# THESES OF THE DOCTORAL DISSERTATION

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MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

# Hungarian University of Agricultural and Life Sciences

# Transcriptomic analysis of grapevine noble rot

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The candidate has fulfilled all the requirements of the Doctoral Regulations of the Hungarian University of Agricultural and Life Sciences and has taken into account the comments and suggestions made during the debate when revising the thesis, and therefore the thesis may be submitted for the defence procedure.

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# BACKGROUND TO THE WORK, OBJECTIVES

*Botrytis cinerea* is a well-known plant pathogenic fungus, capable of infecting more than 1,200 different host plants, and can cause significant economic damage to agriculture. In the case of grapes (*Vitis vinifera* L.), it is also known as a two-faced fungus, as it is one of the most important pathogens of grapes, but it is also thanks to *Botrytis cinerea* that noble rot occurs, resulting in the production of, for example, Tokaji aszú wine, the earliest protected wine product in the world. The uniqueness of Tokaji aszú wine is due to the high quality of the raw material, which is made from a single harvest of aszú grapes. In Hungary, the grapes are called 'aszú grapes' when they are fully stalked, but have a fleshy texture, are purple in colour and do not have any mushroom-like fungi on the surface.

Under the current regulations, almost ten different wine products can be produced from six grape varieties in the Tokaj-Hegyalja wine region, of which seven can be made from partially or fully enriched raw materials.

However, there are some wine regions, such as Champagne in France, where even the slightest appearance of *Botrytis* can have a major impact on the organoleptic qualities of the sparkling wine produced there, so farmers often practically adjust the harvest date to the appearance of grey mould.

The fact that the occurrence of *Botrytis cinerea* in different wine regions of the world is perceived so differently is prompting researchers to study the infection process and its relationship with environmental factors in more detail. Current knowledge suggests that the differences between grey rot and noble rot lie in the conditions of the process, with noble rot being caused by a combination of biochemical processes in the metabolism of the fungus and physical changes in the grape's stalk (Hungarian, 2011), (Fournier, et al., 2013) (Blanco-Ulate, et al., 2015).

The infection process of *B. cinerea* on grapes is generally divided into the following phases: penetration of the host surface, destruction of host tissue/formation of primary lesion, extension of lesion/tissue maceration and sporulation (van Kan, 2006). As a necrotrophic and polyphagous pathogen, *B. cinerea* secretes nonspecific phytotoxins that destroy cells of a wide spectrum of plants. Among the many metabolites isolated from fermentation media (Collado, et al., 2007) the best known is sesquiterpene botrydial, which is produced during plant infection, (Deighton, et al., 2001) induces chlorosis and cell collapse, which appears to

facilitate both penetration and colonisation (Colmenares, et al., 2002).

The highly complex microbial diversity of terrestrial communities makes it very difficult to gain a comprehensive understanding of their interactions and relationships with plants (Francioli, et al., 2018). In addition to the soil microbiome, the study of the microbial community on plants is of particular importance, especially for standing crops such as vines and other woody perennial crops. The study of the microbiota on plants allows us to understand their impact on plants and can also serve as an indicator of significant changes in plant life, which, in addition to their importance for plant conservation and plant health, can reveal to researchers the details of the impact of climate change on plants (Francioli, et al., 2021).

Today, the peer-reviewed literature (Crandall, et al., 2020) is full of exciting new tools and techniques that are being applied in all areas of biology and medicine. Transcriptomics, proteomics, and more recently metabolomics are three techniques that have implications for the phytochemical characterization of fungi. Used individually, each of these techniques can generate a data set that can occupy a research team for years, and when used in combination they have the potential to comprehensively investigate a system at the transcriptional and translational level. Transcriptomics, i.e. quantitative gene expression profiling, is arguably the most widely used technique for characterising fungi from a plant pathological perspective.

Plant diseases caused by *Botrytis* have been described in detail in several studies and summaries over the last 50 years (Elad, et al., 2004). Biological control is a very important part of the control literature, and when its biological and ecological impact is fully understood, effective control strategies against the pathogen can be developed.

Using the latest laboratory technologies, researchers have developed a number of 'omics' tools to study the *B. cinerea* and the virulence of the fungus, which include transcriptomics (the entire set of coding and non-coding RNAs), proteomics (proteins from the genome) and metabolomics (proteins from the cell, tissues or organs), and using high-throughput DNA and RNA sequencing and high-resolution mass spectrometry and nuclear magnetic resonance (NMR), will allow such data to be developed to a systems biology level (AbuQamar, et al., 2016).

The microbial population on the surface of grapes infected by *Botrytis cinerea* and its dynamics have been investigated in

several previous studies (Viannini & Chilosi, 2013), (Hungarian, 2011). Apart from fungi and yeasts, one of the most notable results is the identification of *Starmerella bacillaris* (formerly *Candida zemplinina*) in isolates collected from aszú berry samples from Tokaji, a yeast species that has since been used as a cofermentation species in wine practice (Englezos, et al., 2017).

Two important factors determine the chemical composition of the grapes during the process of noble rot. One is the increase in solute concentration due to water evaporation, the other is the appearance and accumulation of secondary metabolites of *Botrytis cinerea.* These two factors are complemented by the metabolites of other microbes living on the grapes, which are also important, such as acetic acid produced by Acetobacter species or wine bugs caused by *Penicillium* species, which produce a bitter taste.

The questions raised and the theses identified in this work will add new knowledge of practical relevance to the field and may also serve as a basis for new research by our research team and members of the scientific community.

The uniqueness of the relationship between *Botrytis cinerea* and wine grapes (*Vitis vinifera* L.), as one of the most important pathogens causing yield loss worldwide, but also as one that can produce high quality, high value-added wine raw material under specific environmental conditions, allows a more complex study of the plant-fungus relationship, providing a more detailed picture of the microbiological, physiological, physical, and chemical steps of the infection process. In our work, we have put forward several simple hypotheses to prove or disprove them, step by step, to provide information on the elements of grey rot and defoliation, so that not only the microbiological and plant physiological relationships but also the external conditions that determine them can be understood.

