Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Posgrado en Ciencias de la Vida
con orientación en Microbiología

Understanding the relationship between heat stress and pathogenicity in the fungus *Lasiodiplodia theobromae*, a causal agent of botryosphaeria dieback in grapevine

Tesis

para cubrir parcialmente los requisitos necesarios para obtener el grado de
Doctor en Ciencias en Ciencias de la Vida

Presenta:

Marcos Paolinelli

Ensenada, Baja California, México
2016

Tesis defendida por

Marcos Paolinelli

y aprobada por el siguiente Comité

Dra. Rufina Hernández Martínez
Directora de tesis

Dra. Clara Elizabeth Galindo Sánchez

Dr. Philippe E. Rolshausen

Dra Meritxell Riquelme Pérez

Dr. Alfredo Heriberto Herrera Estrella

Dra. Clara Elizabeth Galindo Sánchez
Coordinador del Posgrado en Ciencias de la Vida

Dra. Rufina Hernández Martínez
Directora de Estudios de Posgrado

Resumen de la tesis que presenta **Marcos Paolinelli** como requisito parcial para la obtención del grado de Doctor en Ciencias en Ciencias de la Vida con orientación en Microbiología.

Entendiendo la relación entre el estrés por calor y la patogenicidad en el hongo *Lasiodiplodia theobromae*, agente causal del decaimiento por botriosferias en vid

Resumen aprobado por:

_____

Dra. Rufina Hernández Martínez
Directora de Tesis

Lasiodiplodia theobromae es un hongo fitopatógeno con un amplio rango de hospederos, capaz de afectar el sistema vascular en vid. Se ha sugerido que el estrés por calor aumenta su virulencia, por lo que en el presente trabajo se evaluó cómo afecta éste, a la regulación transcripcional de genes asociados a patogenicidad. El análisis transcripcional de *L. theobromae,* al ser expuesto a estrés por calor, se llevó a cabo *in vitro* en la presencia de madera de vid. Un total de 19,860 transcritos fueron ensamblados *de novo*; 9,731 de estos tienen homología con genes de *Neofusicoccum parvum* y *Macrophomina phaseolina*, 399 poseen homología con genes involucrados en procesos patogénicos y 394 pertenecen a familias de genes que muestran expansión en los genomas de otros hongos capaces de causar enfermedades vasculares en vid. Genes asociados al metabolismo de compuestos fenólicos, que codifican enzimas involucradas en la degradación de ácido salicílico y precursores de la vía de fenilpropanoides en planta, mostraron inducción *in vitro* en respuesta al estrés por calor y a la presencia de madera de vid. Estos resultados sugieren funciones en el hongo dirigidas a manipular la respuesta del hospedero, debido a que los compuestos fenólicos son fundamentales en la respuesta defensiva de la vid. La inducción de salicilato hidroxilasa, intradiol dioxigenasa de ruptura de anillo y fumarilacetoacetato hidrolasa durante el proceso de infección apoya esta hipótesis. Por otro lado, la expresión diferencial de salicilato hidroxilasa en plantas bajo estrés, sugiere que el hongo podría sacar provecho del catabolismo de ácido salicílico, el cuál es producido en mayor cantidad en vid como respuesta al estrés abiótico. Por otro lado, una pectato liasa mostró inducción durante la infección, mientras que una amilasa fue reprimida, lo cual podría corresponder con un crecimiento intercelular del hongo en las primeras etapas de la colonización de la planta. En general, los resultados sugieren que el estrés por calor facilita la colonización, debido a la capacidad de *L. theobromae* de utilizar, como fuente de carbono, compuestos que la planta produce como estrategia de defensa. Este trabajo sienta las bases para el futuro desarrollo de estudios de la biología molecular de *L. theobromae*. El modelo propuesto de la interacción del hongo con la planta, servirá para diseñar estrategias de control de esta enfermedad.

Palabras clave: enfermedades vasculares en vid, Botryosphaeriaceae, RNAseq, transciptómica, RT-qPCR, expresión génica, genes de referencia, decaimiento por botriosferias, genes de patogenicidad, melanina, catabolismo de tirosinas, fenilpropanoides.

Abstract of the thesis presented by **Marcos Paolinelli** as a partial requirement to obtain the Doctor of Science degree in Life Science with orientation in Microbiology

Understanding the relationship between heat stress and pathogenicity in the fungus *Lasiodiplodia theobromae*, a causal agent of botryosphaeria dieback in grapevine

Abstract approved by:

_____

Dra. Rufina Hernández Martínez
Thesis Director

Lasiodiplodia theobromae is a highly virulent plant pathogenic fungus that causes grapevine vascular disease. It has been suggested that heat stress increases its virulence. The aim of this work was to study the fungal transcriptional regulation of pathogenicity genes in response to heat stress. Transcriptional regulation analysis of *L. theobromae* exposed to heat stress (HS) was first carried out *in vitro* in the presence of grapevine wood (GW). A total of 19,860 *de novo* assembled transcripts were obtained, 9,731 of which showed homology with *Neofusicoccum parvum* or *Macrophomina phaseolina*; 399 have homology with genes involved in pathogenic processes and 394 belonged to expanded gene families in others fungal grapevine vascular pathogens. Gene expression analysis showed changes in fungal metabolism of phenolic compounds; where genes encoding for enzymes that degrade salicylic acid (SA) and plant phenylpropanoid precursors were up-regulated during *in vitro* HS response, in the presence of GW. These results suggest that the fungus remove phenolic compunds and evade the host defense response. The *in planta* up-regulation of salicylate hydroxylase, intradiol ring cleavage dioxygenase and fumarylacetoacetase encoding genes, further supported this hypothesis. The differential induction of salicylate hydroxylase in HS-stressed plants, suggest that fungus takes advantage through the SA catabolism, which is produced in higher amount in plants under stress. Pectate lyase was up-regulated while a putative amylase was down-regulated *in planta*, this could be associated with an intercellular growth strategy during the first stages of colonization. Our results support the hypothesis that heat stress facilitates fungal colonization, because of the fungus ability to use the phenylpropanoid precursors and SA, both compounds known to control the host defense. This work provides the basis for future studies of the molecular biology of *L.* theobromae. The proposed model for the interaction with the plant, being corroborated, will help to design control strategies for botryosphaeria dieback in grapevine.

Keywords: vascular diseases, botryosphaeriaceae, RNAseq, transciptomic, RT-qPCR, gene expression, reference genes, grapevine, botryosphaeria dieback, pathogenicity genes, melanin, L-tyrosine catabolism, phenylpropanoids.

Dedicatoria

A mi amor Mari, a mi familia y a la memoria de mis estrellas: Alfio, Mica, Porota y Lili.

Agradecimientos

Al Centro de Investigación Científicay de Educación Superior de Ensenada (CICESE). Al CONACYT por la beca que me permitió realizar este trabajo y por el apoyo económico para realizar la estancia en LANGEBIO. Gracias a UC-MEXUS por la ayuda económica para realizar la estancia en UCRiverside y por la financiación del proyecto que me permitió llevar a cabo la última etapa de este trabajo.

Muchas gracias Rufina por aceptar a este desconocido del sur, por arriesgarte a aceptar el enorme desafío de otorgarme la libertad para construir mi propio camino. Gracias por tu aliento constante a confiar en la curiosidad de mi espíritu. Eternamente agradecido por tu entrega sincera.

Gracias al comité de tesis por evidenciar desde temprano los puntos débiles en mi proyecto y sugerir los cambios que permitieron que esto sea posible. A Alfredo por darme la oportunidad de estar en tu maravilloso grupo y compartir tu visión de la ciencia con pasión. A Clarissa por ayudarme en los primeros pasos en el trabajo con el RNA y la expresión génica, por abrirme la puerta de tu laboratorio desde el primer día. Sin tus recomendaciones iniciales, nada hubiera ocurrido. A Philippe por permitirme la entrada a tu laboratorio, por afrontar la valiente decisión de mirar para este lado de la frontera. Eternamente agradecido por toda tú ayuda en la redacción y por brindarme tu amistad. A Meritxell por estar en los momentos más importantes de la tesis, por tus consejos e insistencia para mejorar mis presentaciones. Gracias por tu apoyo, que fue fundamental en la redacción del artículo y la tesis.

Gracias a Raúl Llera por iniciarme en el maravilloso mundo del uso de Linux y la bioinformática.

Gracias a los compañeros de ruta. A todos los tacuaches. Los primeros, Lili, Paco, Lolis, Nancy; los que me acompañaron durante los cursos: Lupita, Sarita y Cande. Los que siguieron: Carla, Maricela, Habib, Gaby Q, Gaby M, Montana y Guimel. A Clau, Edel y María Fernanda por apoyarme durante las últimas instancias del doctorado, los consejos y la apertura para escucharme en todo momento. Fue un gusto enorme compartir el laboratorio con ustedes.

Gracias a Guillermo González por la gran predisposición a ayudarme en todo momento. A Jerome por su gran ayuda con el cultivo de las plantas de vid en Riverside y por su amistad.

A todos los que me guiaron y con quienes compartí grandes momentos en Irapuato: Martita, Sole, Miguel, Liz, Pedro y a todo el equipo del laboratorio de Alfredo. Especialmente a Manu y Fabricio por compartir su conocimiento y por la ayuda de corazón sincero en este trabajo.

A Laura, Edgar, Oscar, Eddy, Leo, Lluvia, Adri, Arianne y a todo el grupo de Microbiología. A Pao, Naho, y todos los Metagenómicos.

Y por supuesto, esta tesis no hubiera sido posible sin el acompañamiento de mi esposa Mariela, el pilar más importante, sólo ella sabe el sacrificio que significó para ambos este trabajo de tesis. Gracias por el apoyo incondicional. GRACIAS AMOR!

Gracias a la familia que desde lejos me apoyaron como siempre. Especialmente a Mamá por acompañarme en este momento tan importante. A mi viejo y a mis hermanas.

Gracias a todos los integrantes de Pelicarrones y Quimera Shaolin, que ayudaron con los partidos de fútbol y así evitaron que me vuelva loco en el camino.

Tabla de contenido

Lista de figuras

Lista de tablas

Chapter 1. Introduction

The grapevine is one of the most important fruit crops in the world. In Mexico, the main producers of grapevine are the States of Sonora, Zacatecas and Baja California. Together they harvest 284,682 tons per year, which represents a net economic gain of 283,239 dollars per year (SIAP, 2014). The more extended harvested grapevine cultivar is *Vitis vinifera* due to its advantage in quality production, but it has the inconvenient to be highly susceptible to diverse diseases (Salazar and Melgarejo, 2005; Keller, 2010).

Among the diseases affecting grapevine, there are those caused by fungi that affect the vascular system, named grapevine vascular diseases (GVD, a.k.a. grapevine trunk disease). These are caused by taxonomically unrelated fungi that have in common their capacity to survive on the woody trunk. The causal agents are the Ascomycetes: Botryosphaeriaceae spp., Diatrypaceae spp., *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp. and the Basidiomycete: *Cylindrocarpon* spp. (Bertsch *et al*., 2013).The symptoms produced by Botryosphaeriaceae and Diatrypaceae are similar, with the main being a wedge-shaped necrotic lesion that is observed in a cross section of the affected zone (Úrbez-Torres *et al*., 2011) (Figure 1)

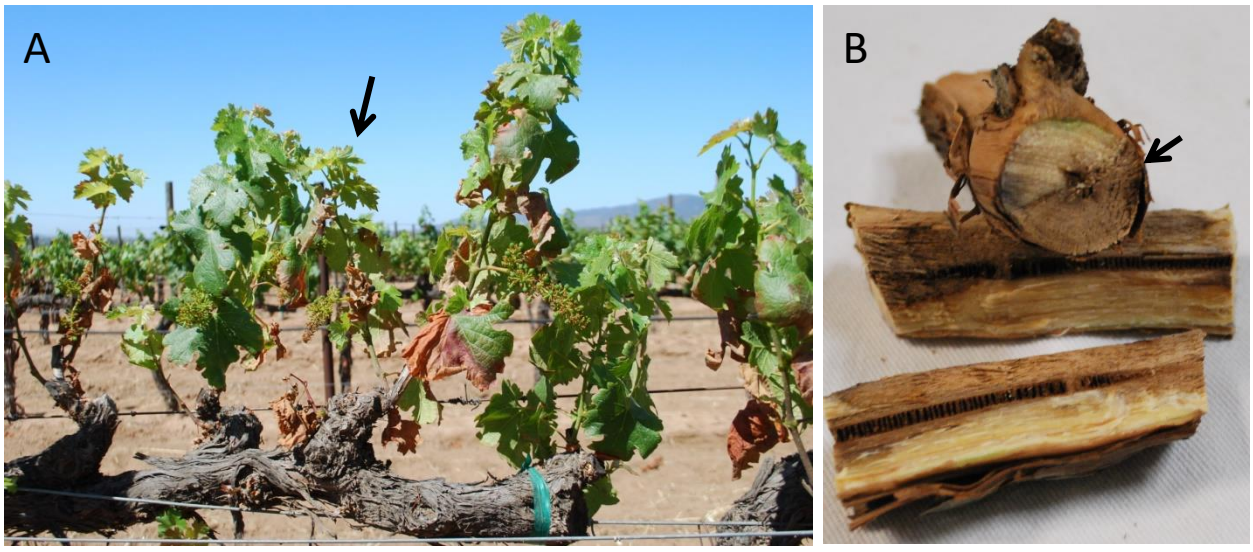

Figure 1. Botryosphaeria dieback symptoms. A. Infected plant showing Botryosphaeria wood dieback in the field. B. wedge-shaped canker.

Botryosphaeriaceae is a fungal family which has acquired importance over the last 15 years as the causal agent of botryosphaeria dieback disease in grapevine. This disease have been recognized as the main threat to vineyards worldwide, once the pathogen is established, it cause a progressive dieback and even plant death. There is no an effective treatment (Fussler *et al*., 2008), which increase even more the concern among growers for this disease. At least, twenty one species of Botryosphaeriaceae have been associated as causal agents of this pathology. The recognized genera are *Diplodia, Dothiorella, Fusicoccum, Guignardia, Lasiodiplodia* and *Neofusicoccum* (Úrbez-Torres, 2011; Taylor *et al*., 2005; Úrbez-Torres *et al*., 2009; van Niekerk *et al*., 2004). In Mexico, *L. theobromae, D. seriata, D. corticola, N. australe* and *N. vitifusiforme* have been associated to botryosphaeria dieback (Candolfi-Arballo, 2009; Úrbez-Torres *et al*., 2008).

Recent studies showed that there are geographic differences on Botryosphaeriaceae distribution, which is mainly attributed to climatic variability (Taylor et al., 2005; van Niekerk *et al*., 2006; Úrbez-Torres *et al*., 2006; Úrbez-Torres *et al*., 2008). For example, *L. theobromae* shows preference for warm and dry climates (Taylor *et al*., 2005; Úrbez-Torres *et al*., 2008; Úrbez-Torres *et al*., 2006); in contrast, *B. dothidea* and *N. parvum* are prevalent species in vineyards growing in cold climates. *D. seriata* is the only Botryosphaeriaceae isolated from all the viticultural regions in the world. This, might be related to the differential capacity of the pycnidiospores of Botryosphaeriacea, to germinate in a wide range of temperatures (from 10 °C to 40 °C) (Úrbez-Torres, 2011). The pycnidiospores or conidia are asexual spores that are released from pycnidia when impacted by rain drops (Úrbez-Torres, 2011). Those are produced inside pycnidia, fruiting bodies developed on death tissue. While it is widely accepted that the inoculum source comes from close infected grapevines, it can not be discarded that in some cases, the primary inoculum source is released by an alternative host that surrounds the vineyard. In this regard, it was observed that *Botryosphaeria ribis* isolated from *Ribes* sp. was highly virulent on grapevine (van Niekerk *et al*., 2004).

Botryosphaeriaceae members infect grapevine through wounds; therefore pruning of trunks and suckers wounds promote the infection (Úrbez-Torres, 2011; Makatini, 2014). In *N. luteum* the presence of wounds on grapevine leaves or stems is needed for successful conidial germination, which suggests that unwounded tissue might have inhibitors of this process, that the wax covering epidermal cells impede the adhesion of the conidia and therefore their germination, that wounds produce germination stimulants (Amponsah *et al*., 2012) or that the saprophytic mycelium growing in the bark could gain access into the xylem in plants under stress (Billones-Baaijens *et al*., 2014). However, the observation that *B. dothidea* produces appresoria on the epicarp of apple fruit forcing its penetration (Kim *et al*.,

1999), suggest that this capacity could be used for active penetration of grapevine tissues, altough this has never been observed. Recently, *Botryosphaeria* spp. were isolated on silver oak seeds suggesting another possible strategy of fungal propagation (Gardefa, 2015), which is coherent with their endophytic behavior.

1.1 Grapevine vascular diseases

Since 2004, several studies have been conducted with the aim to evaluate the pathogenicity of GVD. Such studies allowed to establish the relative relevance of the Botryospaheriaceae members causing damage on grapevine was established. *Lasiodiplodia* spp. and *Neofusicoccum* spp. were reported as highly virulent, while *B. dothidea* and *Diplodia spp.* showed intermediate virulence and *Dothiorella spp* and *Spencermatinsia viticola* were recognized as slightly virulent (Úrbez-Torres, 2011).

Colonization of grapevine has been fairly studied. Microscopical studies indicated that *L. theobromae, D. seriata* and *D. corticola* showed preference for xylematic tracheas and tracheids but also all fungi were found in the phloem (Obrador-Sanchez, 2011). In the case of *L. theobromae,* plant cell wall degrading enzymes (PCWDEs) activity was inferred at the tip of hyphae, promoting the degradation of xylematic vessels and gaining access to parenchyma cells. *D. seriata* and *D. corticola* did not show PCW degradation, but mycelial growth was evident in xylem indicating an endophytic stage (Obrador-Sanchez, 2011). Colonization of cashew tree by *Lasiodiplodia theobromae* was studied by microscopy, revealing that the fungus grew as endophyte, since it did not trigger plant defense responses (Muniz *et al*., 2011). The fungus takes advantage of the weakening of the PCW in pit fields (on plasmodesmata) for cell–to-cell propagation. Only when it cannot access the pit fields, it degrades the PCW to continue with the colonization (Muniz *et al*., 2011). The first carbon sources used by *L. theobromae* in the endophytic stage are starch, proteins and free sugars available in the extracellular space (Encinas and Daniell, 1996); when those resources are depleted, the fungus starts PCW degradation on parenchymatic cells. A combination of hydrolytic and oxidative enzymes are produced and secreted to degrade the complex PCW. Additionally, a marked change on hyphal diameter during colonization has been observed, and the fungus showed preference for colonization of parenchymatic tissue in rays and vessels (Muniz *et al*., 2011). *B. dothidea* infects apple tree through lenticels and the infection is mainly restricted to the cortex (immediately below the epidermis), and while it could well grow both intercellularly and intracellularly, the first is preferred (Han *et al*., 2016).

