the yearly supply is rather erratic due to climatic and political instability in the producing regions. This prompted the development of natural production methods of vanillin, using biotransformation of natural vanillin precursors into vanillin, using microorganisms. Additionally, more research is now being done aiming at finding flavor compositions that could match better the natural *V. planifolia* Vanilla flavor (Gleason-Allured 2014). Vanillin produced by biotransformation of known natural precursors by microorganisms is labelled as a natural product (Korthout and Verpoorte 2007). However, no naturally occurring microorganism is reported in literature to produce vanillin *de novo*, rather microorganisms are used to biotransform fed precursors into vanillin (Converti et al. 2010, Gallage et al. 2014). *De novo* vanillin production by microorganisms has only been possible through genetic transformation. In this way, two yeast species *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were metabolically engineered with genes from mold (*Podospora pauciseta*), bacteria (*Nocardia* sp.), humans (*Homo sapiens*) and plants (*Arabidopsis thaliana*) to enable conversion of glucose (primary metabolite) to vanillin (Hansen et al. 2009). Vanillin yield can decrease by conversion into vanillyl alcohol. To prevent this, the alcohol dehydrogenase ADH6 genes of both yeast species were disabled by gene knockout. *Escherichia coli* was also engineered to produce vanillin *de novo* from primary metabolites such as glucose and tyrosine (Ni et al. 2015).

Fungal endophytes are microorganisms that co-exist in the plant but do not cause visible symptoms to the host (Ludwig-Müller 2015). Endophytes are known to participate either partially or completely in biosynthetic pathways of plant secondary metabolism (Ludwig-Müller 2015). Additionally, the host plant and the endophyte may induce changes in the metabolism of its partner (Hardoim et al. 2015). *Vanilla* fungal endophytes may thus contribute in different ways to Vanilla flavor in *Vanilla* plants. Apart from vanillin, microorganisms are also known to produce metabolites associated to Vanilla flavor (Gu et al. 2015). Thus it is of interest to learn if microorganisms inside *Vanilla* plants could participate in the production of Vanilla flavor metabolites.

As plants harbor endophytes that may participate in the biosynthesis of various plant secondary metabolites, it is of interest to learn more about the role of endophytes in *V. planifolia* plants. In particular, whether those endophytes participate in the formation of Vanilla flavor in the pods. From sources other than *V. planifolia* plants, several fungi have already been isolated that are capable of producing vanillin from various precursors. It is thus possible that *Vanilla* plant endophytes have similar traits. After the isolation of *Vanilla* endophytes, these can be tested for *de novo* production of vanillin and vanillin precursors or for the biotransformation of plant vanillin precursors into vanillin. This could lead to fungi that can be used to make natural vanillin from readily available precursors or plant material. Additionally, we aimed at finding endophytes that may contribute to the formation of Vanilla flavor components other than vanillin.

1.2 <u>AIM OF THE THESIS</u>

These questions were the basis of the research described in this thesis: proving the presence of endophytes in *Vanilla*, and to study their capacity for chemical modifications related to Vanilla flavor compounds (vanillin biosynthesis and other flavor compounds). To find answers the following approaches were used:

- To isolate and identify endophytes unique to *Vanilla* pods.
    - Pod unique endophytes are important given only pods contain Vanilla flavor. Thus, pod unique endophytes may be associated with Vanilla flavor. Pods from clones of the same Vanilla species (*V. planifolia*) vary in flavor across Reunion Island. This difference may be attributable to variations in microbial communities in pods, across cultivation regions in Reunion Island. Hence, plants and pods from different cultivation sites were used for endophyte isolation to find possible regional species differences in pod fungal endophyte communities.

- Can endophytes produce vanillin or any of their precursors?

To test *de novo* production of vanillin and other flavor compounds, the isolated endophytes were cultured on a common growth medium. To test for possible bioconversions, the endophytes were grown on media based on pod materials. Moreover some pure vanillin precursor compounds that might be involved in the vanillin biosynthesis, were fed to the endophytes. This approach may also lead to new insights into the biosynthesis of vanillin as in literature: contradictory results have been reported (Korthout and Verpoorte 2007; Gu et al., 2017).

1.3 ANALYTICAL METHODS AND DATA PROCESSING

To study the biosynthetic properties of the endophytes, one should be able to analyze the compounds involved in the biosynthetic network both qualitatively and quantitatively. For this purpose, both targeted analytical chemical methods and non-targeted metabolomics analysis were used. The instrumental techniques employed were non-targeted ^{1}HNMR spectroscopy, including 2D NMR (culture media pod, leaf, leaf with precursors, axenic plants, calli materials), targeted HPLC-UV (vanillin and precursors in culture medium pod material) and GC-MS (volatiles in culture medium pod material).