Our research aims to test and confirm the hypotheses formulated in the thesis. To this end, we formulate the following research objectives:

1) Determining the transcriptomic differences between noble and grey rot, two processes with opposite outcomes
2) Analysis of the correlation between grape texture and carbohydrate-degrading genes expressed by *Botrytis cinerea*
3) Describing the role of other microbes besides *Botrytis* in the process of noble and grey rot
4) Determining the effect of these microbes on the aroma composition of aszú wines

# MATERIALS AND METHODS

The sampling site is in the Betsek vineyard on the outskirts of the village of Mád in the Tokaj-Hegyalja wine region. The vineyard is considered one of the 42 first-class vineyards on the outskirts of Mád.

The sampling plots were in two vineyard fields, the northernmost field containing furmint and the southernmost field containing hárslevelű varieties. Both vineyard plots were planted in 3 m x 1 m spacings on the resistant rootstock variety Teleki-Kober 5BB. The furmint variety is clone type T85 and the hárslevelű variety is type T311. In terms of cultivation, both fields are medium-high cordon cultivated with 4-5 plots, which have been established several years ago and are worked with 2 bud spur pruning.

Sampling was carried out at several times and for several study purposes, with samples collected over two vintages in 2016 and 2017. It is important to emphasise that when choosing the sampling date, we cannot be sure that there will be sufficient quantity and quality of drought in each vintage, so we have calculated more sampling dates than necessary, which we have updated each time as the weather has changed. The exact sample collection dates are given in Table 1.

1. Table 1: exact date of sample collections, types of samples collected and their use

| Sample collections | Exact date | Collected from sample types | Number of repetitions |
|---|---|---|---|
| 2016 | | | |
| September | 8 September. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |
| October | 11 October. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |
| November | 10 November. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |
| 2017 | | | |
| September | 7 September. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |
| October | october 10. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |
| November | 14 November. | Phase I, II, III, IV, VI | 5-5 biological replicates for RNA and DNA amplicon sequencing, 100 replicates for texture analysis |

To measure the textural properties of the grapes, a TAxT2i (Stable Micro System, Surrey, UK) texture analyser with HDP 90 platform and 30 kg maximum load was used.

Grapes collected and stored frozen were ground to powder in a sterilized ceramic pestle in a liquid nitrogen bath, and the powders were returned to -80° C in 2 ml eppendorf tubes per sample to avoid thawing. After digestion of the samples, total DNA was extracted using a plant DNA extraction kit, Qiagen DNEasy Plant mini kit (Qiagen Gmbh., Hilden, Germany).

For total RNA extraction, we further developed a less advanced but very efficient method to extract RNA from our samples relatively efficiently and with good quality. The main steps of RNA extraction were as follows:

- Extraction of cetromonium bromide (CTAB) with polyvinylpyrrolidone (PVP-40) and spermidine
- Denaturation with mercaptoethanol
- Extraction with chloroform isoamyl alcohol
- Sodium acetate cleaning
- TE buffer cleaning
- Lithium chloride cleaning
- DN-induced digestion

The extracted DNA was sequenced on next-generation amplicons under a contract by Eurofins BIOMI Kft. of Gödöllő, Hungary. The extracted RNA was performed by next-generation metatranscriptomic sequencing under a contract by UD Genomed Kft., Debrecen, on Illumina NextSeq500 platform at 14 Mread/sample.
The raw DNA sequences were extracted using the dada2 package implemented in the R software environment (R Development Core Team 2023) (Callahan, et al., 2016) which is designed to detect subtle variation in DNA sequences.
The quality of RNA reads was assessed using FastQC v0.11.5 (Babraham Bioinformatics, Cambridge, UK). The sequencing reads were then filtered according to their quality using FASTX-TOOLKIT. Low abundance fragments of high coverage reads were then removed. The resulting high quality reads were aligned to the reference genomes of *B. cinerea, A. alternata, A. pulullans, E. nigrum* and *R. graminis* (van Kan, et al., 2017), then using a Python script with Salmon version 1.3.0 (Patro, et al., 2017) to the corresponding genes. Basic statistical analyses were performed in the R program environment using the R Studio user interface (R

version 4.2.0, R Studio "Prairie Trillium" 2022.02.1). To compare changes in functional gene composition between the noble rot phases, pairwise differential expression (DE) analysis was performed between phases I-II, II-III, and III-IV using the R package DESeq2 (Love, et al., 2014). The dataset of expressed genes was sorted into modules using network analysis. To analyze all functional genes of *B. cinerea* and other selected fungi and to compare the expression levels of functional genes with berry texture data, the WGCNA R package (Langfelder & Horvath, 2008) using Weighted Gene Coexpression Network Analyses (WGCNA). The selected hub genes were also functionally analysed, as they play an important role in the transcriptomic co-expression network. The Uniprot database was used for the functional identification of the genes (UniProt, 2015).

We characterize fungal communities determined by sequencing DNA amplicons on noble and grey rot berries, and use these results to select the most abundant filamentous fungi and yeasts for which we perform transcriptomic analysis.

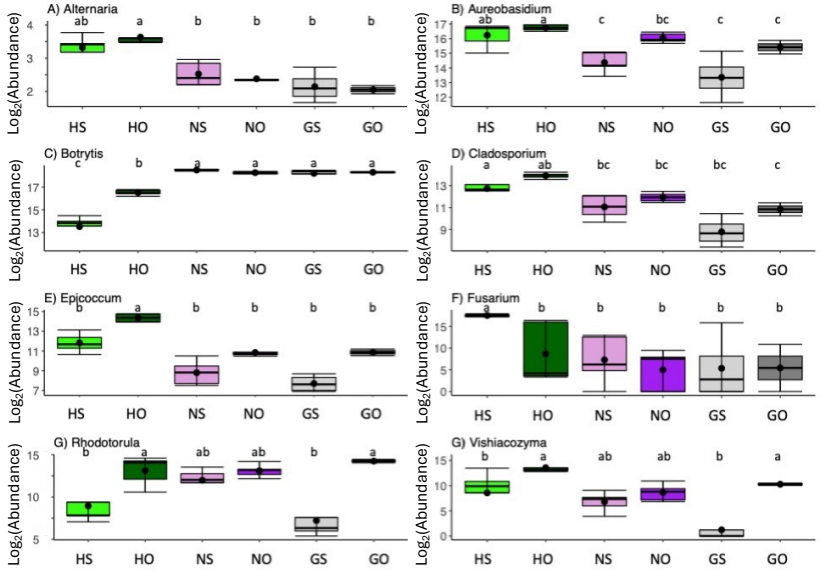


Figure 1: Abundance distribution of the eight genera with the highest overall abundance (plotted on a base two logarithmic scale) in berries from three phases (healthy - H, senesced - N and grey rot - G) and two months (September - S and October - O) of DNA amplicon sequencing data. The lower-case letters above the boxes indicate the significance groups determined by Tukey HSD test.