The host cells in cortex surrounding hyphae, showed a marked change characterized by the disintegration of organelles even when the PCW remained intact, suggesting that the fungus injects compounds that promote organelles degradation, and then absorbs nutrients from dead cells. After some time, the production of PCW is also impaired, suggesting the secretion of PCWDEs by the fungus. Interestingly, when those changes are occurring, the hypha becomes thicker and gets surrounded by a dark electrodense shield. Three months after inoculation, symptoms in apple wood become evident and microscopical images indicate that in the parenchymatic cells, only the PCW remains, devoid of cytoplasm, nucleus and organelles. Hyphae surrounded by the dark electron-dense material showed increase of lipid bodies. Nevertheless, it is still unknown how that material is composed and how is the relationship with lipid bodies. The main defensive response of the host is to construct a peridermal barrier with death cells to separate healthy from infected tissue, but this is unsuccessful, because eventually the pathogen finds the way to break down the barrier and continues with the colonization (Han *et al*., 2016).

From the microscopical analysis of the disease progress of *N. luteum* in grapevine (Billones-Baaijens *et al*., 2014), it was proposed that Botryosphaeriaceae fungi can get access to the cortex in grapevine through lenticels or cracks in the epidermis. Then they could remain alive through the β-oxidation of lipids stored in lipid bodies, such as those observed on *B. dothidea* (Kim *et al*., 2004, Kim *et al*., 2001; Han *et al*., 2016). This might allow the fungus to grow without causing any damage to the host tissue, which corresponds to the latency stage observed in Botryosphaeriaceae, and once the pathosystem is exposed to stress, the fungus takes advantage and colonizes the xylem (wood) of the grapevine, as it was observed for *N. luteum* (Amponsah *et al*., 2014).

1.2 Endophyte to pathogen transition

An interesting aspect of Botryosphaeriaceae is their capacity to behave as endophyte for years, without causing any symptom in the plant host, until an unknown cause triggers its pathogenic behavior. The first report analyzing this phenomenon was carried out in dogwood, where a clear difference in cankers produced by *L. theobromae* under drought stress was observed (Müllen *et al*., 1991) In recent studies, it has been observed that the exposure to high light levels triggers *Diplodia mutila* pathogenicity in the tropical palm *Iriarte deltoidea*, while low light levels favor an endosymbiotic development (Álvarez-Loayza *et al*., 2011).

On another hand, in grapevines grown under water stress, colonization and disease expression produced by Botryosphaeriaceae are more severe (van Niekerk *et al*., 2011). Some studies have evaluated water stress as a predisposing agent in the host to Botryosphaeriaceae infection (van Niekerk *et al*., 2011, Ma *et al*., 2001). However, few efforts fave focused on the behavior of the fungi during abiotic stress events. Thermal stress has not received much attention. It is already known that conidia of *D. seriata* germinate within a range of 10-40 °C, while the optimum temperatures for mycelial growth and germination of *L. theobromae* are in the range of 30-40 °C. These disparities in optimum temperature correlate with the different geographical distribution of the species. While *D. seriata* has a worldwide distribution,; *L. theobromae* is limited to the warmest regions. It has also been suggested that temperature affects the virulence. In grapevine greenshoots infected with *L. theobromae*, more damaged tissue was observed at 35 °C in comparison to temperatures below 30 °C (Úrbez-Torres *et al*., 2011). This suggests a higher rate of colonization, maybe promoted by a higher growth rate or higher pathogenicity/virulence factors secretion.

1.3 Disease caused by Botryosphaeria spp. increasing as effect of global warming

The botryosphaeriaceae-associated disease in European forest increased after the heat and drought wave of the summer 2003 (Desprez-Loustau, 2006), suggesting that this stress favors the botryosphaeriaceae pathogenicity. In this sense, recently, Allen *et al*. (2015) prognosticated that higher mortality of trees would occur as effect of global climate warming, because of the increase of hotter drought events. Extreme events of short duration will cause even more stress, because the plant does not have physiological changes for adaptation and therefore facilitates the growth of the pathogenic microorganism with a higher capacity and flexibility to adapt to those changes (Allen *et al*., 2015). Several models have been proposed to explain the reasons for tree mortality, and there is still an intense debate about what is occurring. A model moderately accepted is, that the combination of abiotic and biotic stresses exacerbate the effect. The nonstructural carbohydrate depletion resulting from dealing with abiotic and biotic stress, reaches levels in which the transport on vascular tissue is severely affected and, in extreme cases, even produces the collapse of the tree (Olive *et al*., 2014).

The increase on the incidence of tree mortality has also been related to the exacerbation of fungal virulence under stress. Examples of these interactions were observed in *Cryphonectria parasitica* on

chestnut, where disease incidence increased under drought and heat waves (Waldboth and Oberhuber, 2009). In this sense, *B. dothidea* causes serious damage only on weak or stressed hosts. Thus, due to its recognized behavior as endophyte it is more likely that the major effect of climate change in the pathosystem is to trigger the fungal pathogenicity in stressed hosts. *Diplodia quercina*, a fungus of the Botryosphaeriaceae recognized as a beneficial endophyte in mediterranean oak, turns to a pathogenic behavior when reiterated drought episodes disrupt the equilibrium that maintain the mutualistic interaction (Moricca and Ragazzi, 2008). The cause of fungal behavioral change is unknown. For some endophytes it has been suggested that their pathogenic stage is limited by the oxygen and nutrient availability, thus when the environmental conditions change, also the interior of the host tissues changes, which favors the development of the pathogenic stage (Krabel *et al*., 2013). On the other hand, it has been suggested that fungal mycelium grows until it reaches a threshold, and then the pathogenic phase is activated producing reproductive structures (Krabel *et al*., 2013),, as it was observed in pine needles (Sieber, 2007). It is possible that grapevines are under the same vulnerability as the forest trees, and therefore similar models could explain the increase of vascular diseases observed in vineyards worldwide.

1.4 Botryosphaeriaceae pathogenic life styles

Based on the fungal strategy of infection, phytopathogenic fungi have been classified in three main groups: biotrophs, hemibiotrophs and necrotrophs. The biotrophs have absolute dependency of the host plant; they cannot live outside of the host. Non-obligate pathogens only require the plant at a stage of their life cycle. In the case of hemibiotrophs, most of their life cycle occurs on the host tissue; while necrotrophs, most of the time live as saprotrophs and occasionally contact the plant killing the host to take nutrients from death tissue (Agrios, 2005). Recently, this classification of pathogenic life styles has been questioned, proposing that a complex fluctuating behavior occurs even simultaneously (Kabbage *et al*., 2015). Based on this new conception and on the behavior of Botryosphaericeae, they can be classified as hemibiotrophs (or latent pathogens) with a biotrophic stage with a duration time that depends on the environmental conditions.

1.5 Grapevine defensive response to Botryosphaeriaceae infection

Currently, there are no studies that evaluate the Botryosphaeriaceae-grapevine interaction; but in grapevine infected with *Eutypa lata*, another trunk disease-associated pathogen, the main response of the plant was the lignification of its cell wall (Rolshausen *et al*., 2008). Symptoms observed in the trunk of grapevine infected with *E. lata* are quite similar to those caused by Botryosphaeriaceae, suggesting a similar plant response. If lignification occurs, it can be assumed that there is an oxidative burst response and the Botryosphaeriaceae fungus must cope with it and with the toxic phenolics precursors of lignin.

Cobos *et al*. (2010) analyzed the differential proteomic response in *D. seriata* during growth in medium with or without carboxymethylcellulose, and identified the induction of an antioxidant enzyme, suggesting that oxidative stress occurs during polysaccharide degradation.

There is little knowledge about the Botryosphaeria-grapevine interaction. However, we can get inferences of what are the roles of the partners in the interaction, from the generalized response of grapevine to vascular pathogens. It has been postulated that the main susceptibility of grapevine to the infection of vascular pathogens occurs when a wound is produced; this implies that the rate of healing is a keystone in the fungus-plant interaction. The main responses include the accumulation of phenols, phytoalexin production, synthesis of hydrolytic enzymes, and cell wall reinforcement with phenolic polymers such as suberin and lignin (Hawkins and Boudet, 1996). Within the vascular tissue the wound response implies tylose production, a balloon-like structure eventually lignified, that blocks the vessels in vascular tissue (Pratt, 1974). The pathogen needs to overcome those barriers for a successful colonization. In a previous study, it was observed that grapevine produces tylose in tracheas in response to *D. seriata*, *D. corticola* and *L. theobromae* infection, but it seems to be ineffective to block fungal growth in vascular tissue, because the fungus finds other vessels to achieve colonization (Obrador-Sanchez, 2011). In the first stage of grapevine infection, *L. theobromae* showed PCW degrading capacity and colonization of the pith parenchyma, but in *D. seriata* and *D. corticola* this behavior was not observed (Obrador-Sanchez, 2011).

Up to date, there are scarce studies that analyze the grapevine response to infection by Botryosphaeriaceae. Spagnolo *et al*. (2014) studied the differential proteins produced between regions with black/brown strip indicative of Botryosphaeriaceae dieback disease compared to healthy tissue (Spagnolo *et al*., 2014). Responsive proteins (PR2, PR5 y PR17) involved in general defensive response, were also produced to alleviate biotic and abiotic stress. Further, proteins involved in the antioxidant

system (Glutathione-S-Transferase (GST) and Cys peroxiredoxine, were differentially expressed in symptomatic tissue. The levels of phenolics compounds were higher on the necrotic tissue. The influence of phenolics in the grapevine response to *N. parvum* and *D. seriata* infection was studied, indicating that mainly stilbene was produced as an attempt to restrict advance of the pathogen. However, it only has a fungistatic effect, because eventually the pathogen recoveed from the toxicity of these compounds and continued the infective process (Lambert *et al*., 2012). The production of detoxifying enzymes and phenylpropanoids in grapevine increased in response to the exposure to Botryosphaeriaceae extracellular compounds (Ramírez-Suero *et al*., 2014).

A polyphenol oxidase (PPO) was induced in the symptomatic region of grapevine infected by causal agents of Botryosphaeria dieback. This enzyme is involved in the defensive phenylpropanoid pathway and therefore is associated with phytoalexin, phenolics and lignin production (Spagnolo *et al*., 2014).

E. lata produces eutipines, which are toxins with protonophore activity on chloroplasts. It was observed that 10 out of 91 genes associated with the absence of symptoms in grapevine, an indicative of resistance, are involved in light absorption and electrons transport in chloroplasts, indicating an attempt to restore the impairment of chloroplasts caused by fungal toxins. Grapevine requires maintaining the level of photosynthate production, and despite the response to alleviate the stress in chloroplasts and to keep the redox balance to reduce the symptoms, the response is not enough to revert the disease process (Camps *et al*., 2010).

From studies of the physiological response of grapevine to the fungus that cause vascular diseases, several important factors were identified. The velocity to heal the pruning wound in grapevine is important to restrict fungal infection; grapevine has low resources/reserves to have a successful response to biotic and abiotic stress. The size of cells surrounding the grapevine vessels or thickened-lignin deposits could explain the differences in tolerance among varietals (Mundy and Manning, 2011).

It is probable that the incapacity of grapevine to establish a successful defense response to Botryosphaeria-caused impairments is due to the difficulty to contend with fungi that have coevolved with the plant (Mundy and Manning, 2011). This close relationship might involve a specific signaling process that could explain how the pathogen evades the plant attempts of compartmentalization.

1.6 The pathogenic resources of Botryosphaeria spp.

1.6.1 Plant Cell Wall Degrading Enzymes (PCWDEs)

Plant Cell Wall Degrading Enzymes (PCWDEs) have a fundamental role in pathogenicity, both for breakage of host structural tissue, which allow the advance of colonizing hyphae, and at the same time to provide carbon source that sustains fungal growth. Their role in pathogenicity has resulted difficult to establish, mainly because they are redundant and act as a consortium (Tudzynski and Sharon, 2002). Despite this limitation, a transcriptomic study in *Magnaporthe oryzae* showed that during the colonization of rice tissue, PCWDEs are among the main differentially expressed genes respect to a control grown *in vitro* (Mathioni *et al*., 2011).

In Botryosphaeriaceae, several PCWDEs have been reported. *L. theobromae* produces celullases, □-glucosidase, pectinases, laccases, □-1,3 glucanases, cutinases, esterases, poligalacturonases, proteases, xylanases and lipases (Umezurike *et al*., 1979; (Saldanha *et al*., 2007; Dekker *et al*., 2007; da Cunha *et al*., 2003; Dickman and Patil, 1986; Ayllón-Gutierrez and Hernández-Martínez, 2011) (Table 1).

1.6.2 Growth regulators

Plant growth is regulated by compounds acting as hormones. The most important types of these compounds are auxins, giberelins, cytokinins, ethylene, jasmonic acid (JA), salicylic acid (SA) and growth inhibitors. Plant pathogens produce similar or antagonic compounds, to manage the plant growth, which benefits the pathogen favoring its colonization (Agrios, 2005).

JA is produced in high quantities by *L. theobromae* (Aldridge, 1970), suggesting the capacity of this fungus to manipulate the host defensive response.

Table 1. Botryosphaeriaceae pathogenicity factors

Pathogenic factors (main classification)	**Names**	**Pathogen**	**References**
Enzymes	Cellulases and β-glucosidases	*L. theobromae*	Umezurike *et al*., 1979
	Pectinases, laccases and β-1,3-glucanases	*L. theobromae*	Saldanha *et al*., 2007
	Polygalacturonases, proteases, xylanases and lipases	*L. theobromae*	Ayllon-Gutierrez and Hernández-Martínez, 2011
	Esterases and cutinases	*L. theobromae*	Dickman and Patil, 1986
	Peroxiredoxin-1 (PRX1) and alcohol-deshydrogenase (ADH)	*D. seriata*	Cobos *et al*., 2010
Polysaccharides	Exopolysaccharides (EPS)	*N. parvum* *N. luteum*	Martos *et al*., 2008
	Lasiodiplodan	*L. theobromae*	Alves da Cunha *et al*., 2012
	Botryosphaeran	*Botryosphaeria spp.*	Barbosa *et al*., 2003
Toxins	Melleins	*D. seriata* *L. theobromae*	Andolfi *et al*., 2011Aldridge *et al*., 1970
	Hydroxymellein, isosclerone and tyrosol	*N. parvum* *L. theobromae*	Andolfi *et al*., 2011; Aldridge *et al*., 1970
	Lasiodiplodine	*L. theobromae*	Aldridge *et al*., 1970
Hormones	Jasmonic Acid	*L. theobromae*	Aldridge *et al*., 1970

1.6.3 Phytotoxic metabolites

Fungal toxins could affect cellular functions or even kill the plant host cells before or during the infection stage (Agrios 2005). The wood and foliar symptoms on grapevine affected by botryosphaeria dieback suggest fungal production of toxins. Several studies proved that grapevine vascular pathogens produce toxic bioactive metabolites, which are responsible of disease symptoms (Tabacchi *et al*., 1999; Mahoney *et al*., 2003; Rolshausen *et al*., 2008). These cytotoxic metabolites are separated in low and high molecular weight.

1.6.4 Low molecular weights toxins

N. luteum and *N. parvum* produce phytotoxins of low molecular weight with lipophylic and acidic characteristics (Martos *et al*., 2008). In recent studies, it was found that *N. parvum* released compounds with toxicity on tomato plants identified as (3R,4R)-(-)-4-hydroxymellein, (3R,4S)-(-)-4-hydroxymellein, isosclerone and tyrosol (Evidente *et al*., 2010; Andolfi *et al*., 2011). Hydroxymellein and isosclerone showed the highest toxicity (Evidente *et al*., 2010; Andolfi *et al*., 2011). On other hand, *D. seriata* produced mellein, (3R,4R)-4-hydroxymellein, (3R)-7-hydroxymellein, (3R,4R)-4,7-dihydroxymellein and tyrosol, which has phythotoxicity because of their oxidant properties (Andolfi *et al*., 2011).

1.6.5 High molecular weight toxic compounds (polysaccharides)

The botryophaerans are produced by *L. theobromae* and they were the first exopolysaccharides (EPS) identified in Botryosphaerias. These are constituted by cross-linked β-(1→3; 1→6)-D-glucans (Barbosa *et al*., 2003). Later, botryosphaeran-like compounds were found being produced by *D. seriata*, *N. luteum*, *N. parvum* and *S. viticola*. They are also exopolysaccharides like botryosphaeran, mainly constituted by glucose, mannose and galactose (Martos *et al*., 2008).

Several metabolites, including EPS, have been suggested to act directly on the plant metabolism and specifically to affect chloroplast functionality. The deleterious effect of EPS on chloroplast has been suggested because of EPS accumulation on the leaf mesophyllic cells. On other hand, it has been proposed that EPS affect vascular transport because of their effect on the blockage of xylem vessels (Andolfi *et al*., 2011).

1.6.6 Antioxidants

The exposure of fungal pathogens to oxidative stress during infection demands an antioxidant response capacity to survive inside the host. Among several antioxidant fungal mechanisms, an important function has been proposed for melanin (Jacobson, 2000). Several studies have found that melanin in some phytopathogens is essential for appresorium formation and host tissue penetration (Perpetua *et al*.,

1996). Recently, genome sequencing and transcriptomic studies of fungal pathogens, has suggested alternative roles for melanin during post-penetration stages.The majority of the members of botryosphaeriaceae have dark pigmented mycelium, both in stationary-phase, during growth and also in mature conidia, suggesting the presence of melanin. The role of melanin in botryosphaerias remains to be analyzed, especially during abiotic stress response and during the pathogenic stage.