The chemical analyses produce large amounts of data. To deal with all variables and the large number of data, multivariate data analysis methods (MVA) were applied. Principle component analysis reduces the full data set into 2D or 3D graphs showing the maximal separation of all samples. Possible grouping of samples will show similarities and differences for the samples.

1.4 OUTLINE OF THE THESIS

The thesis begins with a review of fungal endophytes in the context of *Vanilla* (**Chapter 2**). First of all, the production of Vanilla flavor from the plant *Vanilla planifolia*, the original source of the flavor, is described. This is followed by describing the production of artificial Vanilla flavor. Afterwards, a short introduction to endophytes follows. To understand the role of endophytes in Vanilla flavor production, one needs to know the biosynthesis of vanillin. This is described by showing the network of different pathways that may lead to vanillin, and summarizing the still ongoing debates about which pathway is the real one leading to vanillin. The biotransformation of different vanillin precursors by various microorganisms is also discussed to show what type of bioconversions are already known that might occur in endophytes. Finally the existing knowledge of microorganisms in *Vanilla* is briefly discussed.

Fungal endophyte presence in *V. planifolia* from different isolation regions and pod specific endophytes are described in **Chapter 3**. In the same chapter, endophyte species that participate in the vanillin biosynthesis pathway are identified.

Chapter 4 focuses on volatiles from pod endophyte biotransformation reactions or *de novo* synthesis, which are related to Vanilla flavor.

Biotransformation reactions catalyzed by selected pod endophytes are reported in **Chapter 5**. Specifically, precursors which are associated with products, after biotransformation, on the vanillin biosynthesis pathway are reported in this chapter.

In **Chapter 6**, the aim is to gain more insight into the axenic *Vanilla* plant and *Vanilla* pod fungal endophytes interaction and the potential effect on Vanilla flavor related metabolites.

Chapter 7 gives a general discussion and future prospects based on the studies described in the thesis.

1.5 REFERENCES

Anderson N. (2016) Cell Wall-bound p-Coumaric and Ferulic Acid Analysis. Bio-protocol 6:e1955. DOI: 10.21769/BioProtoc.1955.

Converti A., Aliakbarian B., Dominguez J.M., Bustos V., Perego P. (2010) Microbial production of biovanillin. Brazilian Journal of Microbiology 41:519-30.

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

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.

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)

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 75:9 2765-74.

Hardoim P. R., van Overbeek L. S., Berg G., Pirttilä A.M., Compant S., Campisano A., Döring M., Sessitsch A. (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiology and Molecular Biology Reviews 79:293–20.

Havkin-Frenkel D., Belanger F.C. (2016) Biotechnology production of vanillin. In: Havkin Frenkel D., Dudai N. (Eds) Biotechnology in Flavor Production. (2nd ed.). West Sussex: John Wiley & Sons, Ltd. Pp.165-86.

Hoffman P.G., Zapf C.M. (2011) Flavor, Quality, and Authentication. In: Havkin-Frenkel D., Belanger F. (eds.) Handbook of Vanilla Science and Technology. West Sussex: Blackwell Publishing Ltd. Pp.162-82.

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

Labuda I. (2011) Biotechnology of Vanillin: Vanillin from Microbial Sources. In: Havkin-Frenkel D., Belanger F. (eds.) Handbook of Vanilla Science and Technology. West Sussex: Blackwell Publishing Ltd. Pp.301-27.

Mariezcurrena M.D., Zavaleta H.A., Waliszewski K.N., Sánchez V. (2008) The effect of killing conditions on the structural changes in vanilla (Vanilla planifolia, Andrews) pods during the curing process. International Journal of Food Science & Technology 43:1452–7.

Medina J.C., Jiménes G.C.R., García H.S., Zarrabal T.L.R., Alvarado M.A.G., Olvera V.J.R. (2009) Vanilla: Post-harvest operations. Food and Agriculture Organization of the United Nations (FAO). INPhO - Post-harvest Compendium.

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.

Rain P. (2004) Vanilla: The cultural history of the world's most popular flavor and fragrance. NY: Jeremy P. Tarcher Inc. Penguin Group. P.8.

Ramachandra S.R., Ravishankar G.A. (2000) Vanilla flavour: production by conventional and biotechnological routes. Journal Science Food Agriculture 80:289–304.

Reineccius G. (1994) Natural Flavoring Materials. In: Reineccius G. (ed.) Source Book of Flavors. 2nd Edition. Dordrecht: Springer Science+Business Media. Pp.176-364.

Sinha A.K., Verma S.C., Sharma U.K. (2007) Development and validation of an RP-HPLC method for quantitative determination of vanillin and related phenolic compounds in *Vanilla planifolia*. Journal of Separation Science 30:15–20.

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. (eds.) Handbook of Vanilla Science and Technology. West Sussex: Blackwell Publishing Ltd. Pp.183-219.

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