DNA amplicon sequences were divided into amplicon sequence variants (ASVs). The individual ASVs were assigned to which genus they belonged to, resulting in a data set divided into genomes, with the base 2 logarithm of the abundance of each genus for each sample type (rot type and sample collection month) plotted on a box plot (Figure 1).

Based on these results, we arbitrarily chose two genera of filamentous fungi and two yeasts for the subsequent transcriptomic data analysis, for which suitable reference genomes (GTF files) are available in public databases (NCBI) and their abundance is as high as possible. Thus, the genera *Alternaria, Aureobasidium, Epicoccum* and *Rhodotorula* were chosen.

In the next section, we discuss the results obtained from extracted and sequenced total RNA data. First, the process of enrichment, i.e. phases I-IV, will be investigated, including the genes annotated to the reference genome of *Botrytis cinerea.*

The abundance and frequency distribution of *B. cinerea* transcripts showed a significant increase between phase I (healthy) and all other phases (II, III, IV) of enrichment, but no significant change between later phases.

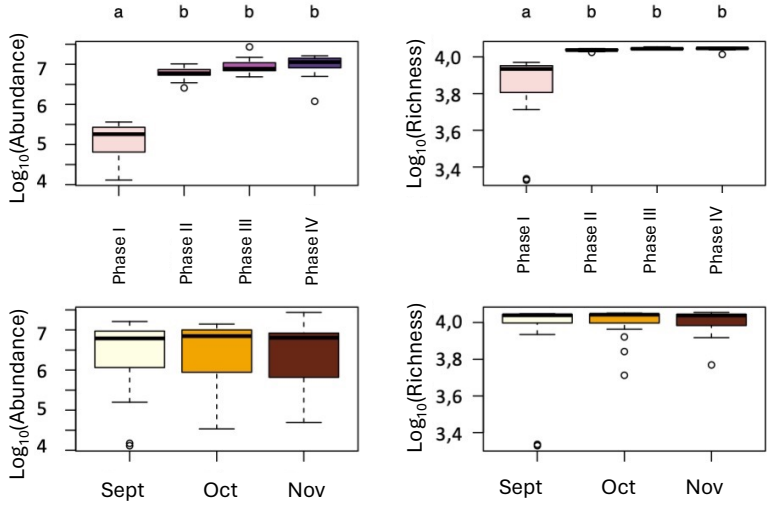


Figure 2: The figure shows the sum of abundance of expressed genes of *Botrytis cinerea* in different groups of samples at different phases of enrichment and at different sample collection times. It is observed that there is only significant difference between phase I and the other phases, both in terms of abundance and frequency.

The ordination diagrams plotted by NMDS showed a strong separation between the phases of enrichment, while sampling time had a minor effect on the separation between samples. The PERMANOVA test showed a strong separation between the phases of enrichment, while sampling time had a smaller effect. Phases accounted for 26% of the variance ($F = 19.712$, $P < 0.01$, $r^2 = 0.26$), while sampling time accounted for 6.6% ($F = 4.01$, $P < 0.01$, $r^2 = 0.066$).

In the following, we describe the properties of the co-expression network of *Botrytis cinerea* genes expressed during enrichment and compare them with the texture parameters measured on berries. In order to understand the internal structure of the network of genes expressed by *Botrytis cinerea,* WGCNA clustering was performed to classify the co-expression of functional genes into modules. Among the modules, the *turquoise, blue brown* and *red* modules showed the most significant negative correlation with texture parameters, as shown in the so-called "heat map" part of Figure 3. The *blue, brown, green, pink, red, yellow* and *turquoise* modules are referred to as NRCM modules, which showed an intrinsic gene expression profile defined by the nobel rot phases or sample collection months, which was clearly correlated with it, while the other six modules showed a distinct and uncorrelated intrinsic gene expression profile.

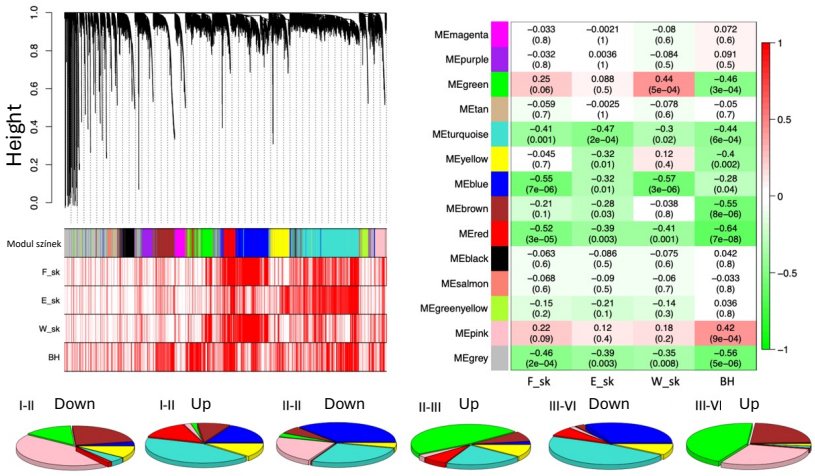


Figure 3: Analysis of the co-expression network of *Botrytis cinerea* genes expressed in four noble rot phases. The hierarchical clustering tree shown in the upper left figure was constructed based on the correlation of genes, under which functional genes are organized into 13 modules with a module size of 100 or more and a coexpression threshold of 0.25. Red bars corresponding to textural parameters indicate significant ($p < 0.05$) correlation with functional genes. The heatmap on the right shows the correlation between the 13 module eigengenes and the different textural parameters (F_sk: berry skin hardness, E_sk: berry skin elasticity, W_sk: work required to break through the berry skin, BH: berry hardness) and the corresponding p-

values in parentheses. The pie charts illustrate the differential gene expression results, i.e. the proportion of functional genes up- or down-regulated between different adjacent phases and distributed between each NRCM module.