In several fungal-plant interactions a correlation between higher tolerance to environmental abiotic stress in a microorganism and its higher capacity to infect the plant has been observed, regardless of the establishment of a mutualistic or parasitic interaction. This association is not well understood, but it has been suggested that abiotic stress mimics the adverse environment sensed by microorganism during infection (Singh *et al.*, 2011). In *Magnaporthe oryzae*-rice and *Ascochyta rabiei*-chickpea interactions, it has been clearly established that the transcriptomic response to infection is similar to that caused by carbon and nitrogen starvation and oxidative stress (Mathioni *et al*., 2011; Singh *et al*., 2012)

1.7 Botryosphaeria dieback control strategies

The strategies commonly employed for Botryosphaeria dieback management on vineyards are, aspersion of chemical or biological compounds, vineyards sanitation and modification of cultural practices (e.g. change from single to double pruning) that help prevent disease propagation (UC Pest Management guidelines http://www.ipm.ucdavis.edu/PMG/r302100611.html).

However, it does not exist an effective control strategy that really helps to impede the disease progression. The major problem that growers face is the diversity of species involved in development of the disease and the use of symptomless infected material to establish a new vineyard. (Úrbez-Torres *et al*., 2011). The current treatment is to perform a reconstitutive surgery which consists on pruning and removing the symptom-expressing parts of the plants and then retraining from the wound. In severe cases, the whole plant needs to be removed from the vineyards to impede inoculum production, avoiding the risk of infection of healthy plants (Epstein *et al.,* 2008). Other preventive option is to cover the pruning wounds with latex paint to diminish the risk of infection during this stage of susceptibility. The latex paint could be mixed with boric acid or essential oils (UC Pest Management guidelines http://www.ipm.ucdavis.edu/PMG/r302100611.html).

Among the fungicides evaluated, methyl thiophanate has been the most effective, protecting 80% of pruning wounds (Rolshausen *et al*., 2010). However, several useful fungicides were banned in Europe and USA, therefore it is extremely important to develop and implement alternative effective, cheap and environment friendly control methods. For this reason, understanding the fungal-plant interaction becomes essential.

1.8 Justification

L. theobromae is one of the most virulent fungi causing vascular disease. In grapevine, the disease it produces is called botryosphaeria dieback and it affects vineyards worldwide, including Mexico. There are no effective control strategies, thus the growers must use huge economic resources in remotion, burning and substitution of plants showing symptoms. The symptoms of the disease are more defined in plants under abiotic stress. Considering all the above, this thesis evaluates the *L. theobromae*-grapevine pathosystem, with a focus on understanding the correlation of abiotic stress and fungal pathogenicity. Studying the fungal transcriptional response to heat stress could help to infer biochemichal mechanisms of adaptation that also could have a role during infection. On other hand, it will also allow the identification of pathogenicity genes important for fungal establishment in the plant. The information generated in this research will help in the design of long-term and effective control strategies.

1.9 Hypothesis

Heat stress induces the expression of pathogenicity factors in the fungus *L. theobromae* promoting its virulence in grapevine.

1.10 General objective

To understand the impact of heat stress in the fungus *L. theobromae* at the transcriptional level, and how it affects its pathogenicity and the expression of symptoms in grapevine.

1.11 Specific objectives

- To identify *in vitro* putative pathogenicity genes in the fungus *L. theobromae* expressed in response to heat stress.

- To evaluate the expression of selected putative pathogenicity genes during fungal colonization in stressed and unstressed grapevines.

Chapter 2. Quantitative real-time PCR normalization for gene expression studies in the plant pathogenic fungi *Lasiodiplodia theobromae*

2.1 Abstract

Lasiodiplodia theobromae is a highly virulent plant pathogen. It has been suggested that heat stress increases its virulence. The aim of this work was to evaluate, compare, and recommend normalization strategies for gene expression analysis of the fungus growing with grapevine wood under heat stress. Using RT-qPCR-derived data, reference gene stability was evaluated through geNorm, NormFinder and Bestkeeper applications. Based on the geometric mean using the ranking position obtained for each independent analysis, genes were ranked from least to most stable as follows: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), β-tubulin (*TUB*) and elongation factor-1α (*EF1α*). Using RNAseq-derived data based on the calculated tagwise dispersion these genes were ordered by increasing stability as follows: *GAPDH, ACT*, *TUB,* and *EF1*α. The correlation between RNAseq and RTqPCR results was used as criteria to identify the best RT-qPCR normalization approach. The gene *TUB* is recommended as the best option for normalization among the commonly used reference genes, but alternative fungal reference genes are also suggested.

2.2 Introduction

Trunk disease fungi are a severe problem in most countries, causing great economic impact to the grapevine industry (Bertsch *et al*., 2013). Botryosphaeria dieback (a.k.a. bot canker) is a grapevine trunk disease caused by several members of the Botryosphaeriaceae. Currently, *Lasiodiplodia theobromae* (teleomorph *Botryosphaeria rhodina*) has been found to be one of the most virulent spp. (Úrbez-Torres, 2011). *L. theobromae* is extensively distributed in tropical and subtropical areas and it has been associated with approximately 500 hosts (Punithalingam, 1976). Diverse members belonging to the Botryosphaeriaceae frequently occur as endophytes in asymptomatic plant tissues (Slippers *et al*., 2007), that might become pathogenic when hosts are subjected to abiotic stress (Smith *et al*., 1996; Sieber, 2007). Heat and drought stresses seem to promote fungus colonization in grapevine (Úrbez-Torres, 2011;

Slippers *et al*., 2007; Smith *et al*., 1996), as larger lesions were observed in grapevine infected with *L. theobromae* when plants were grown at 35 °C, in comparison with those grown at 30 °C (Úrbez-Torres, 2011). This suggests that a rise in temperature may trigger the pathogenicity of these fungi. To get some insight into this process, one approach is to study transcriptional responses using RT-qPCR. This technique is highly sensitive, but demands great standardization efforts, mainly because stable reference genes to normalize the data are required (Thellin *et al*., 1998; Vandesompele *et al*., 2002; Pfaffl *et al*., 2004; Andersen *et al*., 2004; Bustin *et al*., 2009). Reference gene validation is needed for every experiment because there is no universal or ideal gene that can be used for all possible tissues and conditions (Thellin *et al*., 1998; Vandesompele *et al*., 2002; Pfaffl *et al*., 2004; Andersen *et al*., 2004; Bustin *et al*., 2009; Reid *et al.,* 2006; Vieira *et al*., 2011; Zampieri *et al*., 2014). Alternative to the use of endogenous controls, if the samples are processed identically to minimize variability, data distribution can be analyzed and normalized using geometric means, quantiles or even raw data (Mar *et al*., 2009). Also, the increase in RNAseq data availability allows for the analysis of stability of higher numbers of genes, identifying those more stable than the commonly used "housekeeping" genes (Yim *et al*., 2015; Llanos *et al*., 2015). So far there are no studies of this nature for *L. theobromae*, thus, this work provides the first guide for normalizing gene expression in response to heat stress in the presence of grapevine wood.

2.3 Materials and methods

2.3.1 Fungal growth conditions and collection of samples

L. theobromae (UCD256Ma) was isolated from grapevine cankers (Úrbez-Torres *et al*., 2006), kindly provided by Dr. Douglas W. Gubler from the University of California, Davis and kept at -20 °C in glycerol. The isolate was recovered and grown for three days on Petri plates with Potato Dextrose Agar (PDA (Difco©). A mycelium plug (5 mm) of the fungus was used to inoculate flasks containing 10 ml of Vogel's salts medium, pH 6.0, without or with 1% w/v wood obtained from one-year old branches of grapevine cv. Cabernet Sauvignon. Previously, wood was ground using a blender and then filtered through a 0.5 mm sieve. Flasks with inoculated media were incubated at 28 °C in darkness, without agitation, for 48 h. Thereafter, some flasks were exposed to heat stress (HS) by transferring them to 42 °C for 1 h and then placed back at 28 °C (condition with wood: FWS, and without: FS). Controls were maintained without

stress at 28 °C throughout the experiment (condition with wood: FW, and without: F). Each condition had three biological replicates. Ten min after the HS, mycelia were collected using sterile tweezers, washed with diethylpyrocarbonate (DEPC, Sigma-Aldrich) treated water, transferred to a 1.5 mL tube containing 500 µl of Nucleic Acid Preservation solution (NAP) (Camacho-Sanchez *et al*., 2013), and stored at -80 °C until RNA extraction. For DNA extraction, the fungus was grown in Potato Dextrose Broth PDB (Difco©) for 5 d, the mycelium was collected and directly stored at -20 °C.

2.3.2 Total RNA extraction, genomic DNA (gDNA) removal and complementary DNA (cDNA) synthesis

A modified RNA extraction protocol based on Reid *et al*. (2006) and Vasanthaiah *et al*. (2008) was used in combination with silica columns as follows. The fungal mycelium from the four conditions and their three biological replicates was removed from NAP solution and 90-130 mg was lyophilized. Approximately 30 mg of polyvinylpyrrolidone (Sigma-Aldrich) were added in 2 mL bead-beating tubes pre-charged with 50 mg of 0.5 mm and 100 mg of 1 mm glass beads (Biospec Products). Tubes were beaten on a Mini-Beadbeater for 30 s, immediately chilled on ice, and pre-heated lysis solution was added (guanidinium thiocyanate 2.9 M, sodium citrate 22 mM, β-mercaptoethanol 51 mM, N-lauroylsarcosine sodium salt 0.36 % w:v, pH 8) before subjecting the tubes to a second beating cycle for 30 s. Lysed samples were incubated at 65 °C for 10 min and 500 µl of chloroform:isoamyl alcohol (24:1 v:v) were added and vortexed twice. The tubes were centrifuged at 4,000 rpm for 15 min at 4 °C.

The aqueous phase was transferred to a new 1.5 mL tube and mixed with 1 v of 75% ethanol v:v. The total mix was passed through EconoSpin® (Epoch Life Science Inc.) and centrifuged at maximum velocity for 1 min at room temperature. Thereafter, the column was washed with RPE buffer (Qiagen®) and ethanol 75% v:v. Finally, total RNA was eluted in 48 µL of nuclease-free water (Qiagen®) by centrifugation at maximum velocity. Genomic DNA was removed using RQ1 DNAse (Promega) and RNA was purified through precipitation according to the manufacturer's instructions. The purity and quantity of total RNA was determined using a UV/vis Nanodrop 2000 (Thermo Scientific), and RNA integrity was verified by agarose gel (1.5% w:v) electrophoresis Complete genomic DNA removal was evaluated by PCR amplification of a 554 bp (intron containing) glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene fragment, using 1-FW-GAPDH and 1-RV-GAPDH primers (Table 2). *L. theobromae* genomic DNA was isolated as described before (Murray and Thomson, 1980) and used as positive control.

DNA-free total RNA samples were divided in two. A portion was used for RNA sequencing as explained below. The rest of the RNA for each sample was used to convert 1 µg of total RNA to cDNA using the ImProm-II reverse transcription system (Promega) following the manufacturer's instructions and then used for RT-qPCR.

2.3.3 RNA sequencing and analysis

RNA sequencing was done through Illumina HiSeq2500 to obtain 100 bp pair-end reads at the National Laboratory of Genomics for Biodiversity service facilities (Irapuato, México). RNAseq data with detailed information of the differential expression analysis from this work is available at the Gene Expression Omnibus (GEO) DataSet (GSE75978). Briefly, a *de novo* transcriptome was assembled with all the reads, then the gene expression in each treatment was calculated using the counts per million (CPM) of reads. The treatments under heat stress without (FS) or with GW (FWS) were compared with the samples not exposed to stress (F or FW). Additionally, the effect of the presence of GW was evaluated (FW vs F). The logarithm of fold change (LFC) and the statistical significance for all the comparisons were determined through General Linear Model statistics using the edgeR package version 3.4.2 of Bioconductor (Robinson *et al*., 2010) in the R environment (version 3.0.2, R Development Core Team, 2008).

2.3.4 Obtaining sequences of commonly used reference genes for RT-qPCR

Partial *L. theobromae* sequences of elongation factor 1-α (*EF1α*), actin (*ACT*), *GAPDH* and β-tubulin (*TUB*) genes were retrieved from the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/) nucleotide database. These genes are highly used for fungal gene expression normalization (Mosquera *et al*., 2009, Kim *et al*., 2010; Song *et al*., 2012, Timpner *et al*., 2013). The recovered nucleotide sequences were confirmed for *L. theobromae* (UCD256Ma) using the *de novo* assembled transcripts from RNAseq data (GSE75978), the presence of the predicted protein sequences was verified through a local Blastp (E value<$1x10^{-40}$ and % Identity >60) using the predicted protein-encoding transcriptome as a protein database.

2.3.5 Selection of stable genes using RNAseq data

To identify genes with high expression stability in the conditions under study, the RNAseq (GSE75978) data was used to calculate tagwise dispersion and LFC with edgeR version 3.10.2, Robinson *et al*., 2010) in the R environment (version 3.2.3, https://www.R-project.org/). Genes with low tagwise dispersion values (<0.06) were selected as stable and then were filtered to retain only those with functional annotation (Table 2). With the functional information of recently proposed reference genes for fungi (Llanos *et al*., 2015), a search for associated functions was done in the list of obtained *L. theobromae* stable genes (Table 2).

2.3.6 Primer design for reference and target genes used for qPCR

Using the transcriptomic data, six target genes were selected according to their pattern of expression and functional annotation (Table 2). The selected genes were: sugar inositol transporter (*SIT*); cpo9 protein (*a9c9*); intradiol ring-cleavage dioxygenase (*IRCD*); Fumarylacetoacetase (*FMH*); salicylate hydroxylase protein (*SH*) and thioesterase-domain containing protein (*TDP*). Obtained sequences for reference genes and target genes were used to design specific primers with PerlPrimer software (version 1.1.21) (Marshall, 2004), considering that primers hybridize in the 3' region of coding sequence. All the parameters recommended for qPCR primers were considered during design (Vandesompele, 2009). The list of primers used for qPCR is shown in Table 2.

2.3.7 Quantitative PCR (qPCR)

qPCR reactions were done in a CFX96 thermocycler (Bio-Rad, Richmond, CA). Reactions to evaluate reference gene stability were done using 7 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2-0.3 µM of each forward and reverse primer and 3 µL of the diluted template cDNA. The program used for all the targets was: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The fluorescence was read after this step. At the end of the program, the temperature was reduced from 95 °C to 65 °C at a rate ramp of 0.1 °C/s, which allowed for the evaluation of the melting curves for each reaction.

Reactions for relative target gene expression evaluation were done using 7 µl of a mix of 2.5 mM Magnesium Chloride, 1X Taq polymerase Accustart buffer, 0.2 mM of dNTPs, 0.03 U/µl Taq polymerase

Accustart (Quanta BioSciences, Gaithersburg, MD), 1X EVAgreen (Biotium, USA), 0.2 µM of each primer and 3 µL of diluted template cDNA. The program used was: 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and a final cycle of 72 °C for 5 min. Melting curves were done using the same conditions as described before for SYBR green.

Selected primer efficiency was determined by qPCR of a cDNA (1:5) dilution series of a mixture of all cDNA samples. The points obtained in a graph of quantification cycle (Cq) vs log of quantity graph were adjusted to a linear curve, and the slope was used to determine the efficiency values (E) as $E=10^{-1/slope}$ (Vandesompele *et al*., 2002). The melting curves were used to evaluate primer specificity. All graphs and E values, melting curve, and slope (Y and r^2), were obtained using BIO-RAD CFX manager software, version 3.1.

Table 2. Reference genes, qPCR primers and their performance.

Gene symbol	GenBank accession number (de novo assembled transcript code)	Primer sequences (5'-3')	AL (bp)	EF (%)	LR (r^2)	S	Y	CqR	PMC (°C)
ACT	AY846879(comp6557_c0_seq1)	**FW** *CTTCGAGACCTTCAACGCC* **RV** *TAGATGGGGACGACGTGAGT*	130	90.1	0.999	-3.6	16.2	6.2	83-88
GAPDH	GU251386 (comp6457_c0_seq1)	**1-FW** *TAGCTAACCCTGTGCCACC* **1-RV** *TCAACGATGCCGTACTTGTC*	554	-	-	-	-	-	-
		FW *TAGCTAACCCTGTGCCACC* **RV** *ACGTACAGCATACTTGGGCTC*	91	102.2	0.996	-3.3	19.0	3.5	82-87
TUB	JN607139 (comp6710_c0_seq1)	**FW** GAACGTCTACTTCAACGAGGT **RV** GAGGATAGCACGAGGAACGT	114	85.5	0.997	-3.7	13.3	4.7	73-82
EF1α	EU012399 (comp3481_c0_seq1)	**FW** TCGTCTGGGTTCGGCAAAAT **RV** CGCGTTAGCCATTGCTCGTA	126	98.2	0.997	-3.4	19.2	6.8	82-87
SIT	comp8181_c0_seq1	**FW** ATCAAGAGCTGGAAGATGACGA **RV** CTCCTTTATTGCGTACTGGATCA	98	105.5	0.995	-3.2	26.1	6.0	84-90
a9c9	comp13164_c0_seq1	**FW** GGATGCGAGGTTAACTTCCC **RV** TCCCAACCGCATTATCCTTC	108	121	0.96	-2.9	26.7	6.0	84-90
IRCD	comp4276_c0_seq1	**FW** CCTACAGCACCAACACTCAG **RV** AGAAGATGCCGTCAGAAACC	128	91	0.967	-3.6	22.8	5.5	86.5-92
FMH	comp14342_c0_seq1	**FW** GAACGGGAATTTGACAGCAG **RV** TCCAGCAACACCAATATCGG	114	90.2	0.996	-3.6	23.2	6.0	85-91
SH	comp12473_c0_seq1	**FW** TCCAGCAACACCAATATCGG **RV** TCTCCTCCTTGAAATTGTCTCGT	119	88.6	0.976	-3.6	24.7	6.0	80-86
TFP	comp8066_c0_seq1	**FW** GCCAGCTTGACGATTAACTAC **RV** CATCCACCAACGTCTCCAAC	126	104.6	0.988	-3.2	27.1	5.5	87.5-93

ACT: Actin, cell motility, structure and integrity protein; ***GAPDH:*** Glyceraldehyde-3-phosphate dehydrogenase, glycolytic enzyme; ***TUB***: β-tubulin, cytoskeleton structural protein; ***EF1α:*** elongation factor 1-α, catalyzation of GTP-dependent binding of amynoacyl-total RNA to the ribosome; ***HSP70:*** family chaperone lhs1 protein.***MCO***: multicopper oxidase type 1; ***SIT:*** sugar inositol transporter; ***a9c9***: apo9 cpo9 protein; ***IRCD:*** intradiol ring-cleavage dioxygenase; ***FMH***: Fumarylacetoacetase; multicopper oxidase type 1; ***SH:*** salicylate hydroxylase protein; ***TDP:*** thioesterase-domain containing protein; AL: Amplicon length; EF: Efficiency; LR: Linear regression; S: slope; Y; intercept on Y axis; CqR: Cq range: PMC; peak in melt curve.

