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

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Summary

In **Chapter I** a general background is given about *Vanilla* and vanillin. Vanilla flavor is one of the most sought flavors world-wide. Compared to synthetic vanillin (used since the 1920s), natural vanilla flavor is more complex. Additionally, consumers prefer natural flavors. This contributed to high demands for natural vanilla flavor. *Vanilla planifolia* is the only plant source (since before year 1520) of natural vanilla flavor. Later, "natural" vanillin was synthesized using microorganisms (as from year 1970). The most important aspect of vanilla flavor and aroma is its phytochemical diversity across cured *Vanilla* pods from different cultivation regions. The ratio of different vanilla flavor metabolites in cured pods affects the flavor (non-volatile metabolites) and aroma (volatile metabolites). Because of the shortage of high quality natural vanilla extracts, various efforts are made to improve the production amount of vanillin in the plant or to find new ways for the production of 'natural' vanillin. The latter has resulted in development of biotechnological processes like the conversion of ferulic acid into vanillin by different microorganisms, and the introduction of a vanillin biosynthetic pathway in yeast. Despite quite some research, still at the level of the plant itself there remain many questions, among others the vanillin biosynthesis is still not fully understood. Moreover nothing is known yet about the possible role of microorganisms, such as fungal endophytes, in the production of vanillin and vanilla flavor compounds in the vanilla pods on the plant. Also little is known about a possible role of microorganisms during the curing process. Based on this, the following aim was formulated: 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).

In **Chapter 2** a review is given of the present knowledge of vanillin and related flavor compounds. The biosynthesis of vanillin and other vanilla flavor compounds is reviewed, showing that it is still not completely understood. By understanding completely the pathways and their regulation, one may be able to find ways to better control the production of the flavor and thus increase the commercial value of the pods. Natural 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 will not be able to replace *Vanilla* in high quality products. Customers tend to demand natural flavors but natural *Vanilla* extract is about 200 times more expensive than synthetic vanillin. To produce 'natural' vanillin, fungi are used for the biotransformation of vanillin precursors into vanillin. All plants contain a number of fungal endophytes which do not cause any disease symptoms. Fungal endophytes seem to be plant specific, and they are thought to play a role in the interaction of the plant with its environment. Certain endophytes have been reported to produce secondary metabolites which previously were thought to be produced by the plant. Such fungi might thus also play a role in the biosynthesis of vanillin and vanilla flavor compounds in the plant. This could be in the form of whole pathways or parts of the vanillin pathway in an interaction with the plant's biosynthesis of vanilla flavor compounds. Studies on a possible role of endophytes may thus shed new light on parts of the vanillin biosynthetic pathway that still is under debate. Terroir effects on the flavor, as observed for vanilla pods, might be due to the occurrence of microorganisms in the rhizosphere or in the plant itself. Based on this analysis, it is proposed to study *Vanilla* plants for its endophytes and test these endophytes for the presence of possible vanillin and vanilla flavor biosynthetic reactions.

In **Chapter 3** a study on the potential involvement of *Vanilla* fungal endophytes in the vanilla flavor formation in pods is described. Endophytes were isolated from *Vanilla* leaves of different ages and pods by microbiological methods, and identified at the species level by molecular tools. To gain insight in the overall endophyte effects on vanilla flavor, fungal endophyte species diversity was assessed from plants from different regions of Reunion Island. Twenty three species of fungal endophytes were isolated from V*anilla* plants, using water agar followed by potato dextrose agar, and identified as different Molecular Organizational Taxonomic Units (MOTUs). Of these 57% were isolated from pods, the others from the leaves. The isolated endophytes were each grown on a medium containing green pod material. After growth, the spent growth media were analyzed by ^{1}HNMR spectrometry, analysis by principle component analysis (PCA) showed clear separation from control non-spent media. In all cases, glucovanillin levels were reduced to a very low level or could not be detected anymore. Hydrolysis of glucovanillin yields vanillin. This can be further metabolized to the corresponding alcohol which was indeed found, though at different levels in the various media. With the pathogen *Fusarium oxysporum*, most of the vanillin and its glucoside and vanillyl alcohol were catabolized. The endophyte *Pestalotiopsis microspora* converted most of the vanillin into vanillyl alcohol. This compound is known to have a balsamic flavor, a flavor typical for the bourbon-type *Vanilla* pods. The spent medium after *Diaporthe phaseolorum* growth media contained both vanillin and its alcohol in quite high levels. The differences found in the endophyte spectra and their specific conversions of green pod material compounds indicate that endophytes may play a role in the terroir effect.