Eigengene expression levels for the defined NRCM modules were analysed using ANOVA and Tukey HSD test to determine which phases or sample collection months significantly increase or decrease this value (Figure 4).

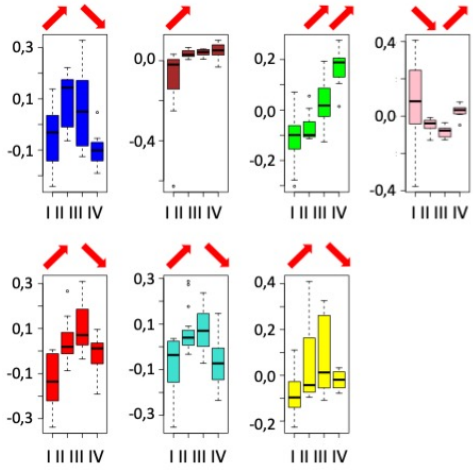


Figure 4: Distribution of the abundance values of the eigengenes associated to the modules between the enrichment phases for the NRCM modules using one-way ANOVA models. Red arrows indicate significant increases or decreases between adjacent phases or months.

We identified a number of enriched genes and pathways in all major phase transitions of all NRCMs. Most of these were related to carbohydrate and protein metabolism, both of which play important roles in the process of enrichment. Among the 17 enriched pathways, genes up-regulated in *brown* module phase I-II, we identified genes for "carbohydrate metabolism", "fructose and mannose metabolism", "glycolysis" and "butanoate metabolism". In the *red* module, genes up-regulated from phases I-II were significantly enriched in "Starch and sucrose metabolism". In the *turquoise* module, genes up-regulated from phase I-II were enriched for "Galactose metabolism",

"Glycolysis", "Other glycan metabolism" and "Starch and sucrose metabolism". Carbohydrate metabolism was also enriched between phases III and IV at the end of the botrytis process, with up-regulated genes for 'Carbon metabolism' and 'Glycolysis' in the *green* module and 'Carbon metabolism' and 'Pyruvate metabolism' in the *pink* module. From phase I to phase II of the *brown* module, up-regulated genes related to protein metabolism include enriched metabolic pathways such as 'Alanine and aspartame metabolism', 'Tryptophan metabolism', 'Valine, leucine and isoleucine metabolism' and 'Ubiquitin-mediated proteolysis'. In addition to the pathways related to amino acid metabolism, the "ketone bodies" pathway was also enriched among the up-regulated genes of the phase I-II *brown* module. With respect to the *green* module, we identified upregulated pathways related to protein metabolism such as "Alanine and aspartame metabolism", "Beta-alanine", "Cysteine and methionine metabolism" and "Glycine, serine and threonine", which were enriched during the later phase III and IV of the noble rot processes.

In addition to carbohydrate and protein metabolism, pathways responsible for the formation of secondary metabolites were detected in most modules. These compounds play a role in the development of the aszubi berry of oenological relevance, such as the formation of aromatic compounds and antibiotic activity. For example, in the *pink* module, both major phase transitions (genes up-regulated from phase I to phase II and from phase II to phase III) contained significantly enriched pathways related to "sulphur metabolism". In the *brown* module, the "Alkaloid biosynthesis" pathway was enriched among up-regulated genes from phase I to phase II. The same pathway in the *green* module was also enriched among genes up-regulated between phases III and IV. A complete list of the enriched pathways can be found in Appendix 3.

In contrast to the previous section, the next section will move from an examination of the process of enrichment to an examination of the differences between noble and grey harvesting. In this chapter we will also focus exclusively on the *Botrytis cinerea* genes. As in the previous sections, we first perform quantitative analyses (ANOVA and NMDS) and then move on to differential expression, gene ontology and further functional analyses.

In RNA isolated from healthy grapes, the abundance of *Botrytis* genes was lower compared to both types of rot. This trend was also true for the two sampling dates studied, September and October.

This shows that by looking only at the sum of the abundance or richness of the genes, no difference between the two rot types can be observed and no significant difference between the sampling months. This finding, which can be read from Figure 5, was confirmed by the PERMANOVA test, where *B. cinerea* functional gene abundance showed a significant difference between both berry rot types (including healthy cases) ($F = 8.8097$, $p < 0.01$, $r^2 = 0.42629$) and sampling months ($F = 2.0978$, $p < 0.01$, $r^2 = 0.06518$).

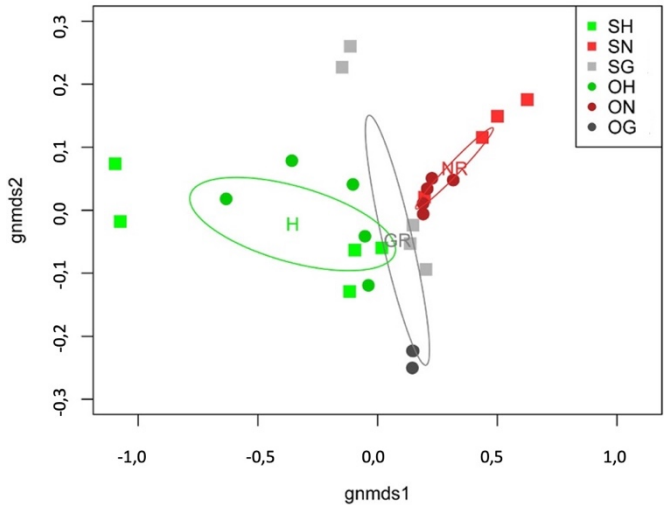


Figure 5. NMDS analysis of functional genes of *Botrytis cinerea* in healthy (H), necrotic (N) and grey rot (G) berries in September (S) and October (O). Ellipses depicting the standard deviation from the centre of ordination of each sample type show little or no overlap, indicating strong differences in the composition of expressed functional genes between berry types and months.

Gene ontology and pathway enrichment studies were performed on berry samples collected in September and October. Of the 18 significantly enriched GOs from the core set of genes identified in the 600 up-regulated in noble rot shared between September and October, one-third were related to metabolism, some of which were associated with noble rot activity, including 'Cellular protein metabolism' and 'Protein metabolism' (Table 3). In the up-regulated September-October common GR core profile of 628 genes, only three GOs were found to be significantly enriched, but none of these were found to be associated with metabolic or physical changes related to noble rot. The identification of

multiple enriched pathways in the set of up-regulated *Botrytis* genes corresponds to the identification of multiple GO expressions. Several enriched pathways were identified in the *B. cinerea* noble rot gene profile. Among these, the three most enriched pathways were "Ribosomal processes", "Metabolic pathways" and "Biosynthesis of secondary metabolites". Like the GO enrichment analyses, no enriched pathways were identified for the basic set of up-regulated GR genes.