2.3.8 Validation of reference genes using RT-qPCR

The stability of reference genes was determined through quantification of 1:5 cDNA dilutions from two biological and two technical replicates. After examination with a quality filter using qBASE+ software (version 2.5), samples showing differences in Cq higher than 0.5 between technical replicates were discarded. Expression stability was evaluated with the average Cq for each target/sample using the applications geNorm version 2.5 in qBASE+ software (Hellemans *et al*, 2007), NormFinder (version 0.953) (Andersen *et al*., 2004) and Bestkeeper (version 1) (Pffafl *et al*., 2004). A general ranking was obtained using the geometric mean calculated from the values for the position assigned in rankings obtained from each separate statistical analysis.

2.3.9 Relative expression of target genes

Target gene expression was determined using raw data without normalization, normalized using the geometric mean or an endogenous control gene through the ΔΔCq strategy in the HTqPCR package (version 1.22.0, Dvinge and Bertone, 2009) in R (version 3.2.3**,** https://www.R-project.org/), The Shapiro-Wilk test was used for analyzing the normality of the data (Shapiro and Wilk, 1965). A Pearson coefficient (Pearson, 1895) was used to estimate the correlation between gene expression obtained with RT-qPCR and RNAseq. The final LFC value was corrected manually with the PCR efficiency calculated for each gene as proposed before (Vandesompele *et al*., 2002).

2.4 Results

2.4.1 Efficiencies and primer specificities

PCR efficiencies were in the range of 85.5% (*TUB*) to 121% (*a9c9*) with a linear regression range from 0.96 to 0.999 (Table 2). All primers were specific, since a unique peak was obtained in the melting curve graphs (Figure 2).

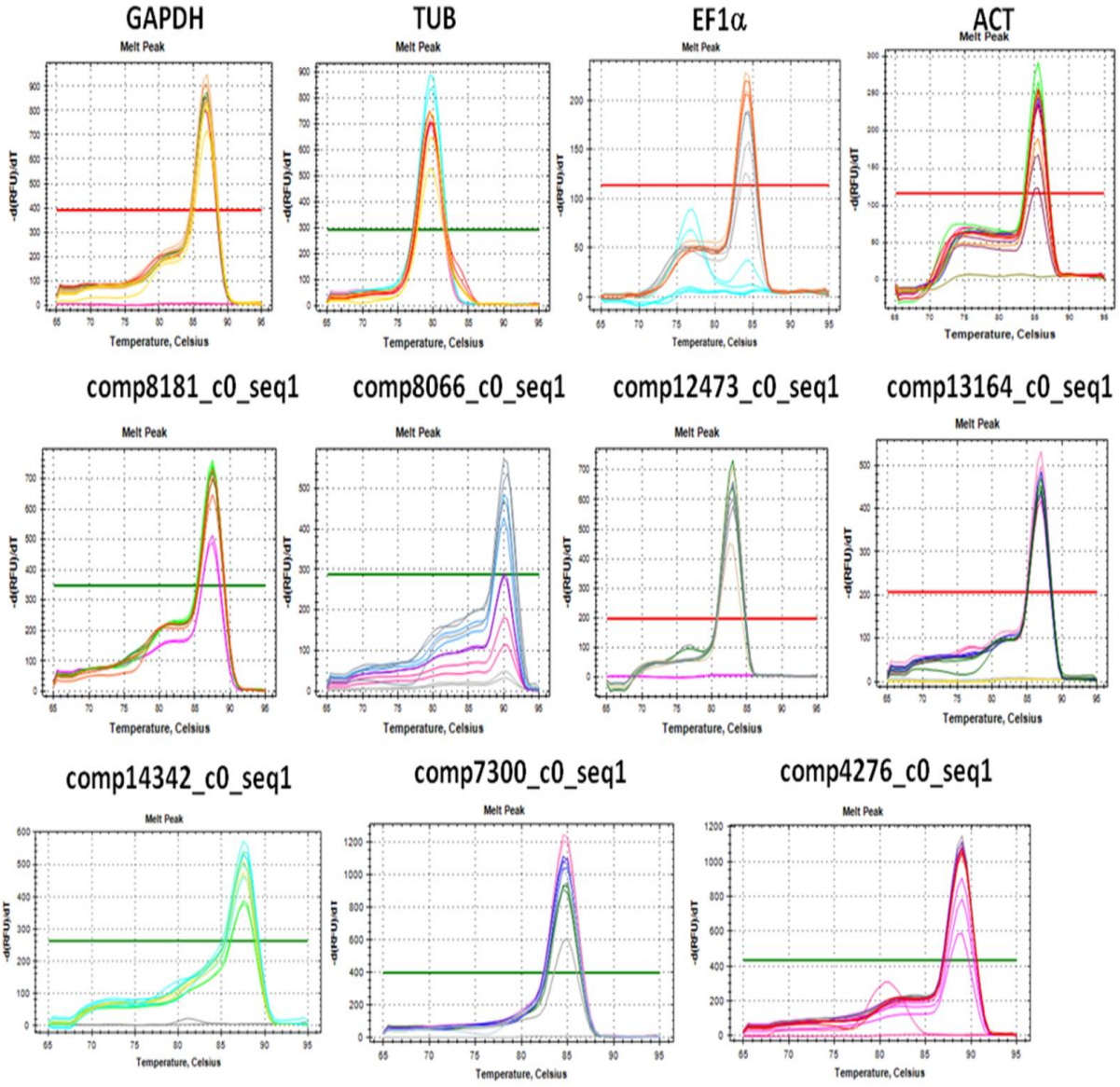

Figure 2. Specificity of primers. After the cycles for PCR amplification, the temperature was reduced from 95 °C to 65 °C at a rate ramp of 0.1 °C/s, registering the fluorescence at every change of temperature. Images were generated using BIO-RAD CFX manager software (version 3.1). The PCR amplification was done using a mix of cDNA for all the samples to be evaluated.

2.4.2 Reference gene stability

EF1α and *ACT* were the most stable genes according to geNorm analysis, and the M values were slightly higher than the recommended threshold of 0.5 for homogeneous samples (Figure 3A). The pairwise variation analysis of the V value versus the Vn/Vn+1 ratio was slightly higher than the recommended threshold of 0.15 (Figure 3B). The analysis of all samples using two reference genes (*EF1α* and *ACT*) showed lower pairwise variation (0.174) compared to the use of three reference genes (0.217) (Figure

3B), indicating that increasing the number of reference genes did not improve stability. Thus, according to geNorm the *EF1α* and *ACT* pair was the best strategy for normalization. In contrast, analysis of all linearized Cq by NormFinder, which considers both intra and intergroup variability, revealed *EF1α* and *TUB* as the most stable genes (Figure 3C). According to Bestkeeper, when all data were analyzed together, the most stable genes were *GAPDH* and *TUB* (Figure 3D).

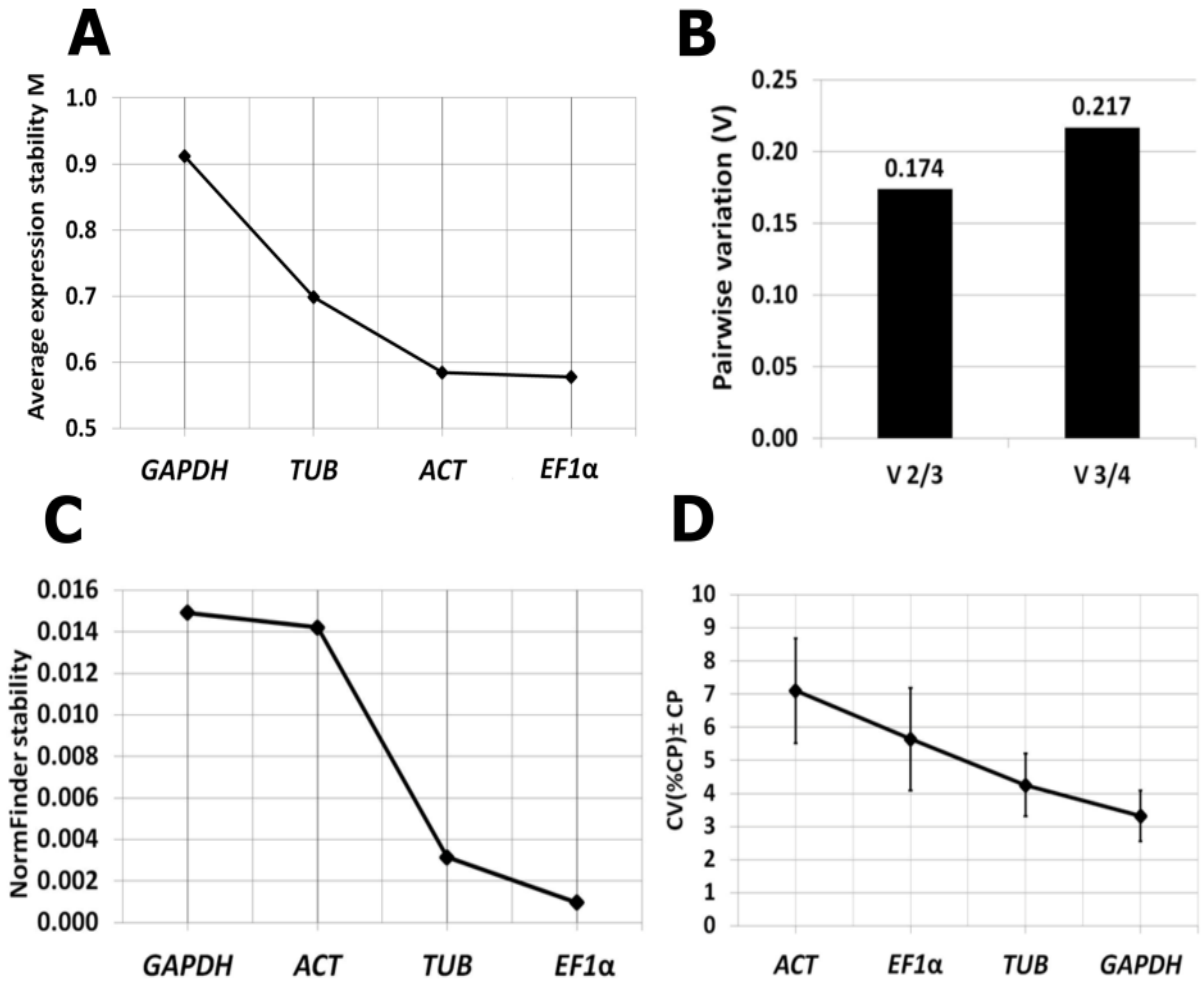

Figure 3. Reference gene stability for *L. theobromae* growing in the presence of grapevine wood and in response to heat stress. M values graph (A) and pairwise variation (B) obtained with geNorm, NormFinder (C) and Bestkeeper (D) analysis. *ACT*: actin, *TUB*: *β*-tubulin, *EF1α*: elongation factor 1*α* and *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

Using the number of position for each gene in the ranking generated by independent analysis, analyzing the data together, a geometric mean was calculated and a general ranking was obtained. From lower to higher values, indicating more to less stability, genes were ranked as follows: *EF1α, TUB, ACT* and *GAPDH* (Figure 4). Similar results were obtained when an independent analysis was done for each condition; the

only exception was observed when the fungus was grown in the presence of GW, where the most stable gene was *EF1α*, followed by *TUB, GAPDH* and *ACT* (Figure 5).

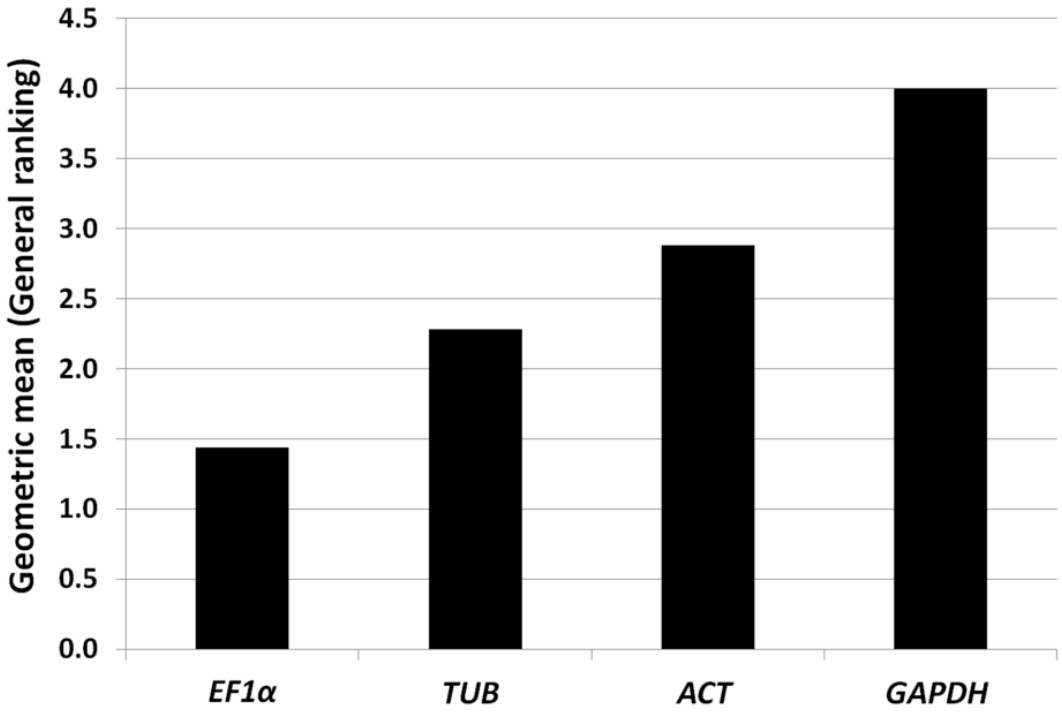

Figure 4. **General ranking of reference genes stability for *L. theobromae* growing in the presence of grapevine wood and in response to heat stress**. Geometric mean of the general ranking generated with the combination of geNorm, Normfinder and Bestkeeper values in independent rankings. *ACT*: actin, *TUB*: β-tubulin, *EF1α*: elongation factor 1*α* and *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

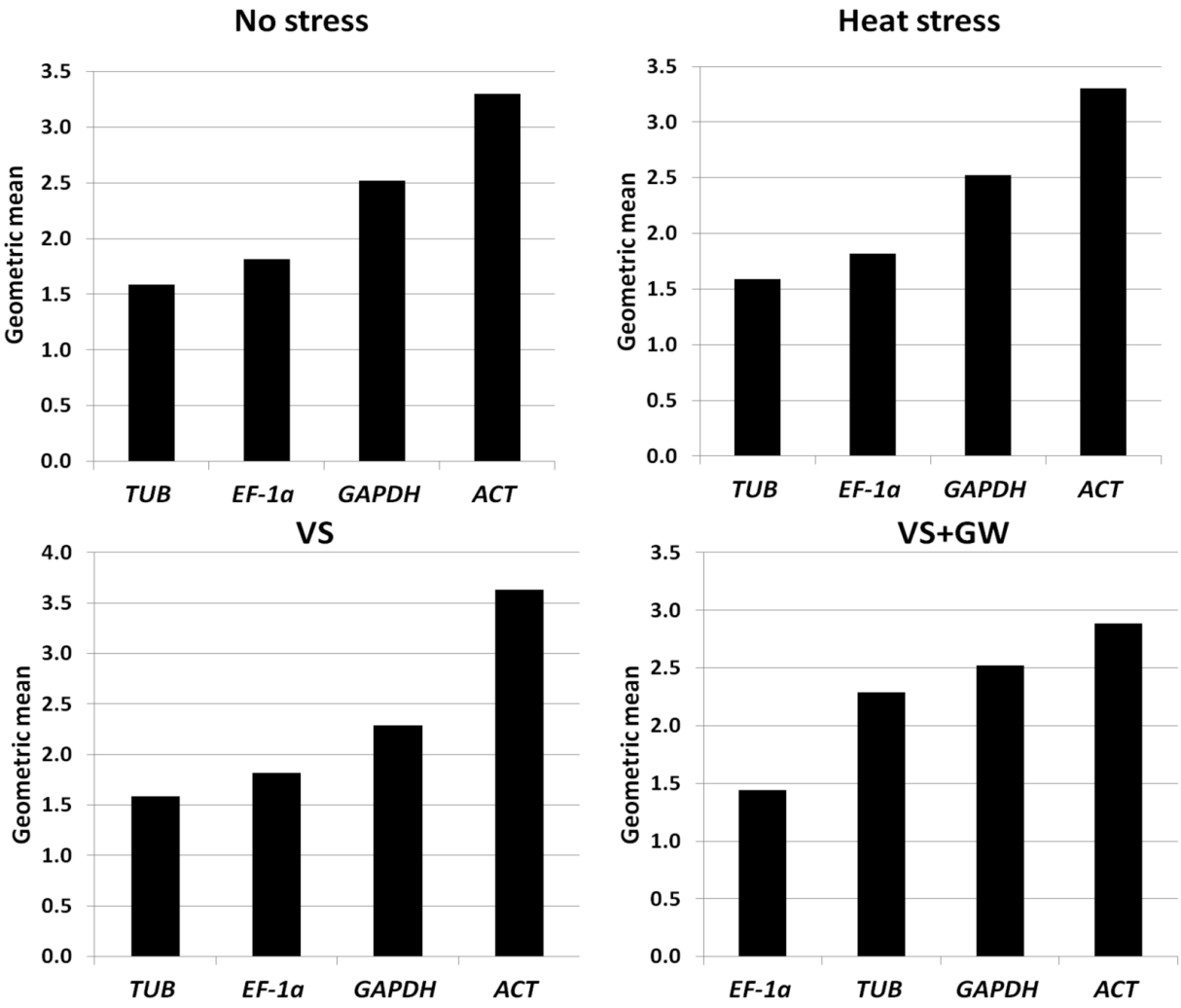

Figure 5. Ranking of stability generated using the number of position in independent rankings produced through GeNorm, BestKeeper and Normfinder. The ranking was obtained using only no stressed samples (No stress), only heat stressed samples (Heat stress), only growing in Vogel's salts (VS) or only in the presence of GW (VS+GW).