In **Chapter 4**, endophytes that increased vanillin levels the most in the media (**Chapter 3**) were further studied for their possible contribution to the vanilla aroma, and in particular to the volatiles. Two endophytes, *Diaporthe phaseolorum* and *Pestalotiopsis microspora,* previously isolated from green vanilla pods, and one newly isolated fungus from pods after scalding, *Hypoxylon investiens,* were grown on different media. To measure the production of any vanilla aroma metabolite by the fungi themselves, they were grown on a potato dextrose agar medium. The results were compared with the fungi grown on media containing ground green vanilla pod material or vanilla pod waste after extraction with 40% ethanol (extracted cured pod medium). After growing the fungi on the media, static headspace GC-MS analysis was performed on the media. Several volatiles known to contribute to the vanilla aroma were found in the media: *p*-xylene, α-phellandrene, 3-carene, α-terpineol, *p*-hydroxybenzaldehyde, α-cubebene, β-caryophyllene, vanillin, and vanillyl alcohol. All were produced by the fungi growing on *Vanilla* material containing media at levels higher than in the control media without fungal growth. Volatiles α-phellandrene, α-terpineol, *p*-hydroxybenzaldehyde, α-cubebene, β-caryophyllene, vanillin, vanillyl alcohol were absent from potato dextrose medium after fungal growth. The type of media and the fungal species used contributed significantly to the type and abundance of volatiles, whereas the growth period had no significant effects. The production of these volatiles could be due to *de novo* biosynthesis by the endophytes, or biotransformation of precursors from the *Vanilla* pod materials. The fungus *Hypoxylon investiens* recovered after pod scalding, shows promise for the re-use of extracted cured pods. It seems that this fungus can convert ferulic acid into vanillin. The results support the hypothesis that endophytes play a role in vanilla flavor metabolite biosynthesis.

The biosynthesis of vanillin and related flavor compounds in *Vanilla planifolia* plants is still not completely clear. Various endophytic fungi occur in *Vanilla* pods where the vanilla flavor compounds accumulate. From literature, several fungi have been shown to be able to produce vanillin from ferulic acid. This raises the question if endophytic fungi could play a role in the production of vanillin and vanilla flavor related compounds, i.e. particularly looking at the non-volatiles which is the aim of **Chapter 5**. The endophytes *Hypoxylon investiens*, *Diaporthe phaseolorum*, *Pestalotiopsis microspora*, and the *Vanilla* pathogenic fungus *Fusarium oxysporum* f.sp. *vanillae* were grown on media containing *Vanilla* leaf material to probe for *de novo* vanillin biosynthesis. Moreover the following possible intermediates of vanillin biosynthesis were fed, through addition to the same media, to each of the fungi: *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic aldehyde, ferulic acid, and vanillin. After fungal growth, the media were analyzed with ^{1}HNMR spectroscopy. Tyrosine and glucose, two early vanillin precursors, are present in *Vanilla* leaf agar medium, but no *de novo* vanillin production was observed after fungal growth. All endophytes tested showed conversion of vanillin into vanillyl alcohol. Vanillin and vanillyl alcohol were found in the media containing ferulic acid after cultivation of *H. investiens* whereas *p*-coumaric acid was not converted, pointing to a specific conversion of ferulic acid. *p*-Hydroxybenzoic acid was produced by *D. phaseolorum* after feeding *p*-coumaric acid. But this fungus did not show a similar reaction after feeding ferulic acid. None of the fungi was capable of doing the complete *de novo* biosynthesis of vanillin, although they covered some of the steps. Only the formation of the 3-methoxy-4-hydroxy substitution of the aromatic ring is not found in any of the fungi. As the *H. investiens* strain was collected from pods after scalding, it is of interest for further exploration for biotechnological vanillin production.