The virulence of BLAST analyses (Cuzick, et. al., 2023) detected the presence of six virulence factors in the differentially expressed gene profile in the noble rot, while nine were found in the grape rot (Table 6) The identified NR virulence factors contribute to the ability of *B. cinerea* to colonise the grape skin by various mechanisms, including bypassing the plant host defence responses (BCIN_03g03390) (Rolke, et al., 2004), (BCIN_09g02390) (Rui & Hahn, 2007), and, is involved in cell wall degradation (BCIN_08g04530) (Ren, et al., 2018) (BCIN_16g03950) (Peterson & Nevalainen, 2012). The virulence profile of grey rot was mainly composed of oxidoreductases, which provide protection against host defence mechanisms (BCIN_02g07640, BCIN_12g06380, BCIN_05g03550, BCIN_01g07190), (Yu, et al., 2020) in addition, genes involved in membrane transport (BCIN_01g09910) (Hayashi, et al., 2002), genes contributing to cell wall degradation (BCIN_14g00850) (Micheli, 2001), and genes that attack plant tissues, namely BCIN_15g03390 (Schumacher, 2012) and phytotoxin synthesis genes BCIN_01g00060 (Colmenares, et al., 2002), (Tani, et al., 2005) and BCIN_12g06380 (Pinedo, et al., 2020) were also found to be significantly enriched.

In the next section, the role of other previously selected filamentous fungi and yeasts is examined. First, the differences between the noble and grey rot samples will be analysed, first from a quantitative point of view (ANOVA, NMDS) and then also from a functional point of view (differential gene expression, pathway enrichment, fungus-plant relationships).

In the case of *Alternaria,* when looking at abundance, all three berry types are distinct from each other in both months, while when looking at the frequency distribution, this difference is not significant for the healthy berry and the grey moth. In the case of *Botrytis*, the transcript distribution of healthy berries shows a significant difference with both rot types. *Epicoccum* shows a similar pattern to *Alternaria*. In the case of *Aureobasidium, the*

transcripts determined on healthy berries are well separated from those of the two rot types, while in the case of *Rhodotorula,* distributions similar to *Botrytis* were obtained (Figure 6).

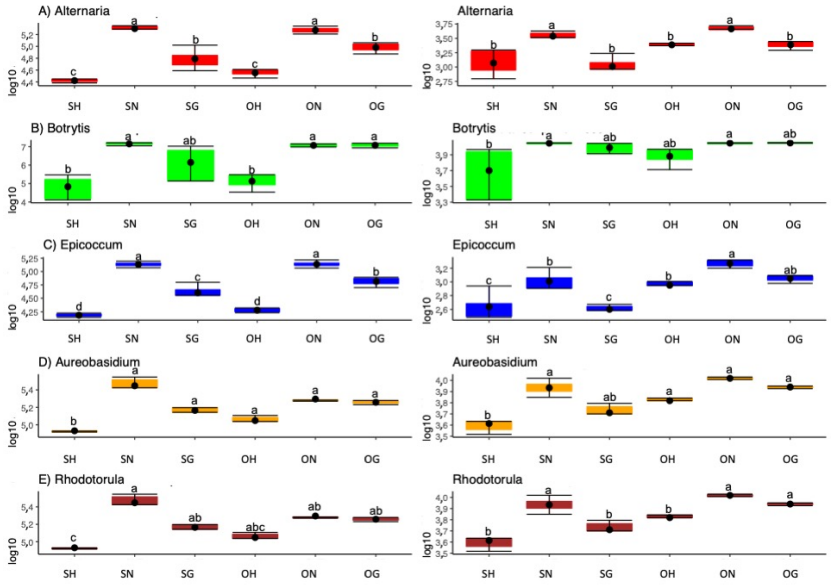


Figure 6. Comparison of filamentous fungi A) *Alternaria alternata*, B) *Botrytis cinerea*, C) *Epicoccum nigrum* and yeast D) *Aureobasidium pullulans* and E) *Rhodotorula graminis* transcript abundance (left) and richness (right) values between healthy berry (H), non-rotten berry (NR) and rotten berry (GR) types in September (S) and October (O) using ANOVA. Pairwise differences calculated by Tukey's HSD tests are indicated by the different superscripts, lower case in Latin.

We identified an enriched gene set of metabolically related gene ontological expressions in the gene pool of *A. alternata*, *B. cinerea*, *E. nigrum* and *A. pullulans* up regulated in differentially expressed noble rot berries in both months studied, some of which are related to the process of aszú development and cell division. In the case of *A. alternata,* this affects mainly genes related to nitrogen metabolism, such as 'Peptide metabolism', and cellular processes such as 'Translation'. In the *B. cinerea* gene set, we identified 18 gene ontology terms, one third of which were related to metabolic activity, including "Cellular protein metabolism" and "Protein metabolism". We also identified three enriched terms for *E. nigrum*, but these were mainly related to cellular processes such as "Intracellular transport". For *A. pullulans*, the twelve enriched terms were mainly related to fatty acid metabolism, such as "Fatty

acid metabolism process", "Fatty acid biosynthesis process" and "Monocarboxylic acid metabolism process".
Considering BLAST to identify virulence factors, three genes were identified in the up-regulated gene pool of *A. alternata*, including a 1,3,8-trihydroxynaphthalene reductase (CC77DRAFT_528893), a 26S proteasome-like protein regulatory subunit (CC77DRAFT_968321) and a mitogen-activated protein kinase (CC77DRAFT_1016542). Three genes were identified in the up-regulated noble rot gene pool of *E. nigrum*, including an unidentified protein (B5807_08936), a protein containing the PKS_ER domain (B5807_02028), and a 5-aminolevulinate synthase (5-aminolevulinate synthase). Finally, we identified a single virulence gene, namely an ATP-dependent RNA helicase, eIF4A (RHOBADRAFT_46472), in the up-regulated grey matter gene pool of *E. nigrum.* RNA helicases are involved in all processes involving RNA, from transcription to degradation (Adbelkri, et al., 2020), so this gene may play an important role in the regulation of adaptation to stressful environments in *R. graminis.*

In the next phase, the genes identified during the enrichment process, i.e. in the phase I-IV samples, were analysed, namely those annotated to the reference genome of one species of each of the 4 selected genomes (*Alternaria, Aureobasidium, Epicoccum, Rhodotorula*).
In the gene expression distributions of the phases of the genera studied (*Alternaria, Aureobasidium, Botrytis, Epicoccum* and *Rhodotorula*), 36%, 47%, 26%, 49% and 39% of the significant separation observed in the ordination plot between genera can be explained by phase. Like the analysis of variance, the ordination plots also show that the date of harvest determines the separation of samples to a much lesser extent than the phase (Figure 7).