2.4.3 Stable genes selected using RNAseq data analysis

Using the criteria of low LFC variation and low tagwise dispersion among conditions evaluated from RNAseq data-derived results, the commonly used reference genes were ranked from higher to lower stability as: *EF1α*, *ACT*, *TUB* and *GAPDH* (Table 3).

Table 3. Stability of selected reference genes ranked according to its tagwise dispersion

Ranking position	Assembly Trinity code	Tagwise dispersion	Average logCPM	LFC FWS_vs_FW	LFC FS_vs_F	LFC FWS_vs_F	LFC FW_vs_F
1	*EF1α* (comp3481_c0_seq1)	0.064	5.39	0.52	1.26	1.47	0.95
2	*TUB* (comp6710_c0_seq1)	0.069	9.51	-0.79	-0.32	-1.06	-0.27
3	*ACT* (comp6557_c0_seq1)	0.070	9.18	-0.34	-0.11	-0.47	-0.12
4	*GAPDH* (comp6457_c0_seq1)	0.146	11.61	0.00	0.60	0.31	0.31

ACT: Actin, cell motility, structure and integrity protein; ***GAPDH:*** Glyceraldehyde-3-phosphate dehydrogenase, glycolytic enzyme; ***TUB***: β-tubulin, cytoskeleton structural protein; ***EF1α:*** elongation factor 1-α, catalyzation of GTP-dependent binding of amynoacyl-total RNA to the ribosome; **logCPM**: logarithm counts of reads per million; **LFC**: logarithmic fold change (ratio of expression between two conditions); **FWS**: Fungus growing in grapevine wood and under heat stress; **FW**: Fungus growing in grapevine wood; **FS**: Fungus under heat stress; **F**: fungus growing just in Vogel´s salt medium.

The search for alternative reference genes (*DUF221*, *UBCB, ADA*, *FIS1*, *CU-ATPASE*, *PSM1*, *SPO7-LIKE*, *SPT3*, *DUF500*, *SAC7*, *AP-2 B* and *NPL1*) labeled from R1 to R12 by Llanos *et al*., (2015) revealed that 6 (50%) of those have functionally associated genes in *L. theobromae* and are found as the most stable (tagwise dispersion < 0.06, Table 4). In contrast, all the commonly used reference genes (*ACT, APSC*, *COXV*, *GAPDH*, *GLKA*, *G6PDH*, *ICDA*, *PFKA*, *PGIA*, *SARA*, *EF1α* and *TUB*) labeled from C1 to C12 genes in Llanos *et al*., 2015, showed lower stability (tagwise dispersion > 0.06). Therefore it is suggested that genes shown in Table 4 are a better alternative to the commonly used reference genes for RT-qPCR normalization of *L. theobromae.*

Table 4. List of stable genes according to its tagwise dispersion and showing shared functions with alternative fungal reference genes suggested by Llanos *et al.*, 2015

Trinity assembly code	Blastx hit on NCBI-NR database	Pathway common to genes in Llanos *et al.*, 2015	TD	logCPM	LFC FWS/FW	LFC FS/F	LFC FWS/F	LFC FW/F
comp9359_c0_seq1	Ras GTPase	Involved in signal transduction (R10)	0.047	5.06	-0.385	-0.297	-0.382	0.003
comp8098_c0_seq1	Ubiquitin/SUMO-activating enzyme E1	Involved in the ubiquitin mediated proteolysis (R2)	0.048	5.72	-0.383	0.033	0.219	0.601
comp8751_c0_seq1	Ubiquitin-conjugating enzyme E2	Involved in the ubiquitin mediated proteolysis (R2)	0.049	5.31	-0.246	0.222	-0.079	0.166
comp3953_c0_seq1	putative ubiquitin conjugating enzyme protein	Involved in the ubiquitin mediated proteolysis (R2)	0.051	5.26	-0.107	-0.004	0.049	0.155
comp9282_c0_seq1	Clathrin adaptor mu subunit	Involved in chlatrin-dependent endocytosis (R11)	0.051	5.26	-0.061	0.673	0.274	0.335
comp9037_c0_seq1	Clathrin/coatomer adaptor adaptin-like protein	Involved in chlatrin-dependent endocytosis (R11)	0.052	5.65	0.135	0.326	-0.065	-0.200
comp3137_c0_seq1	Sporulation/nuclear morphology Spo7	Involved in the spore formation process (R7)	0.053	5.19	0.589	-0.148	0.559	-0.03
comp6397_c1_seq1	putative copper-transporting atpase protein	copper-transporting atpase protein (R5)	0.054	6.12	0.106	-0.274	-0.397	-0.504
comp5642_c0_seq2	putative cohesin complex subunit protein	Involved in chromosomes segregation during mitosis (R6)	0.057	4.98	0.153	-0.392	-0.176	-0.329

logCPM: logarithm counts of reads per million; **LFC**: logarithmic fold change (ratio of expression between two conditions); **FWS**: Fungus growing in grapevine wood and under heat stress; **FW**: Fungus growing in grapevine wood; **FS**: Fungus under heat stress; **F**: fungus growing just in Vogel´s salt medium, TAC: Trinity Assembly Code; TD: Tagwise Dispersion.

2.4.4 Gene expression according to normalization strategies using RT-qPCR results

To test the aforementioned normalization strategies, the relative expression of target genes was determined using a well-ranked gene according to its stability (*TUB*), and compared to the lowest ranked (*GAPDH*) in a RT-qPCR normalization. Normalization using the geometric mean was also tested and all the strategies were compared with non-normalized data. Slight differences between normalization strategies were obtained on data distributions as shown in Figure 6, although a more homogeneous distribution is distinguishable when *GAPDH* was used as a reference gene, as shown in Figure 6D, and as indicated by its higher p-value in the Shapiro-Wilk normality test (Table 5).

Table 5. Effect of RT-qPCR normalization strategy on data distribution and final relative expression results

Normalization strategy	Shapiro Wilk test (W)	Shapiro Wilk test (p-value)	RNAseq/RT-qPCR results Pearson correlation (r)	RNAseq/RT-qPCR results Pearson correlation (p-value)
Raw data	0.9468	2.617e-05	0.8509	5.701e-05
Endogenous control: *TUB*	0.94322	1.382e-05	0.8747	2.112e-05
Endogenous control: *GAPDH*	0.96902	2.389e-03	0.8677	2.883e-05
Geometric mean	0.95901	2.753e-04	0.8132	2.022e-04

Using the criteria that more coincident expression levels between RNAseq and RT-qPCR yields more reliable results, the Pearson correlation between results using both methods was evaluated, and the effect on correlation when using different normalization strategies was noted (Table 5). A lower correlation was obtained when using the geometric mean for normalization compared to the use of raw data, indicating that a bias is introduced using this strategy (Table 5).

In contrast, an improvement in the Pearson correlation was obtained using either *TUB* or *GAPDH* as an endogenous control, as compared to non-normalized data. *TUB*-normalization generates RT-qPCR gene

expression results with the highest correlation to the RNAseq derived results (highest regression coefficient (r) Table 5).

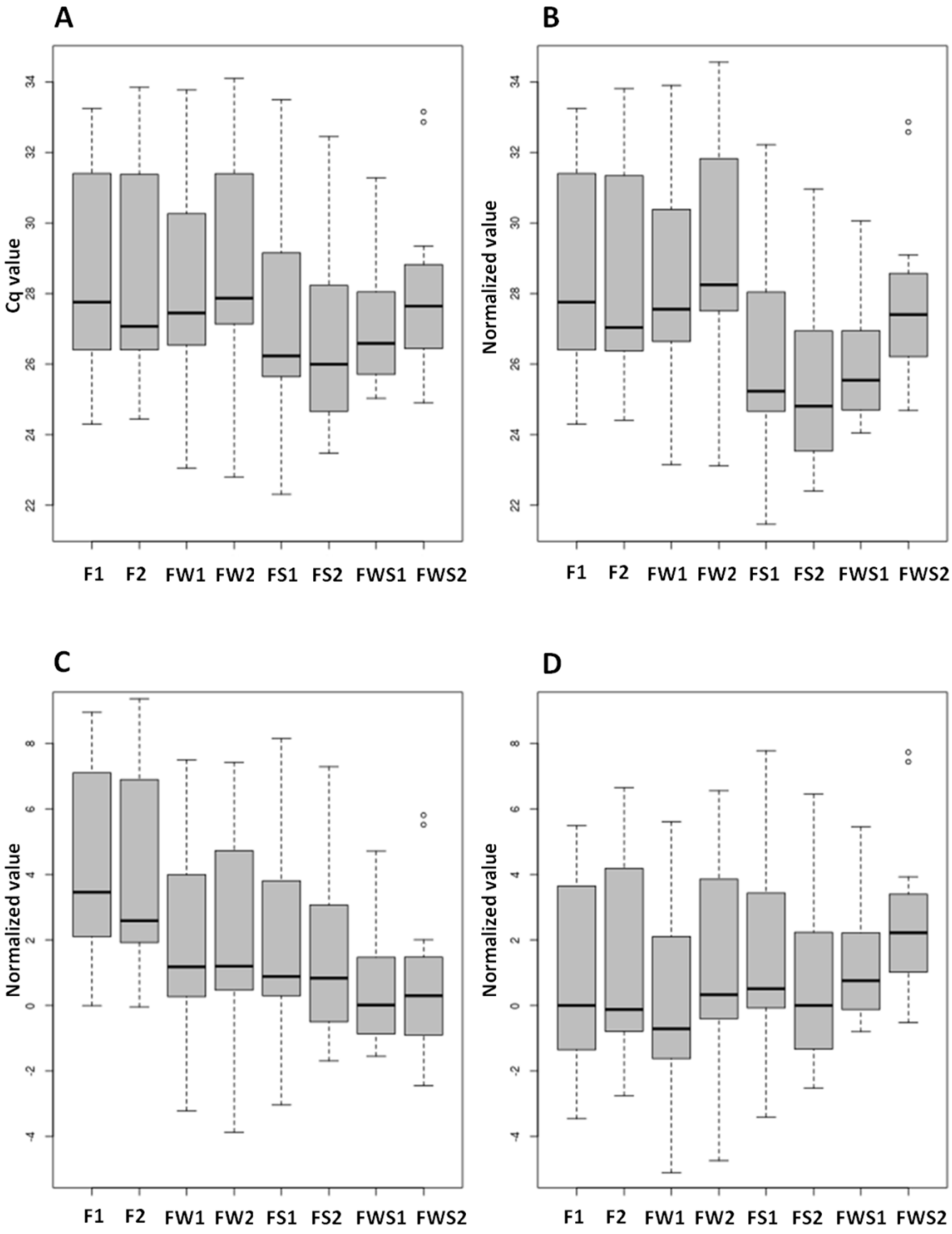

Figure 6. **Boxplot of data distribution based on the strategy used for normalization**: Raw data distribution (A), normalization through geometric mean (B), using *TUB* (C) or *GAPDH* (D) for endogenous control normalization.

2.5 Discussion

This work provides a framework for identifying an adequate normalization strategy for *L. theobromae* gene expression using RT-qPCR. Various statistical methods for gene stability analysis through RNAseq (tagwise dispersion) and RT-qPCR (normFinder, BestKeeper, geNorm) derived data were used. Contrasting results on the ranking of gene stability were obtained when using different statistical tools (Fig 1A, C and D). The comparison with RNAseq-obtained data was used to define the best strategy. Of the well-ranked genes, TUB has similar stability to EF1a, the former having a higher expression level (higher logCPM in Table 3), a desirable feature for a better normalization within an endogenous control gene. Based on the correlation of results among methods, TUB was recognized as the best reference gene for a normalization approach.

On the other hand, results derived from *GAPDH*-normalization had a lower correlation with RNAseq results than those normalized with *TUB*, however the correlation was better than when using raw data, indicating that despite its low ranking in stability, it still improved the normalization. *GAPDH* showed the best qPCR efficiency and a low Cq range compared to the other reference genes (Table 2), thus its high qPCR performance could compensate for the instability of expression between samples in the evaluated experimental conditions, and probably contributes to better consistency in *GAPDH*-normalized data (Figure 6D). Besides, it has a high expression level, as indicated with the high logCPM observed (Table 3) which also might compensate for its instability.

Alternative to the use of endogenous control genes, several data-driven normalization strategies have been proposed (Mar *et al.*, 2009; Deo *et al*. 2011). When a data-driven strategy using the geometric mean for normalization was used, no marked change was observed in the distribution of data (Figure 4 and Shapiro-Wilk test in Table 5), however, it decreased the correlation to RNAseq expression results (Table 5) compared to the raw data, indicating that a bias is being introduced, and therefore this strategy is not recommended.

According to the general analysis, the use of endogenous genes for normalization is suggested because this strategy improves the correlation with RNAseq-derived results (Table 5), compared to the raw data. Functionally associated genes proposed for normalization in fungi (Llanos *et al*., 2015) showed higher stability than *GAPDH* and *TUB*, based on RNAseq- derived data analysis, and therefore are highly recommended candidates for improving normalization and performance in RT-qPCR experiments.

Still, it is a matter of continuous debate, and no consensus in the scientific community has been established on which strategy for normalization is best to use. In this sense, results here showed inconsistencies in the selection of stable endogenous controls. The growing availability of RNAseq-derived results is challenging this topic, adding even more alternatives when considering RT-qPCR normalization. Much effort is still required from the bioinformatics and statistical experts to design more accurate tools to solve this task. The strategy used by Llanos *et al.* (2015), is a satisfying approach that shows correlation with the genes shown to be stable from our analysis, demonstrating that alternative, more stable genes for fungi could be selected using RNAseq-derived data. Furthermore, as it was shown here, it is critical to try diverse strategies for stable gene selection, in order to compare among them and select those that best fit the particular data under evaluation

2.6 Conclusions

To the best of our knowledge, this is the first work on *L. theobromae* to establish a framework for gene expression studies. Based on our data, RT-qPCR normalization using *TUB* as an endogenous control gene was the best strategy to get reliable gene expression results on this fungus.

Chapter 3. Global transcriptional analysis suggests *Lasiodiplodia theobromae* pathogenicity factors involved in modulation of grapevine defensive response

3.1 Abstract

Lasiodiplodia theobromae is a fungus of the Botryosphaeriaceae that causes grapevine vascular disease, especially in regions with hot climates. Fungi in this group often remain latent within their host and become virulent under abiotic stress. Transcriptional regulation analysis of *L. theobromae* exposed to heat stress (HS) was first carried out *in vitro* in the presence of grapevine wood (GW) to identify potential pathogenicity genes that were later evaluated for *in planta* expression. A total of 19,860 *de novo* assembled transcripts were obtained, forty-nine per cent of which showed homology to the Botryosphaeriaceae fungi, *Neofusicoccum parvum* or *Macrophomina phaseolina*. Three hundred ninety-nine have homology with genes involved in pathogenic processes and several belonged to expanded gene families in others fungal grapevine vascular pathogens. Gene expression analysis showed changes in fungal metabolism of phenolic compounds; where genes encoding for enzymes, with the ability to degrade salicylic acid (SA) and plant phenylpropanoid precursors, were up-regulated during *in vitro* HS response, in the presence of GW.

These results suggest that the fungal L-tyrosine catabolism pathway could help the fungus to remove phenylpropanoid precursors thereby evading the host defense response. The *in planta* up-regulation of salicylate hydroxylase, intradiol ring cleavage dioxygenase and fumarylacetoacetase encoding genes, further supported this hypothesis. Those genes were even more up-regulated in HS-stressed plants, suggesting that fungus takes advantage of the increased phenylpropanoid precursors produced under stress. Pectate lyase was up-regulated while a putative amylase was down-regulated *in planta*, this could be associated with an intercellular growth strategy during the first stages of colonization. *L. theobromae* transcriptome was established and validated. Its usefulness was demonstrated through the identification of genes expressed during the infection process. Our results support the hypothesis that heat stress facilitates fungal colonization, because of the fungus ability to use the phenylpropanoid precursors and SA, both compounds known to control the host defense.

3.2 Introduction

In recent years, global climate change has had a devastating impact on crop productivity. Drought and heat stress have resulted in an increased of tree mortality worldwide (Allen *et al.,* 2010; Teskey *et al.,* 2014; Allen *et al.,* 2015). This phenomenon could be attributed in part to endophytic fungi. Fungi inhabiting the vascular system of trees can become pathogenic under abiotic stress. In susceptible plant hosts, this results in the development of wood necrosis and cankers, because trees failed to effectively compartmentalize the pathogen. As the fungus colonizes its host, the plant vascular function becomes increasingly impaired. Prolonged abiotic stress conditions and biotic infections lead to tree decline and eventually death (Desprez-Loustau, 2006; Oliva *et al*., 2014; Moricca and Ragazzi, 2008). Two independent studies have analyzed, at the molecular level, the interaction of trees susceptible to fungal pathogens. Both studies highlighted the adaptation capability of the fungus to metabolize terpenoids and stilbene, the main defensive compounds produced by the host in response to infection, and use them as carbon sources for wood colonization (DiGuistini *et al*., 2011; Hammerbacher *et al*., 2013). Both compounds are produced in response to biotic and abiotic stress in plants (Keller, 2010; Úrbez-Torres, 2011), suggesting that the fungal colonization is favored when plants are under abiotic stress.

Grapes are the world's most economically important fruit crop and have been cultivated in a broad range of environmental conditions (Bertsch *et al*., 2013). Fungal vascular diseases (a.k.a. Eutypa dieback, Botryosphaeria dieback and Esca) are major factors limiting grape productivity and fruit marketability worldwide (Úrbez-Torres *et al*., 2008; Úrbez-Torres *et al*., 2006). Several taxonomically unrelated fungi are known to cause these diseases. *Lasiodiplodia theobromae* [teleomorph *Botryosphaeria rhodina* (Griff. and Maubl., Bull. Soc.Mycol. Fr. 25: 57. 1909)] is one of the causal agents of Botryosphaeria dieback. It is especially predominant in hot climates, and it has been classified as one of the most aggressive vascular pathogen of grapevine (Úrbez-Torres *et al*., 2006; Taylor *et al*., 2005; Mahoney *et al*., 2003; Rolshausen *et al*., 2006). Despite its socio-economic impact, there is still little knowledge on its biology and no genome sequence information is currently available. In comparison, *Eutypa lata* (Pers.:Fr.) Tul. and C. Tul. (syn. *E. armeniacae* Hansf. and Carter), which causes similar wood symptoms, has been extensively studied and its genome has been sequenced (Rolshausen *et al*., 2008, Slippers and Wingfield, 2007; Octave *et al*., 2006; Blanco-Ulate *et al*., 2013, Sakalidis *et al*., 2011). *E. lata* has the capacity to colonize the host vascular system targeting non-structural carbohydrate (i.e. starch) and structural hemicellulosic cell wall glucans for its metabolism (Sakalidis *et al*., 2011).