In the previous chapters, endophyte isolation from *Vanilla* plants was described. Their ability to perform one or more steps of the vanillin biosynthesis was also previously shown *in vitro* but not yet *in vivo*. **Chapter 6** reports the characterization of the interaction of the endophytic fungi used in the previous chapters with *Vanilla* plant material *in vivo*. To measure the effect of each single endophyte on *Vanilla*, axenic *Vanilla* plants and callus cultures of different ages were produced and infected with the *Vanilla* endophytic fungi *Diaporthe phaseolorum*, *Pestalotiopsis microspora*, or *Hypoxylon investiens* as well as with the *Vanilla* pathogen *Fusarium oxysporum*. To find how to infect the *Vanilla* material in the experiments, the usual *in situ* endophyte mode of transmission towards infection was investigated. Endophytes were isolated only from open flowers, but not from closed buds, thus horizontal transmission is possible. Therefore fungal infection was performed by immersing plant material in fungal spore suspension, for the co-culture experiments. Using histological, morphological and ^{1}HNMR-based metabolomics analyses, two different stages of calli development were characterized (15 and 30 days of culture). For *p*-hydroxybenzyl alcohol and *p*-coumaric acid (both vanillin precursors), the highest level was observed in 30 days old calli cultures co-cultured with *Fusarium oxysporum*, and *Hypoxylon investiens* respectively. Overall, it seems that the fungal pathogen *F. oxysporum* does not differ much from endophytes on its effects on plants and calli. Apparently the different plant materials give a different response to co-culturing with an endophyte. Among the tested endophytes, *Hypoxylon investiens* and *Pestalotiopsis microspora* induced highest levels of metabolites associated to steps on the phenolic metabolic pathways related to vanillin biosynthesis in the plant. However, as vanillin is produced in pods, and not in the plant itself, further studies are needed to be able to conclude any effect on the vanillin biosynthesis itself.

Chapter 7 is a brief evaluation of the work of the thesis in connection with the original aim and objectives and includes some perspectives for future work. Considering the aim as described in **Chapter 1**, it can be concluded that *Vanilla planifolia* leaves and pods do contain various fungal endophytes, with a difference in isolated species for pods and leaves, and also regional differences. The isolated endophytes were probed for the production of any intermediates of the vanillin biosynthetic network. Three of these indeed seemed to be able to perform some steps from this network when grown on a medium containing green pod material. Further testing showed that various compounds from the vanilla aroma complex are produced by these endophytes. Full *de novo* biosynthesis of vanillin from early precursors could not be detected, but *Hypoxylon investiens*, an endophyte isolated from cured pods, was able to convert ferulic acid into vanillin, which makes it an interesting candidate for further studies as it may increase vanillin levels during the curing process. Studies with living plant material, in the form of calli or shoots showed that the three endophytes do affect the phenolic metabolome. To prove that the endophytes do really play a role in the vanillin biosynthesis, would require to work with axenic plants that flowered and start to form the pods, but such experiments are time consuming (from shoot to pods will take at least 4 years) and could not be done in the timeframe of this thesis project. So the aim and objectives of the project have been met. But for full proof of the involvement of endophytes in vanillin biosynthesis, further *in vivo* experiments are required. At least the research supports the hypothesis that terroir effects might be related to endophyte diversity and that endophytes may play a role in the formation of intermediates of the vanillin biosynthetic network during pod development and during the curing process of the pods.