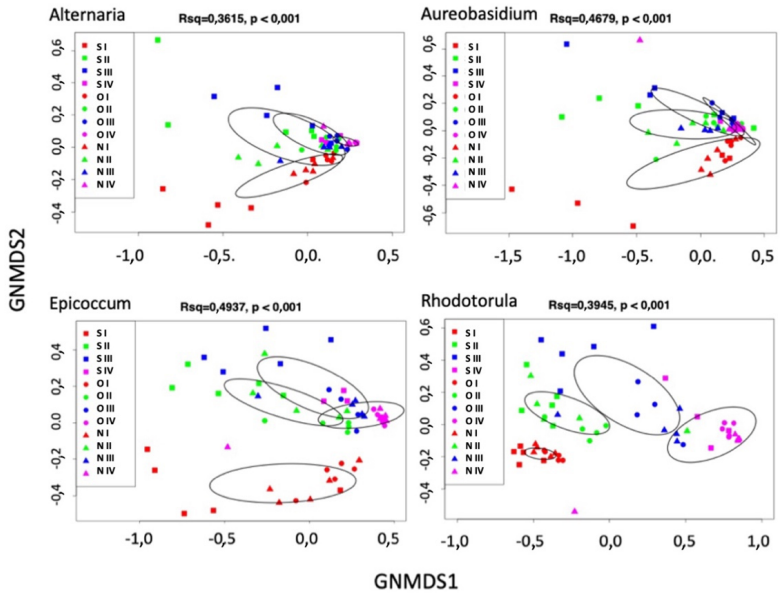


Figure 7: NMDS ordination diagram of the *non-Botrytis* genera studied. Colours indicate the noble rot phase (I, II, III, IV), shapes indicate the sampling month (S - September, O - October, N - November).

The WGCNA cluster analyses identified ten gene module clusters (Figure 21). For distinctness in this analysis, module names are suffixed with a 2 in the module names, such as *blue2*, so that the gene clusters identified for *Botrytis* can be distinguished from the gene clusters identified for the combined analysis. The *turquoise2* module contained more than 95% of the non-Botrytis genes, and ANOVA analyses of the eigengene showed an increase between the four phases, but this increase was only significant between phases III and IV (Figure 8).

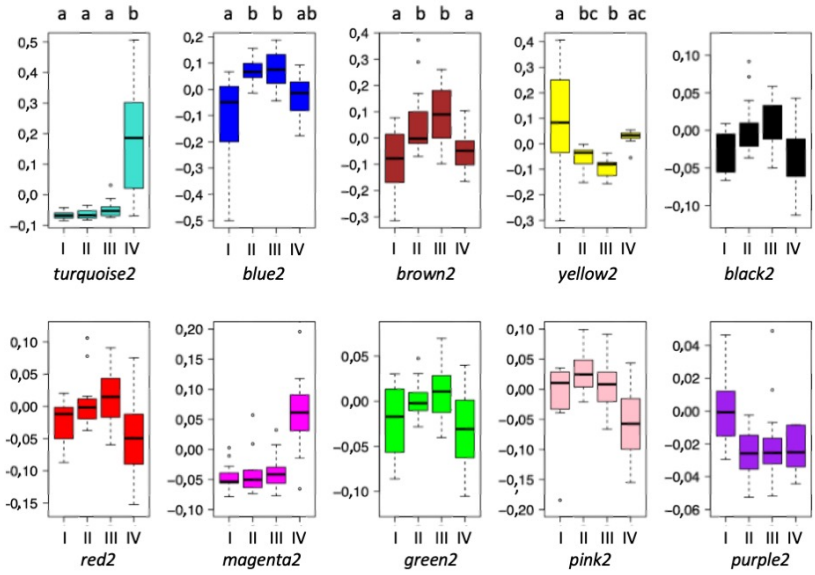


Figure 8: Phase distributions of the different gene expression modules, significant differences are marked in small letters, module colours are shown below each figure.

In the *turquoise2* module, 47, 7, 50, 21 and 21 enriched pathways were identified, among which several pathways were identified that contain enzymes that contribute to the stability and quality of wine or are precursors for the formation of aromatic compounds in wine, in particular for amino acid metabolism (Callejón et al, 2010), carbohydrate metabolism (Han et al., 2020), lipid metabolism and enzymes involved in the formation of secondary metabolites. Genes contributing to the sensory and quality parameters of wines were identified from these enriched pathways, and hub genes with similar functions were identified from the WGCNA clustered modules, which are presented in detail in Appendix 4 of this thesis.

## CONCLUSIONS AND PROPOSALS

Differences in certain visual and measurable physico-chemical properties of the dried berries, used to determine the different phases of the process (Hegyi-Kaló, et al., 2020), (Carbajal-Ida, et al., 2016), implicitly suggested that these changes are at least partly caused by several, as yet unknown, functional genes of *B. cinerea. To* our knowledge, there have been few studies that shed light on the qualitative and quantitative differences in the functional gene profile of *B. cinerea* during naturally occurring ascorbic development under field conditions and how these functional genes are likely to contribute to the physicochemical changes in botrytised grapes, with important implications for winemakers.