L. theobromae and related members of the family Botryosphaeriaceae have been recognized as latent pathogens in many hosts (Müllen *et al*., 1991; Álvarez-Loayza *et al.,* 2011). This pathogen was identified as a causal agent of mortality in dogwood tree under drought stress (Müllen *et al*., 1991). Similarly, Álvarez-Loayza *et al*. (2011) observed that the disease incidence caused by Botryosphaeriaceae, *Diplodia mutila* on tropical palm *Iriarte deltoidea,* had a clear correlation with light availability, higher plant exposure to sunlight results in faster disease progression. Authors suggest that fungal reactive oxygen species (ROS) detoxification and melanin production triggered the imbalance in the endophyte-host interaction.

Biochemical assays have been used traditionally to identify pathogenicity factors produced by grapevine vascular pathogens, including secondary metabolites and plant cell wall degrading enzymes (PCWDEs) (Rolshausen *et al*., 2008; Octave *et al*., 2006; Morales-Cruz *et al*., 2015). The advent of high throughput (OMICs) technologies have allowed for a better understanding of the complex plant/pathogen interactions in grapevine diseases. For instance, the secretome analysis of *Diplodia seriata* identified three necrotic inducible proteins, suggesting that fungi induce a hypersensitive-like response in host grapevine cells (Cobos *et al*., 2010). Recently, the genomes of grapevine vascular pathogens *Neofusicoccum parvum*, *D. seriata* and *E. lata* were obtained (Blanco-Ulate *et al*., 2013a, Blanco-Ulate *et al*., 2013b, Morales-Cruz *et al*., 2015). Through a genomic comparative study, the expansion of dioxygenase (PF00775), pectate lyase (PF03211), major facilitator superfamily (PF07690), carboxylesterase (PF00135) and glucose-methanol-choline oxidoreductase (C-terminal: PF05199 and N-terminal:PF00732) gene families were identified on these pathogens suggesting a role in pathogenicity (Morales-Cruz *et al*., 2015).

The hot and arid viticulture production areas provide a unique place to study how commensal interaction between an endophytic fungus and grapevine become pathogenic under extreme heat conditions. This research aim was to evaluate *L. theobromae* global transcriptional response in both in *in vitro* and *in planta* bioassays. In addition, this work was designed to better understand the impact of heat stress at the fungal transcriptional level, and how it affects pathogenicity and the expression of disease symptom in grapevines.

3.3 Material and methods

3.3.1 Fungal growth condition

L. theobromae UCD256Ma was isolated from grapevine showing symptoms of Botryosphaeria dieback located in Madera County in California, USA by Úrbez-Torres *et al.* (2006) and kindly shared by Dr. Douglas Gubler. The isolate was maintained at – 20 ºC in 20% glycerol in the laboratory of phytopathology at CICESE. Infection and recovery in *Vitis vinifera* cv. Cabernet Sauvignon green shoots was used to reactive it. After recovery, a plug of mycelium of the fungus grown on Potato Dextrose Agar (PDA, Difco) was inoculated in Erlenmeyer flasks containing Vogel's salts (VS) (Vogel, 1956) or Vogel's salts with chips of grapevine wood (GW).Wood samples were obtained from branches of one year old *V. vinifera* cv. Cabernet Sauvignon grown in greenhouse from cuttings, pieces were frozen in liquid nitrogen, ground with a blender (Waring), filtered through a 0.35 mm sieve (Precision Scientific) and autoclaved. After inoculation, flasks were incubated at 28 °C in darkness, without agitation for 48 h. Then, some flasks were exposed to HS by transferring them at 42 °C for 1 h and then returned to 28 °C, while controls were maintained at 28 °C. Ten minutes after HS, mycelia were collected using previously sterilized tweezers, washed with water treated with diethylpyrocarbonate (DEPC, Sigma) and transferred to 1.5 ml tubes with 500 □l of Nucleic Acid Preservation (NAP) buffer (Camacho-Sanchez *et al*., 2013) and stored at -80 °C until RNA extraction.

3.3.2 RNA extraction and sequencing

Total RNA extraction was done through modification of protocols based on Reid *et al*. (2006) and Vasanthaiah *et al.* (2008) as described by Paolinelli-Alfonso *et al.* (2016). RQ1 DNAse (Promega) treatment was done according to manufacturer's instructions. DNA-free total RNA was sent to the National Laboratory of Genomics for Biodiversity service facilities (Irapuato, Mexico) for sequencing. RNA quality was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit according to the manufacturer's instructions. Complementary DNA (cDNA) and the ligation of adapters were done with the TruSeq Paired-End Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instruction. Libraries of cDNA were made from fungus grown in the conditions described in Figure 7. These conditions were: grown in the absence of GW (F), in the absence of GW plus HS (FS), in the presence of GW (FW) and in the presence of GW plus HS (FWS). Three biological replicates for each condition were

sequenced in 1 lane of an Illumina Hiseq2500, to obtain 100 bp pair-end reads. All sequence data are available from the Gene Expression Omnibus DataSets (GSE75978).

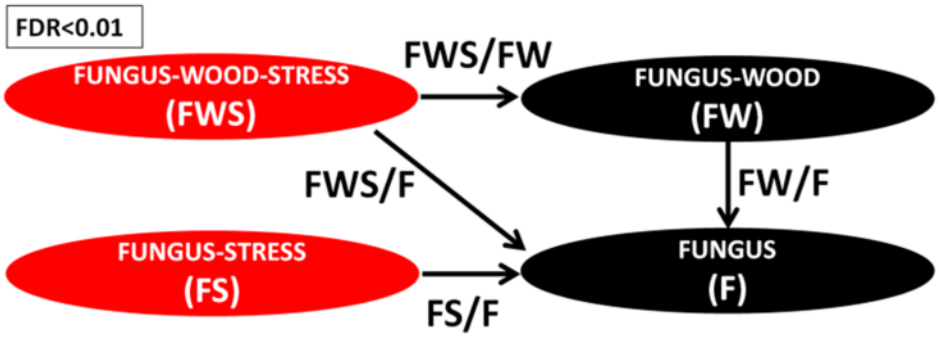

Figure 7. Contrasting conditions evaluated in general linear model statistics to identify differentially expressed genes. The conditions evaluated were from fungus grown in Vogel´s salts without heat stress (F) or under heat stress (FS), Vogel´s salts with 1% of grapevine wood (GW), without heat stress (FW) or under heat stress (FWS). To evaluate the gene expression under the effect of heat stress (HS), the expression of FS was compared to F (FS/F). To test only the effect of HS when GW was present, FWS/FW ratio was measured, while to test both effects at the same time the ratio FWS/F was considered. Finally, fungal gene expression only as effect of GW, was measured evaluating FW/F ratio.

3.3.3 Bioinformatics analysis

The general pipeline used for transcriptome analysis is schematically represented in Figure 8. All scripts employed here are available upon request.

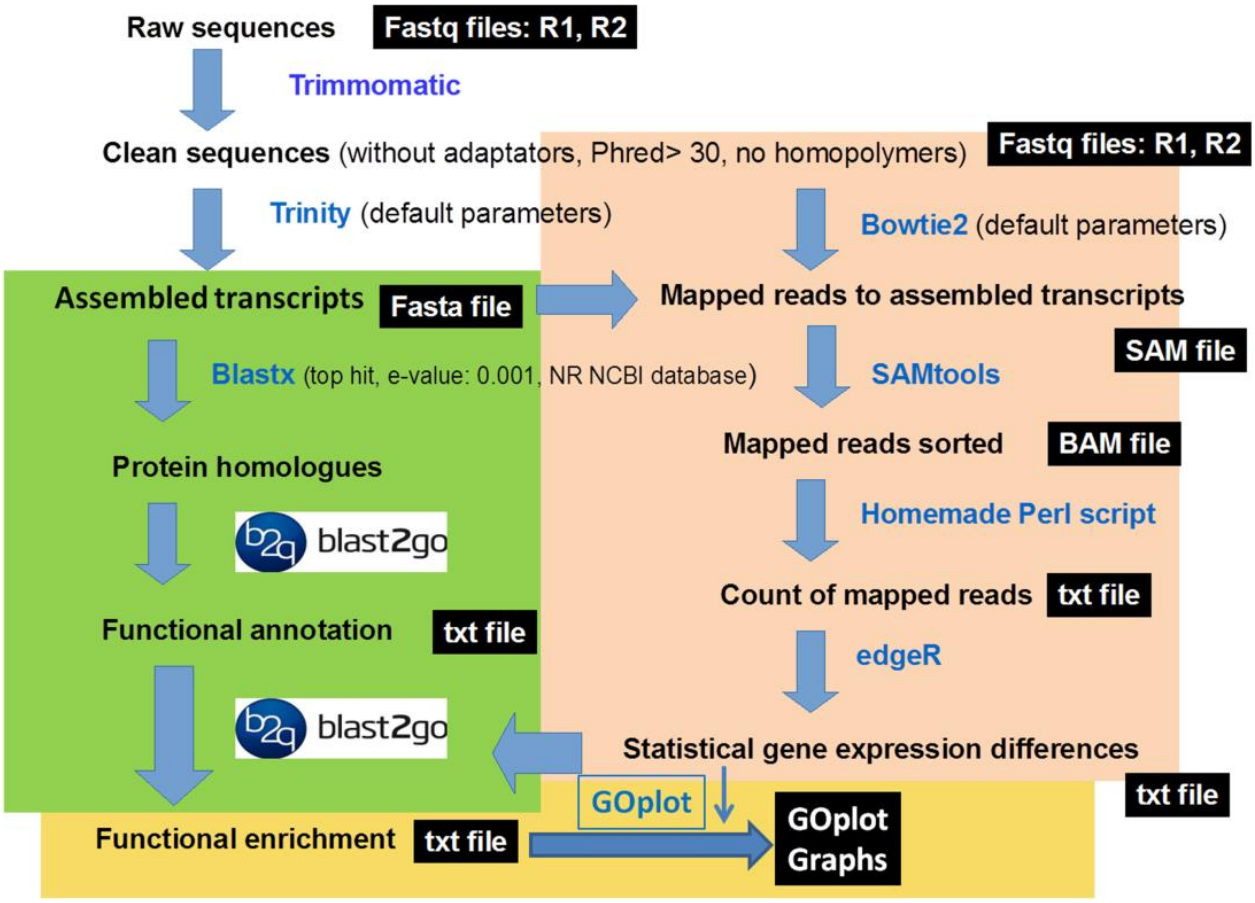

Figure 8. Pipeline employed in bioinformatics analysis.

3.3.3.1 *De novo* transcriptome assembly and functional annotation

All the reads obtained from the Illumina sequencing were filtered using Trimmomatic (Trimmomatic-0.32, Bolger *et al*., 2014) and sequencing quality control program (FastQC, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to select only those showing a PHRED quality higher than Q30, absence of adapters and homopolymers, and those with a length higher than 90 bp. The filtered sequences were used for assembly using Trinity (trinityrnaseq_r20140717, Grabherr *et al*., 2011) with the default settings (kmer=25). The assembly quality check was done with the Perl script (analyze_blastPlus_topHit_coverage.pl) included in the trinityrnaseq_r20140717 package (Grabherr *et al*., 2011). The fasta file output produced by Trinity, containing all possible isoforms, was filtered to retain only the longest using an online available Python script (longest_isoform_filter_trinity_assem.py).

The longest isoform sequences were used as queries in BlastX (version 2.2.29+, Altschul *et al*., 1990) from the National Center for Biotechnology Information (NCBI) using the non-redundant (NR) protein database, retaining the highest scored hit with E value < 0.001. The file with BlastX hits was used to retrieve the functional annotations with Blast2GO software (version 3.0, GO-DB version 2014-09, Conesa *et al*., 2005) using the gene ontology (GO) database updated on September 2014 and default parameters as recommended (Gotz *et al*., 2008). The InterProScan sequence analysis application (Zdobnov and Apweiler, 2001) in the Blast2GO software was used to predict the secretion signal with SignalP 4.0 (Petersen *et al*., 2011). The presence of transmembrane domains was determined with TMHMM (Krogh *et al*., 2001). Domain information was retrieved through PfamA (Finn *et al*., 2010) and HAMAP (Lima *et al*., 2009). Sequences with positive SignalP predictions (Petersen *et al*., 2011) in the N-terminal region and without any transmembrane regions as predicted by TMHMM (Krogh *et al*., 2001) were selected as candidate secreted proteins.

The fasta file of the longest isoform sequences was used to predict the open reading frames (ORFs) through the usage of TransDecoder (TransDecoder_r20140704; Haas *et al*., 2013). The predicted ORFs sequences were used in a local BlastP (version 2.2.28+, Altschul *et al*., 1990) analysis in the Pathogen-Host Interaction (PHI) database (version 3.6, Winnenburg *et al*., 2006).

3.3.3.2 Differential expression and functional enrichment analysis

The raw reads of both ends obtained for each output file were aligned to the longest isoform transcripts using Bowtie 2 (version 2.1.0; Langmead and Salzberg, 2012) with default parameters. The resulting output in SAM format was converted to BAM format through Samtools (version 0.1.19-96b5f2294a; Li *et al*., 2009). Using the sorted bam file, the number of alignments were counted for each transcript and sample using a homemade Perl script specially designed (count_filebam.pl and count_names.pl) to output a tab delimited table. The statistical analysis was done using edgeR package version 3.4.2 (Robinson *et al*., 2010) of Bioconductor (http://www.bioconductor.org) in the R environment (version 3.0.2; R Development Core Team, 2008). Normalization was done using counts of reads per million (cpm) and filtered to retain transcripts that had at least 3 cpm in more than 2 samples. The statistical analysis was done using a General Linear Model (GLM) approach. The conditions compared are shown in Figure 7. The false discovery rate (FDR) < 0.01 was set as the threshold to consider genes as differentially expressed.

Differentially expressed genes (DEGs) for any of the contrasting conditions evaluated were arranged in clusters based on their expression pattern through Pearson correlations in edgeR. The list of genes for each cluster was used as test and all the differentially expressed genes were used as references in an enrichment analysis (Fisher's exact test, FDR<0.05) using Blast2GO software (version 3.0, GO-DB version 2014-09; Conesa *et al*., 2005). The GOplot package (version 1.0.1; Walter et al., 2015) in the R environment was used to generate the graph GOcluster and GOcircle in order to obtain a more detailed view of co-regulated genes in relationship with functional enrichment.

3.3.4 Evaluation of *L. theobromae* growth in benzoate

To evaluate the capacity *of L. theobromae* to grow in benzoic acid (BZA) as the sole carbon source, the fungus was inoculated in agar plates containing Vogel's Minimal Medium (VMM) (Vogel, 1956) supplemented with 0, 15 or 30 mM of BZA. Photographs were taken after 8 days of incubation at 28 °C.

3.3.5 *In vitro* and *in planta* fungal gene expression of selected targets

Twelve genes that were found to be differentially expressed in the RNAseq analysis were selected for validation and relative quantification of gene expression through reverse transcription-quantitative PCR (RT-qPCR), both *in vitro* and *in planta* (genes selected and primers are shown in Supplementary material 1).

The effect of HS was simulated in growth chambers by exposing the *L. theobromae*-infected plants to a daily temperature cycles changing from higher minimal to a higher maximal. One year-old cuttings *V. vinifera* cv. Merlot, were grown in UC special mix soil in 5 L pots and kept at room temperature at the University of California Riverside lathhouse facilities. Using a sterile razor blade, green shoots from thirty plants were wounded on the second basal internode and inoculated with a 3 mm mycelia plug obtained from a three day-old culture of *L. theobromae*. A total of 6 plants were inoculated with a PDA plug and used as controls. All wounds were covered with paraffin wax film (Parafilm M) following inoculation. Five days later, fifteen plants inoculated with *L. theobromae* and three plants inoculated with PDA were exposed to HS using night-day cycles ranging from 20 °C to 42 °C at 50% humidity on growth chamber

(Environmental Growth Chamber EGC-TC2). The remaining plants were incubated in a growth chamber (Conviron PGR15) with night-day cycles ranging from 10 °C to 30 °C at 50% humidity, and used as controls without HS. At 7, 11 and 15 days post inoculation (dpi) plants were collected, shoots were cut in transversal sections at least 5 mm up or down from the inoculation point, a section was used for stereo microscopy (Leica M125) observation and the remaining were collected in NAP solution and stored at -20 °C until RNA extraction. The RNA extraction was done using a cetyltrimethylammonium bromide (CTAB)-based protocol (details in Supplementary material 2). Total RNA was treated with RQI DNAse (Promega) and the cDNA obtained with the ImProm AM3800 (Promega) kit, according to the manufacturer's instruction. The qPCR was done on a CFX96 thermocycler (Bio-Rad) using 3 µl of cDNA and 7 µl of mastermix containing 2.5 mM MgCl2, 1X Taq polymerase Accustart buffer (Quanta), 0.2 mM of dNTPs, 0.03 U/ul Taq polymerase Accustart (Quanta), 1X EVAgreen (Biotium) and 0.2 µM of each primer. Two biological with three technical replicates were used for *in vitro* and three biological with three technical replicates were used for *in planta* gene expression analyses.

The HTqPCR package (version 3.2; Dvinge *et al*., 2009) for R environment (version 3.0.2; R Development Core Team, 2008,) was used for normalization against β-tubulin mRNA, relative expression quantification and limma statistical analysis. *In planta* fungal gene expression was calculated as fold change in HS-treated host or non HS-treated and infected plants compared to *L. theobromae* growing in *in vitro* conditions. cDNA from non-infected plants was used as negative controls when the specificity was verified through the analysis of melting curves for each gene.

3.4 Results

3.4.1 Transcriptome assembly and functional annotations

Sequencing reads varied in number, ranging from 3 to 6 million per sample, with an average of approximately 5 million reads (Figure 9), showing PHRED quality scores per base of 36. The *de novo* assembly with Trinity (Grabherr *et al*., 2011) produced 29,621 total components (including isoforms), %GC=56 and N50=3,135 bp (details in Table 6).

From the 29,621 total components, the longest isoforms were selected, rendering 19,860 unique transcripts with N50=2,472 bp; the median length was 658 bp and the average contig length was 1,310.58 bp (Table 6).

Table 6. Main parameters of Trinity *de novo* transcriptome assembly

Parameters	All transcript	Only longest Isoform
Number of contigs	29,621	19,860
Contig N10	6,773	5,525
Contig N20	5,345	4,321
Contig N30	4,365	3,572
Contig N40	3,667	2,971
Contig N50	3,135	2,472
Median contig length	1,242	658
Average contig	1,798.4	1,310.58
Total assembled bases	53,270,493	27,680,863

More than 50% of the assembled transcripts showed at least 80% full-length coverage as compared to well-annotated proteins, and 33% showed 100% coverage, indicating that the assembly fulfills the requirements of quality analysis through ortholog hit ratio (O'Neil and Emrich, 2013) which justifies its use to map the reads and analyze gene expression.

Linea	Mtra	ID	Read 1	Total de Reads	%>= Q30	Read 2	Total de Reads	%>= Q30	Gb TOTAL	%>= Q30 (Promedio)
7	1	RH1TL2SS01.02	6,099,622	61,799,612	97.3	6,099,622	61,799,612	96.3	12.5	96.7
	2	RH1TL2SS02.04	5,450,726			5,450,726				
	3	RH1TL2SS03.05	5,623,765			5,623,765				
	4	RH1TL2SS04.06	4,307,433			4,307,433				
	5	RH1TL2SS05.07	4,591,711			4,591,711				
	6	RH1TL2SS06.12	3,948,995			3,948,995				
	7	RH1TL2SS07.13	3,757,416			3,757,416				
	8	RH1TL2SS08.14	4,628,791			4,628,791				
	9	RH1TL2SS09.15	3,948,995			3,948,995				
	10	RH1TL2SS10.16	4,777,110			4,777,110				
	11	RH1TL2SS11.18	5,518,705			5,518,705				
	12	RH1TL2SS12.19	5,772,084			5,772,084				

Figure 9. Numbers and quality of reads obtained through Illumina Hiseq2500 sequencing

When the transcripts were arranged according to the taxonomic information retrieved from BlastX, most hits showed similarity with members of either of the Botryosphaeriaceae family, *M. phaseolina* (5,800 sequences, 29%) and *N. parvum* (3,800 sequences, 19%), the only two Botryosphaeriaceae genomes available in the NR-NCBI database at the time of analysis, or several other fungi from the Ascomycota phylum (Figure 10). The high number of *L. theobromae* sequences homologous to *M. phaseolina* over *N. parvum* was supported by previous phylogenetic analyses showing *L. theobromae* phylogenetically closer to *M. phaseolina* than to *N. parvum* (Phillips *et al*., 2013).

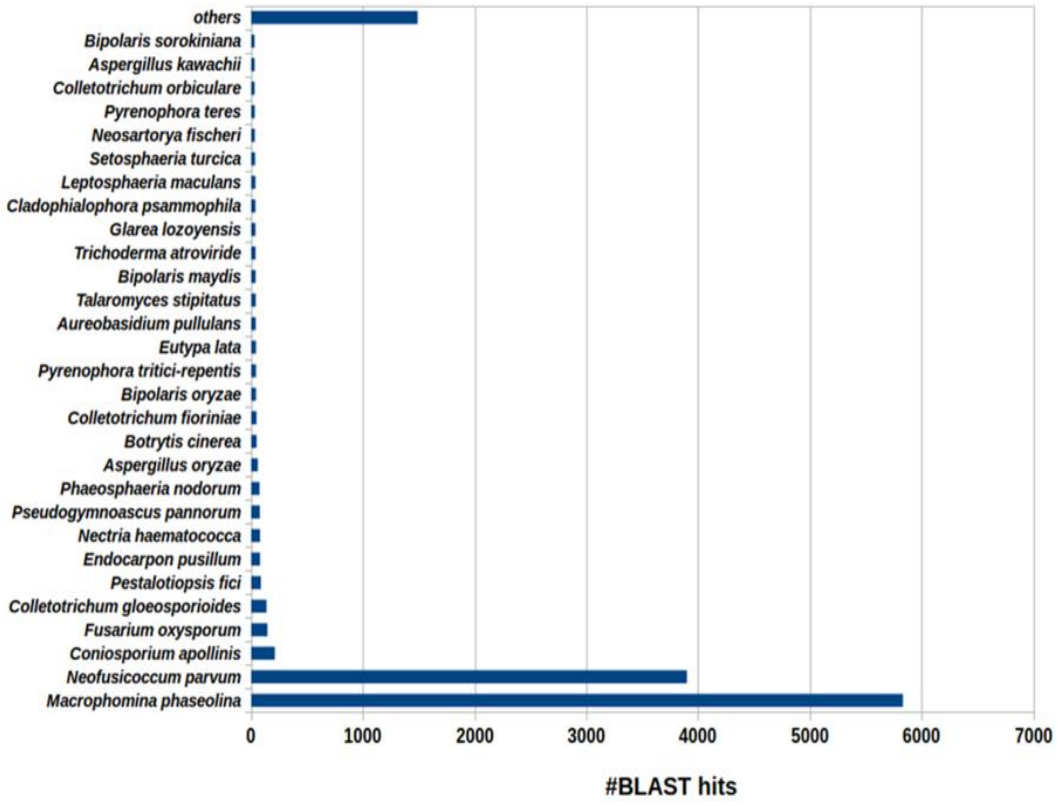

Figure 10. Classification (Kingdom Fungi restringed) of Blastx hits according with the taxonomy information of orthologs. Transcripts were classified according with its taxonomy information through Blast2GO software (version 3.0, GO-DB version 2014-09).

From the 19,860 longest isoforms, 7,547 sequences (38%) had recognizable functional GO annotation (Figure 11). The GO terms associated with each transcript encoded protein were used to associate them according to their function. A wide diversity of functions were identified (details in Supplementary material 3). Among others, in the Biological process in fourth level of gene ontology classification, most of the transcripts were involved in nitrogen metabolism (684), aromatic (665), organic cycles (689) and heterocyclic (676) compounds metabolism. Also there are 589 transcripts involved in oxidation-reduction process.

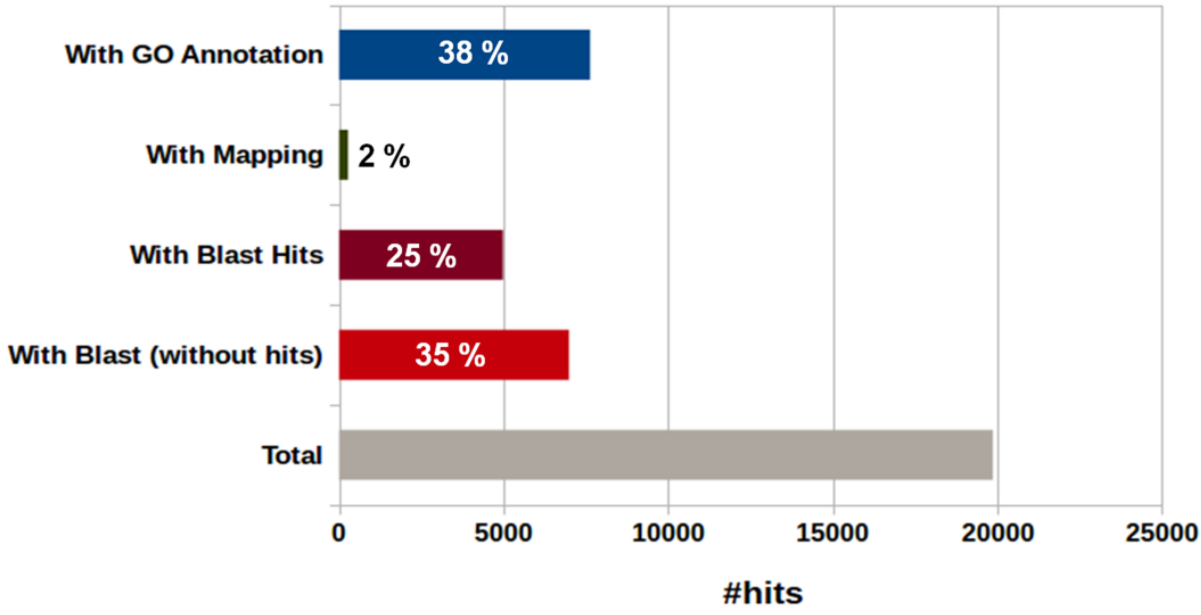

Figure 11. Main results of functional annotation in Blast2GO (version 3.0, GO-DB version 2014-09).

Based on the importance of genes encoding proteins with extracellular functions in the establishment of the interaction of the fungus with its host, transcripts encoding proteins with N-terminal secretion signal were identified. From 15,981 transcripts that contain at least one ORF, 850 were predicted to be secreted.

On other hand, a total of 399 protein-coding transcripts, showing homology with those included in the Pathogen-Host Interaction database (PHI-base) were identified. PHI-base contains experimentally verified pathogenicity or virulence factors, therefore *L. theobromae* transcripts showing PHI-base hits are predicted to be involved in pathogenicity. Based on functional annotation of PHI-base hits, they were classified according to their gene ontology information, and were identified as being involved in protein modification processes, metabolism of phosphate-containing compounds, hydrolase activity on glycosyl bonds, and oxidoreductase on CHOH groups (Figure 12). Additionally, 22 PHI-base hits were predicted to be secreted, and according to their annotation, they could be involved in carbohydrate metabolism, proteolysis and oxido-reduction processes.

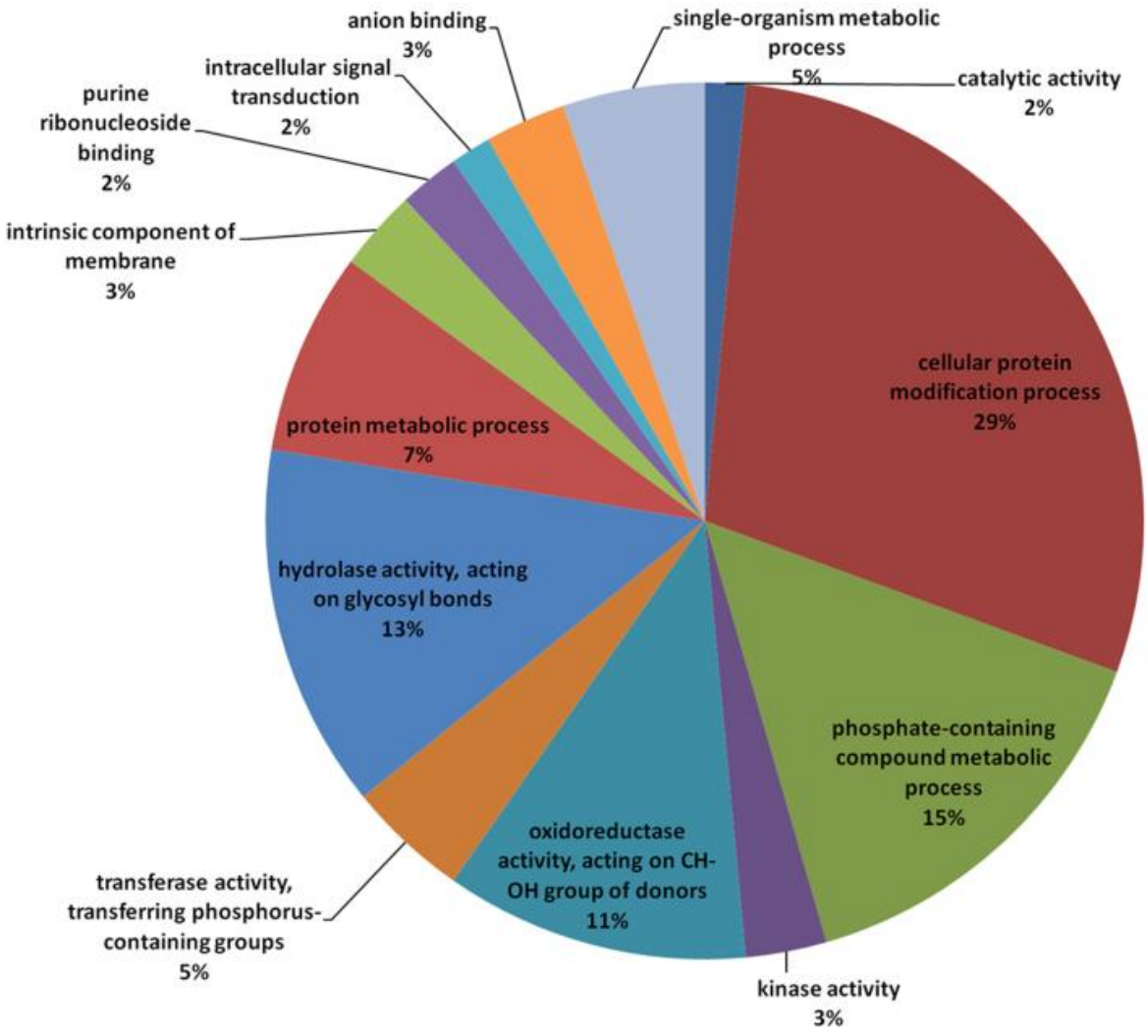

Figure 12. Pie chart describing the main Gene Ontology (GO) of molecular functions categories of transcripts with putative pathogenicity role. The predicted open reading frames encoded by transcripts were used in a local Blastp to find hits in the experimentally verified Pathogen-Host Interaction database (PHI-base). The PHI-base hits were classified based on its GO information.

3.4.2 Differential gene expression, co-regulation and functional enrichment

The analysis of RNAseq data indicated that sufficient differences were achieved among treatments and therefore allowed the identification of DEGs (Supplementary material 4). In total, 2,386 DEGs (FDR<0.01) were identified in any of the contrasting conditions tested (Figure 7). These corresponded to 12% of the total number of transcripts. Different numbers of DEGs were obtained for the different contrasting conditions, FWS/F contrast has the highest number (Table 6). All the contrast generate specific unique

DEGs not shared with the DEGs from the other contrasts (Figure 13). An analysis of their pattern of expression was used to arrange the genes in clusters of co-regulated genes (Figure 14).

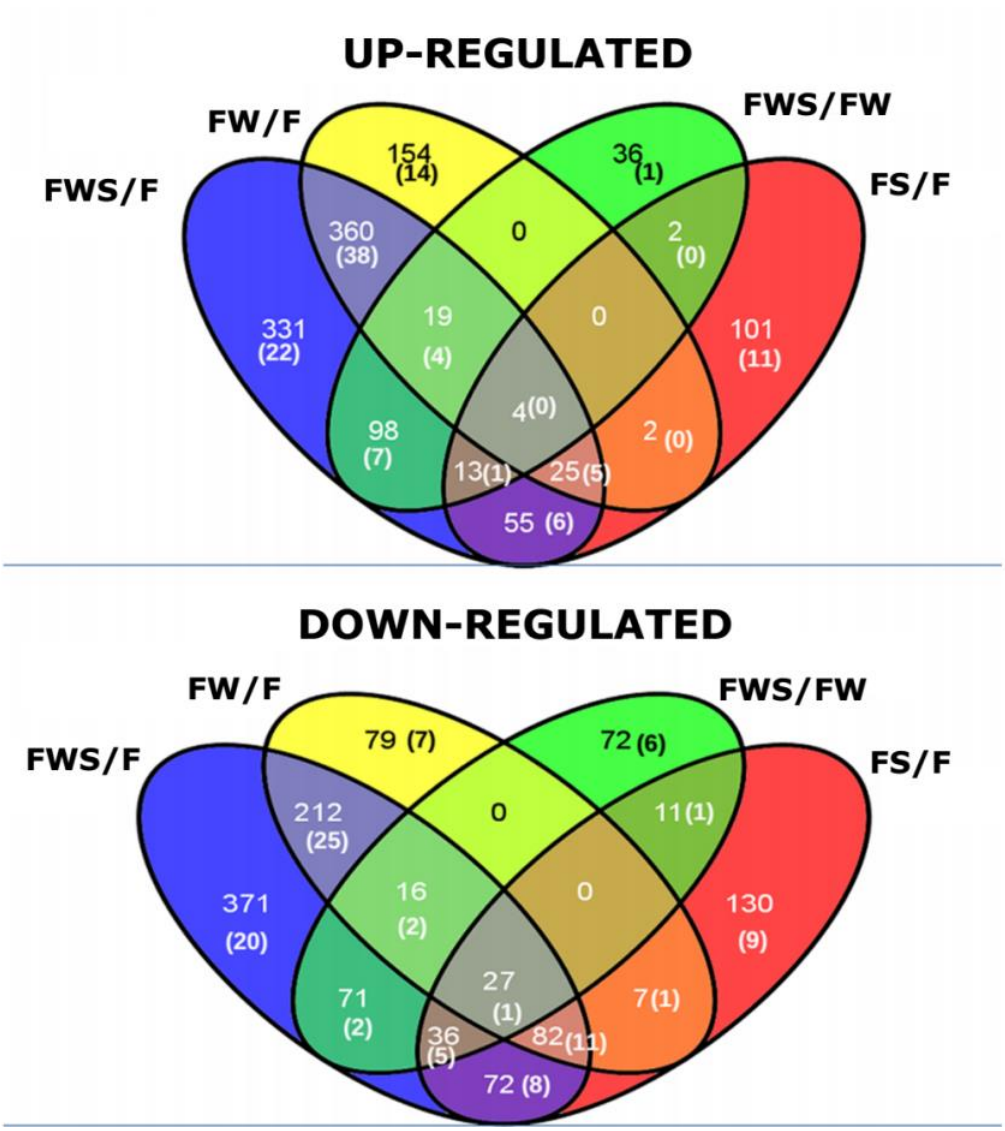

Figure 13. Venn diagram indicating relations among differentially expressed genes and the contrasting conditions evaluated. Between brackets are shown the number of differentially expressed genes that were predicted to be secreted through SignalP 4.0 (Petersen *et al.*, 2011).