The overall similarity of the gene expression profile of *B. cinerea* noble rot in the months studied indicates that the composition of genes expressed in *B. cinerea* is relatively stable over time, implying a degree of predictability. The latter was also confirmed by NMDS analyses, which showed a clear separation of phases II, III and IV when all months were pooled, confirming our expectations. The latter is of crucial importance for winemakers. We therefore conclude that the Botrytis cinerea genes expressed during the enrichment process do not change with sampling time and, although they show higher total abundance in phases II-IV, they are also present at high frequencies on healthy berries.

The skin of *Vitis vinifera* grapes is mainly composed of carbohydrate-based compounds such as cellulose, arabinan, mannan, galactan, xylan and xyloglucan, lignin-structured proteins and proanthocyanidins (Apolinar-Valiente, et al, 2017). Within the NRCM modules, we identified several differentially expressed genes that were significantly correlated with texture parameters, suggesting an enrichment of functional pathways involved in berry skin degradation during the botrytis process. We found that genes differentially expressed during the transition from healthy berries to phase II berries were genes involved in atypical aspen development, as described in other studies (Hungarian, 2011) (Hegyi-Kaló, et al., 2020) (Kallitsounakis & Catarino, 2020).

This was because a significant change in the elastic modulus (E-drop) of the berry shell was observed at this phase, suggesting that cell wall degradation occurs primarily at this early phase of

senescence. The observed correlation of enzymes involved in carbohydrate metabolism with changes in physical parameters of the berry skin in the present study confirms that they play an active role in berry texture changes during the process of desaturation.
In addition to carbohydrate metabolism, the protein metabolism enriched pathways were also predominant in the respective modules. In addition to the structural degradation of the grape skin, the metabolism of nitrogen-containing compounds such as amino acids, peptides, and nucleic acid derivatives play an important role in the metabolism of *B. cinerea* (Lacroux, et al., 2008). This probably explains the identification of nitrogen-releasing enzymes such as omega-amidase (BCIN_14g01750), which is up-regulated in the *brown* module between phase I and II, in the enriched alanine-aspartate pathway (Slocum, 2005) and glutamate dehydrogenase (BCIN_03g07670), up-regulated between phases III and IV in the green module, which is part of the enriched alanine aspartame pathway. The identification of the enriched pathway "ketone bodies" also refers to the degradation of amino acids containing ketone bodies. Thus, we identified significantly enriched carbohydrate metabolism in the early phases of the enrichment, particularly in cell wall degradation processes. In the later phases of the process, we obtained significant enrichment in the nitrogen metabolism pathway, in which genes related to the production of readily available nitrogen were found to be active.
Numerous studies have shown that *B. cinerea* is the fungus with the highest biomass during enrichment (Bene & Magyar, 2004) (Blanco-Ulate, et al., 2015), suggesting that it has a certain competitive advantage over other microorganisms in the grape microenvironment. Sulphur metabolism and subsequent detoxification are therefore important in the plant-fungus interaction model during the noble rot process (Griffith, et al., 2015). In the *pink* module, the pathway of sulfur metabolism is enriched between phases I and II and between phases III and IV. *Botrytis cinerea* is able to reduce sulfur compounds, thereby reducing the amount of these compounds to levels sufficient for survival. It can also convert these compounds into other secondary metabolites that are beneficial for its reproduction. In addition to producing and converting metabolites that promote its reproduction, *B. cinerea* has also been identified as having antagonistic effects against other microorganisms. In the *brown* module, we identified the enzyme catalase (BCIN_03g01920), which belongs to the "secondary metabolites" pathway up-

regulated between phases I and II. This enzyme is part of several enriched pathways, such as the glyoxylate enriched pathway, but is also a key enzyme in the synthesis of alkaloids. This latter group includes compounds with antagonistic activity such as siderophore ferricrochlrocine (Chowdhury, et al., 2020). The antimicrobial substances produced by *B. cinerea* confirm our hypothesis that this species has adaptive properties that allow it to fully dominate during the enrichment process.

In the next section, we interpret the results obtained from the analysis of the differences between the differences in greying and greying mortality. Our analyses revealed significant differences in the composition of expressed genes between grey- and noble rot berries, as well as between different harvest dates. We found significant differences in the composition of expressed functional genes in *B. cinerea* between berry rot types, which is consistent with our hypotheses. Sampling date also showed a strong correlation with the expressed gene composition in all berry rot types, indicating that harvest date has a significant effect on the physiological and biochemical processes of berries. Compared to healthy berries, functional differences in up-regulated genes of *B. cinerea* were more significant in the case of blight than in the case of grey rot, especially in metabolism. In the current study, the identification of several GO terms related to cellular activity, such as "cellular biogenesis" and "cell division", shows the proliferation of *B. cinerea* during the disease. This is Blanco-Ulate et al. (Blanco-Ulate, et al., 2015) where they identified several enriched genes related to cell growth and host tissue organization. Similarly, the study by Lovato et al. (Lovato, et al., 2019) also identified significantly enriched GO terms related to cell proliferation, namely 'cellular biosynthetic process' and 'translation', in berries showing post-harvest noble rot. The identification of the enriched pathway "antagonistic metabolite biosynthesis" in up-regulated genes of necrotic berries suggests that *B. cinerea* has antagonistic activity against other microorganisms, which provides the opportunity for proliferation and dominance. This provides a genetic explanation for our previous observations that, during senescence, *B. cinerea* becomes dominant in aszú berries as other filamentous fungi are reduced. This antibiotic pathway is not enriched in grey rot, so genes expressing antagonistic activity are not significantly active in this type of rot. This is probably the reason why plant pathogenic fungi start to dominate and diversify during grey rot (Barata, et al.,

2012). Thus, our work has characterized the functions of genes expressed during grey and noble rot. Among these, we obtained several active genes for carbohydrate metabolism, protein metabolism and antimicrobial biosynthesis, whose roles have been described in detail.

To the best of our knowledge, our study is the first to analyse the functional gene expression profiles of filamentous fungi and yeasts in addition to *B. cinerea in* grey and noble rot berries, and to show the extent to which these fungi may contribute to the physicochemical changes that occur during the two rots. We also provide novel insights into the possible interaction dynamics and succession of dominant fungi during decay, including the production of certain antagonistic microbe-microbe and microbe-plant genes. Among the fungal species studied, *B. cinerea* has the highest number of differentially expressed genes in its functional gene pool in different rot types at both sampling dates studied, demonstrating its predominance in the two grapevine rot types. This is not surprising since grapes infected by *B. cinerea* were collected. The number of genes differentially expressed in noble rot (>31) compared to grey rot (<5) in other filamentous fungi and yeasts suggests that they may play a more active role in the development of dry berry than in grey rot.

Significant differences were found in the qualitative and quantitative gene expression profiles of filamentous fungi and yeasts in grey and noble rot. GO analysis and pathway enrichment analysis of *A. alternata, B. cinerea, E. nigrum, A. pullulans* and *R. graminis* showed a much more active gene expression profile for aphid than for grey rot, especially in terms of metabolism and cellular activity related to transport and proliferation. The identification of enriched GO terms associated with cell proliferation in the up-regulated gene profile of *A. alternata* and *B. cinerea* is consistent with the findings of Blanco-Ulate et al. (Blanco-Ulate, et al., 2015), who also found enriched pathways related to cell processes such as 'cell growth'. Similarly, enriched GO terms, including cellular biosynthetic process and 'cellular component biogenesis' (Lovato, et al., 2019), and (Otto, et al., 2022) have been identified. The identification of several genes involved in cell proliferation is likely to be associated with an increase in cell biomass in berry aphid in all fungal species analysed here.

The identification of enriched pathways for antagonistic metabolite biosynthesis and the identification of genes related to virulence BLAST-associated factors in up-regulated genes

associated with noble rot in the fungal species studied suggest several cases of microbial interactions. Enzymes related to the synthesis of antagonistic metabolites were also identified that are responsible for the synthesis of compounds in the enriched "biosynthesis of antagonistic interaction genes" pathway in *A. alternata* and *E. nigrum that* may be responsible for imparting unpleasant organoleptic properties to wine, including sulphide and sulphur (Goode et al. 2008).

In contrast to other filamentous fungi and yeasts, whose antagonistic interaction genes were expressed only in the enriched berries, antagonistic interaction factors were identified in the *B. cinerea* transcript for both types of rots (the enriched "Biosynthesis of antagonistic interaction genes" and virulence genes). The fact that the enriched "Biosynthesis of antagonistic metabolites" pathway was present in the gene pool of all filamentous fungi and *A. pullulans* genes from non-rotten berries underlines that complex interaction dynamics at this phase are likely to characterize the microbiota. This contrasts with the case of grey rot, where there is a lack of antagonism against *B. cinerea* by other microbes.

It can therefore be concluded that genes related to microbial interactions have been identified in the noble rot and grey rot berries. We found that, while *B. cinerea* genes exhibited antagonistic behaviour in both types, they were significantly enriched in the other fungi only on aszú berries.

The present study is, to our knowledge, the first to investigate the active metabolic role of phylloxera and yeasts identified alongside *B. cinerea* during the acidification process and how they contribute to the acidification process, to the formation of grape and wine aromas, aroma-related compounds and their precursors that can influence organoleptic properties and overall wine quality.

The increase in transcript abundance of both filamentous fungi and yeasts during the enrichment process is in contrast to what is known from the literature, namely that filamentous fungi appear early in the process, in contrast to yeasts, which dominate at later phases (Li, et al., 2021). This opposite trend probably reflects the increasing functional role of the aromatic precursors during the four phases of enrichment, especially in the present study. The significant increase in the specific gene associated with the clustered *turquoise* module of WGCNA from phases II-III to phase IV, and the occurrence of the highest number of uniquely up-regulated genes in all phylloxera and yeasts in phase IV quantitatively indicate that other microbial effects on wine flavour

and aroma occur in this phase, in combination with other microbial effects on wine flavour and aroma in later phases of enrichment. The occurrence of several enriched pathways and genes, such as those involved in amino acid and lipid metabolism, associated with aroma formation indicated their direct and indirect roles in aroma formation. The identification of several enzymes from filamentous fungi and yeasts other than *B.* cinerea that are related to aromatization suggests an important but not exclusive role of *B. cinerea in the* latter aspect (see Appendix M4 of this thesis).

## NEW SCIENTIFIC RESULTS

1. thesis

During noble and grey rot infection, *B. cinerea* expresses genes responsible for the degradation of the grape skin as a first step in berry infection, followed by the expression of genes promoting pathogen dominance and colonisation of the berry surface and internal tissues. These genes were found to be more active and significantly more abundant in the case of noble than in the case of grey rot.

2. thesis

Expressed functional genes of *Botrytis cinerea*, during noble rot, showed significantly enriched pathways for biochemical changes that are beneficial for senescence, such as an increase in sugar and protein content and biosynthesis of precursor molecules that determine the so-called botrytis type aroma, while in the case of grey rot, and express genes for evading plant defence responses and producing phytotoxic compounds.

3. thesis

The genes expressed by *Botrytis cinerea are* significantly enriched in the degradative steps of pathways related to carbohydrate degradation during the initial phases of senescence, which contribute to changes in berry skin structure, whereas the metabolic pathways enriched in the later phases of enrichment allow *Botrytis cinerea* to colonise the grape berry ('sulphate metabolism', degradation) and dominate its microbiota ('biosynthesis of antimicrobial substances').

4. thesis

In my work, I investigated the metabolic activity of the most abundant fungi on asafoetida besides *B. cinerea*, such as the yeasts *A. alternata, E. nigrum*, *A. pullulans* and *R. graminis.* I have shown that these microbes play a major role in the process of senescence during noble rot, as they express many up-regulated genes (899 genes), whereas during grey rot they express much fewer (15 genes).

5. thesis

In my study, I have described the key enriched pathways expressed by *A. alternata, A. pulullans, E. nigrum* and *R. graminis* involved in amino acid, lipid and carbohydrate metabolism that activate the synthesis of precursor molecules that contribute to the organoleptic properties of aszú wines, as well as their food quality and biochemical stability.

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**HEGYI, ÁDÁM ISTVÁN**; MARGOT, OTTO; HEGYI-KALÓ, JÚLIA; GEML, JÓZSEF; GEIGER, ADRIENN; SZABADI, KRISZTA LILLA; GORE, RICHÁRD; GOMBA-TÓTH, ADRIENN; THOMAS, CELS; VÁCZY, KÁLMÁN ZOLTÁN a transcriptomic analyses of the role of other fungi in botrytis cinerea caused noble rot of grapevine In: *Verdier, V 12th International Congress of Plant Pathology* (2023)

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