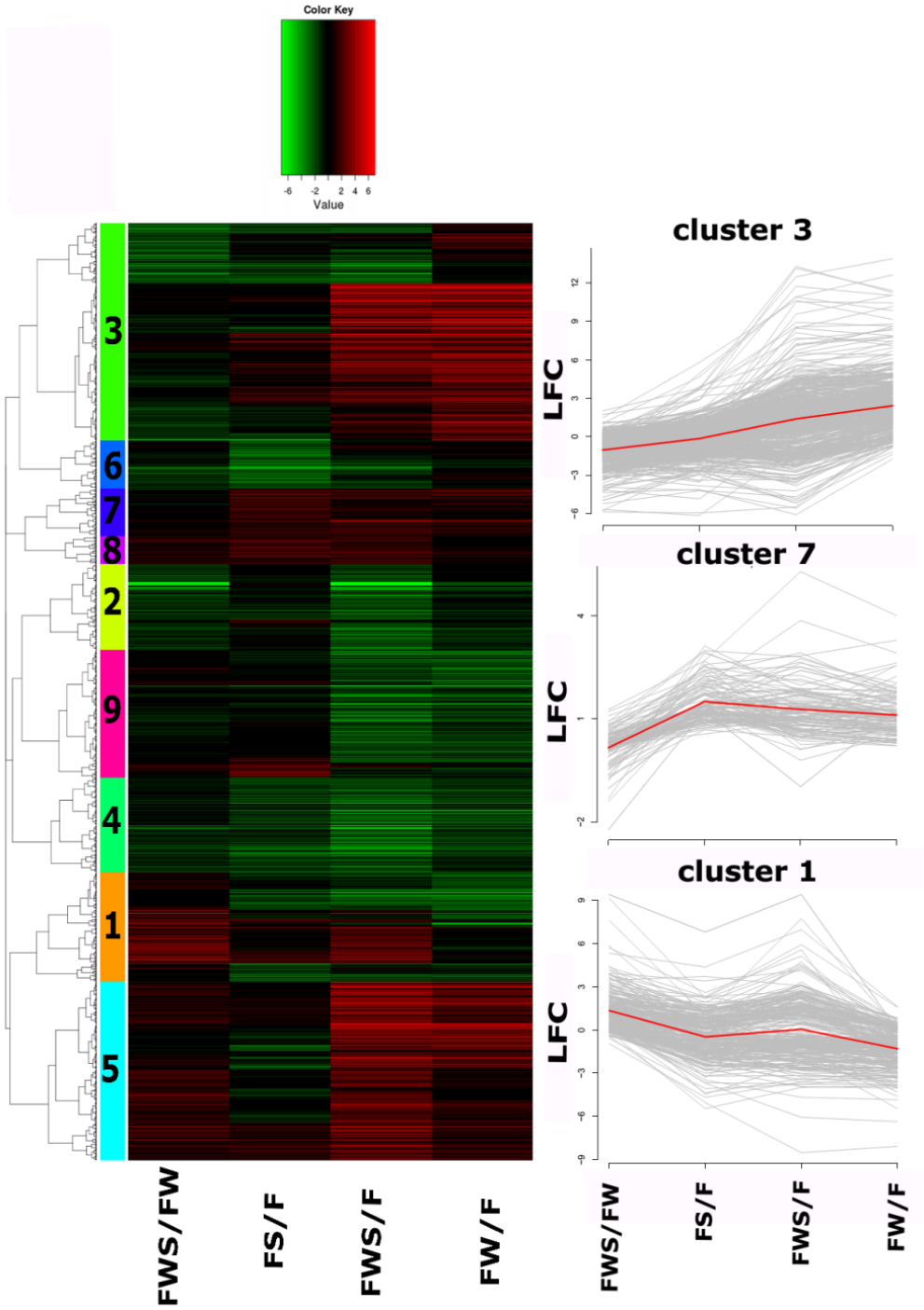

Figure 14. Heatmap showing clusters of co-regulated genes with their expression profiles. The dendrogram indicates the relationship between gene expression profiles as determined by hierarchical clustering (Pearson correlation). The differentially colored boxes at the left indicate different clusters of genes with similar expression profiles. The color key indicates logFC values ranging from bright red for most up-regulated to bright green for the most down-regulated genes, considering a FDR<0.01. The expression pattern for clusters with functional enrichment (clusters 1, 3 and 7) are shown in a graph with gray lines for each gene while the average for all the genes in the cluster is shown in red line. The contrasting conditions evaluated are those indicated in Figure 7. Briefly, FS/F: effect of heat stress (HS); FWS/FW: effect of HS but in the presence of grapevine wood; FWS/F: effect of HS in the presence of grapevine wood and FW/F: effect of only GW.

Table 7. Number of differentially expressed genes (DEGs, FDR<0.01) and enrichment test for secretion for each contrasting conditions

Contrasting conditions	Regulation	Differentially expressed genes (DEGs)	Secreted DEGs	p-value (hypergeometric test for enrichment of secreted proteins)
FWS/F	Up-regulated	905	83	0.156003
	Down-regulated	887	74	0.549571
FWS/FW	Up-regulated	173	13	0.707640
	Down-regulated	233	17	0.770976
FW/F	Up-regulated	565	61	0.012635
	Down-regulated	424	47	0.554427
FS/F	Up-regulated	203	23	0.077195
	Down-regulated	366	28	0.738934

Functional enrichment was obtained in three clusters of co-regulated genes. An enrichment for oxido-reductase activity was obtained for cluster 1 (FDR=$1.2x10^{-3}$), and the shared pattern of expression for the putative genes was mainly the induction in FWS/FW and FWS/F, and repression in FW/F (Figure 14). Cluster 3 showed enrichment in hydrolase activity, mainly involved in the hydrolysis of O-glycosyl compounds (FDR=$2.3x10^{-8}$), and showed an inverse pattern of expression compared to cluster 1, with major protein-coding genes up-regulated in FW/F, but down-regulated in FWS/FW and a lower level of induction or down-regulation in FS/F (Figure 14). Cluster 7 showed an enrichment (FDR<0.01) in translation processes, GTPase and methyltransferase activities. The shared pattern of expression in cluster 7 was mainly due to the induction in FS/F (Figure 14)

On the other hand, the presence of GW resulted in repression of genes encoding enzymes with oxido-reductase activity (GO term: GO0016491) and induction of genes coding for O-glycosyl hydrolyzing enzymes (GO term: GO0004553, Figure 15 and Figure 16). In contrast, after HS, the expression pattern was inverse to the fungus growing in GW: genes for oxido-reductase activity were up-regulated while genes coding for O-glycosyl hydrolyzing enzymes were mainly down-regulated (Figure 15 and Figure 16). The genes involved in translation activities (GO terms: GO0008135 and GO0003743) and methyltransferase activity (GO term: GO0008168) showed primarily up-regulation in both contrasting conditions, FW/F and FWS/FW (Figure 15), indicating the importance of the translation process in regulating the response to either change.

Our results showed that inosine monophosphate (IMP) cyclohydrolase, squalene monooxigenase and protein disulfide monooxigenase GO categories were induced in all the contrasting conditions (Figure 17). According to the established functions of these GO categories, it seems that basic metabolism such as purine nucleotide biosynthesis, sterol biosynthesis and post-translational modifications are important both to sustain growth on GW and under HS. Similar basic mechanisms might be required to deal with both conditions, perhaps because oxidative stress is produced in response to HS, and also required for GW degradation. On the other hand, genes involved in tyrosine and L-phenylalanine metabolic processes were only induced in FWS/FW, while they were highly repressed in all other contrasted conditions, suggesting a specific requirement of this metabolic pathway to deal with HS when in contact with wood components (Figure 17).

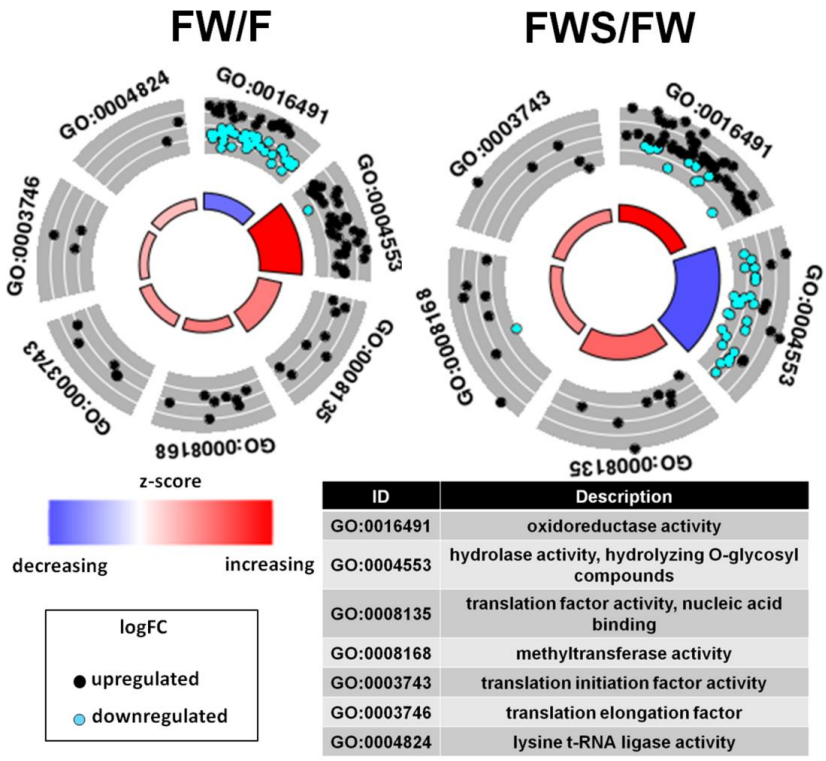

Figure 15. Gene Ontology (GO) of molecular functions categories with enrichment in hierarchical clusters of similar expression profiles. The GO term differential expression is considered through z-score ($\text{zscore}=\frac{(\text{upregulated-downregulated})}{\sqrt{(\text{upregulated+downregulated})}}$, described in Walter *et al.*, 2015). Red color indicates higher proportion of up-regulated genes and blue color corresponds to higher proportion of down-regulated genes. The color key indicates logFC values ranging from golden for up-regulated genes, to cyan for down-regulated genes. The effects of only grapevine wood (FW/F) and heat stress in the presence of grapevine wood (FWS/FW) were evaluated.

Secreted proteins are more prone to interact with the host; therefore we tested whether an enrichment of genes predicted to encode secreted proteins occurred among the DEGs. Among the upregulated genes in GW condition, there was an enrichment of those enconding for secreted proteins (FDR < 0.05 in Table 7), indicating that the presence of wood promotes the expression of secreted proteins.

3.4.3 *L. theobromae* gene expression during the interaction with grapevine

Co-regulated clusters (Figure 14) with functional enrichment indicated a whole fungal transcriptional change when exposed to HS in the presence of GW. Several genes belonging to any of the clusters 1, 3 and 7 (Figure 14) that were identified as having pathogenicity roles, based on their functional annotation, were selected to evaluate their expression by RT-qPCR. cDNA obtained from *in vitro* conditions was used for the validation of RNAseq results, and cDNA from *in planta* conditions in an attempt to elucidate their role in pathogenicity.

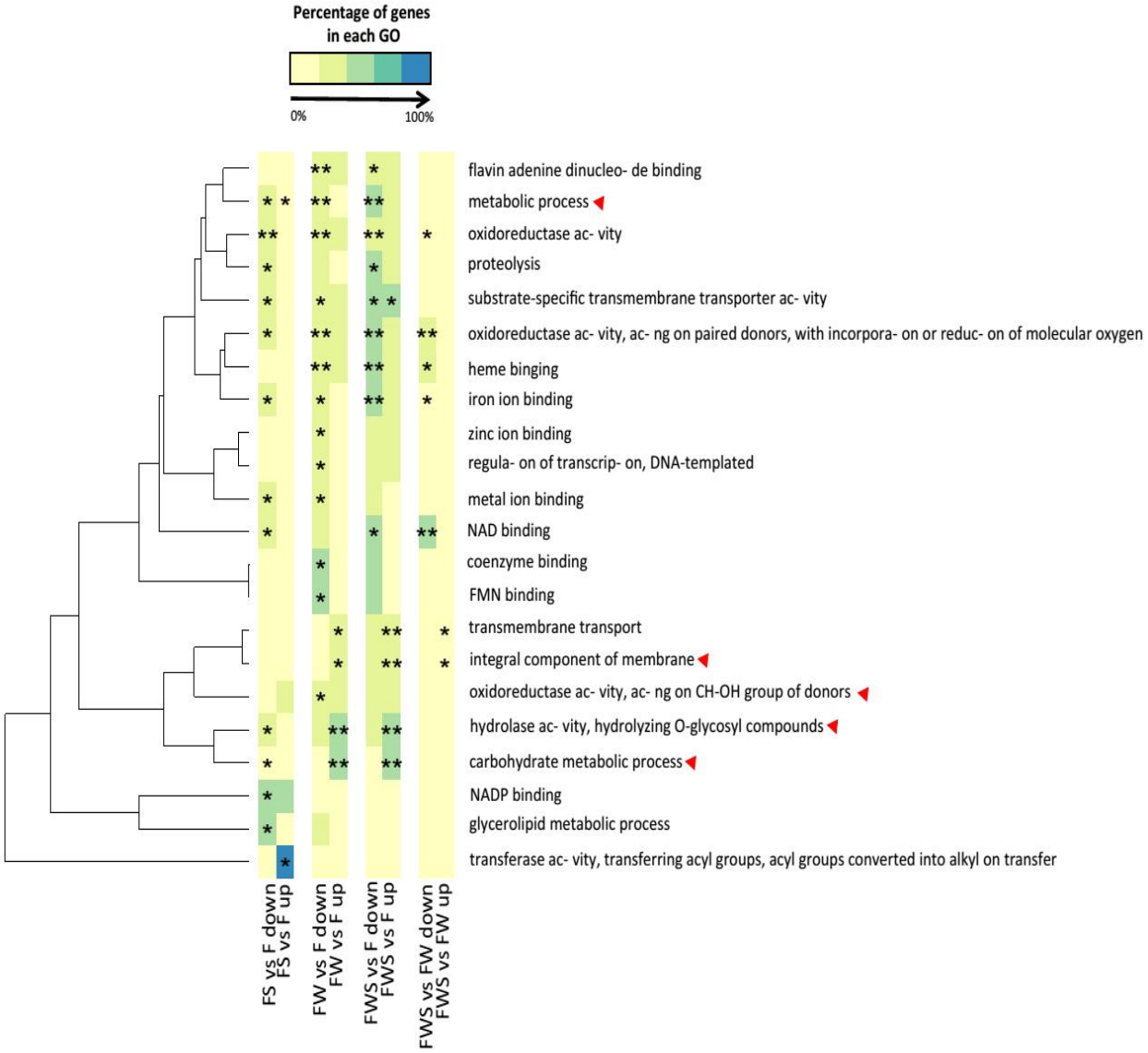

Figure 16 Functional enrichment of Gene Ontology (GO) categories based on the differentially expressed genes in each analyzed contrast. The color key indicate from pale yellow to blue, the increasing percentage of genes representing each functional category. To eliminate general and excessively specific categories, only the functional categories that have at least 5 and maximum 500 genes were considered in the analysis. The contrasting conditions evaluated are those indicated in Figure 7. Briefly, FS/F: effect of heat stress (HS); FWS/FW: effect of HS but in the presence of grapevine wood; FWS/F: effect of HS in the presence of grapevine wood and FW/F: effect of only GW. * FDR < 0.2 and ** FDR< 0.05.

The *in vitro* expression of 7 genes confirmed the contrasting pattern of expression based on the cluster to which each gene belonged and showed a high correlation with the results obtained through RNAseq (R^2= 0.876 and p-value=1.9x10^{-5}, Figure 18).

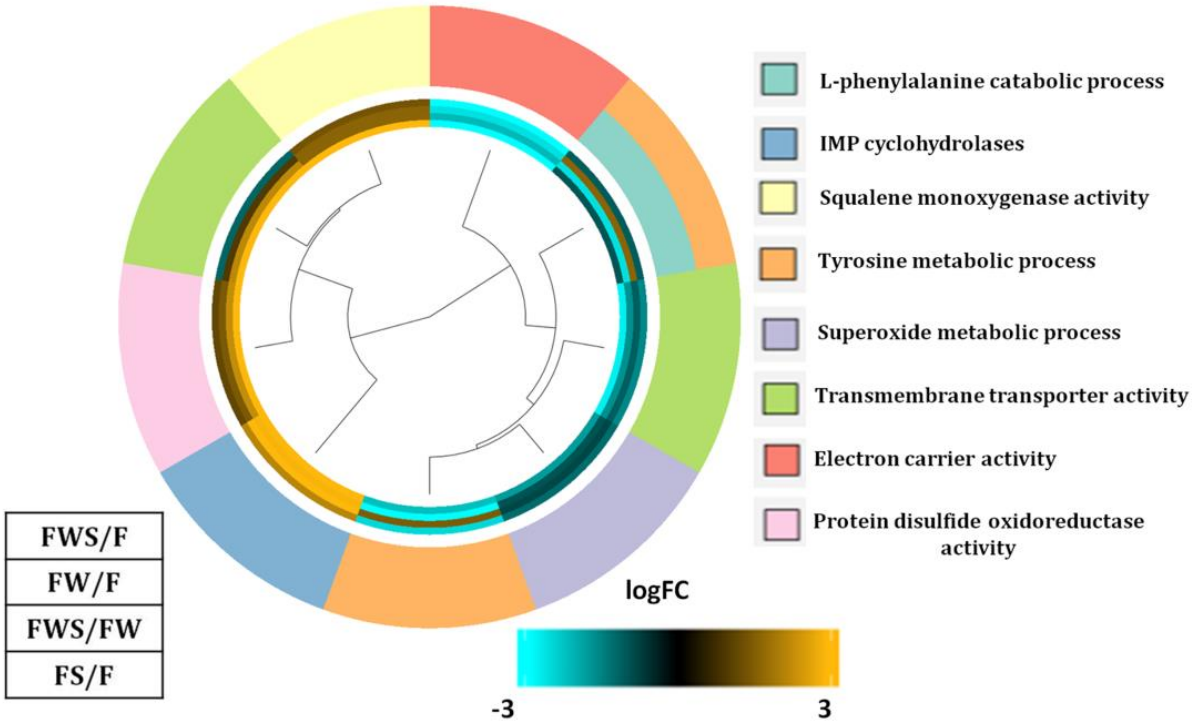

Figure 17. Gene Ontology (GO) of biological process categories with enrichment and showing differentially expressed genes in all the contrasting conditions evaluated (FDR<0.05). The color key indicates logFC values ranging from golden for up-regulated genes, to cyan for down-regulated genes. The dendrogram indicates the relationship through the hierarchical clustering of gene expression (the middle rings show from the center out: effect on *L. theobromae* gene expression of heat stress (HS) and grapevine wood (FWS/F), only grapevine wood (FW/F), only HS (FS/F),and effect of HS in the presence of grapevine wood (FWS/FW). The Biological Process GOterms are indicated in different colors on the outermost circle.

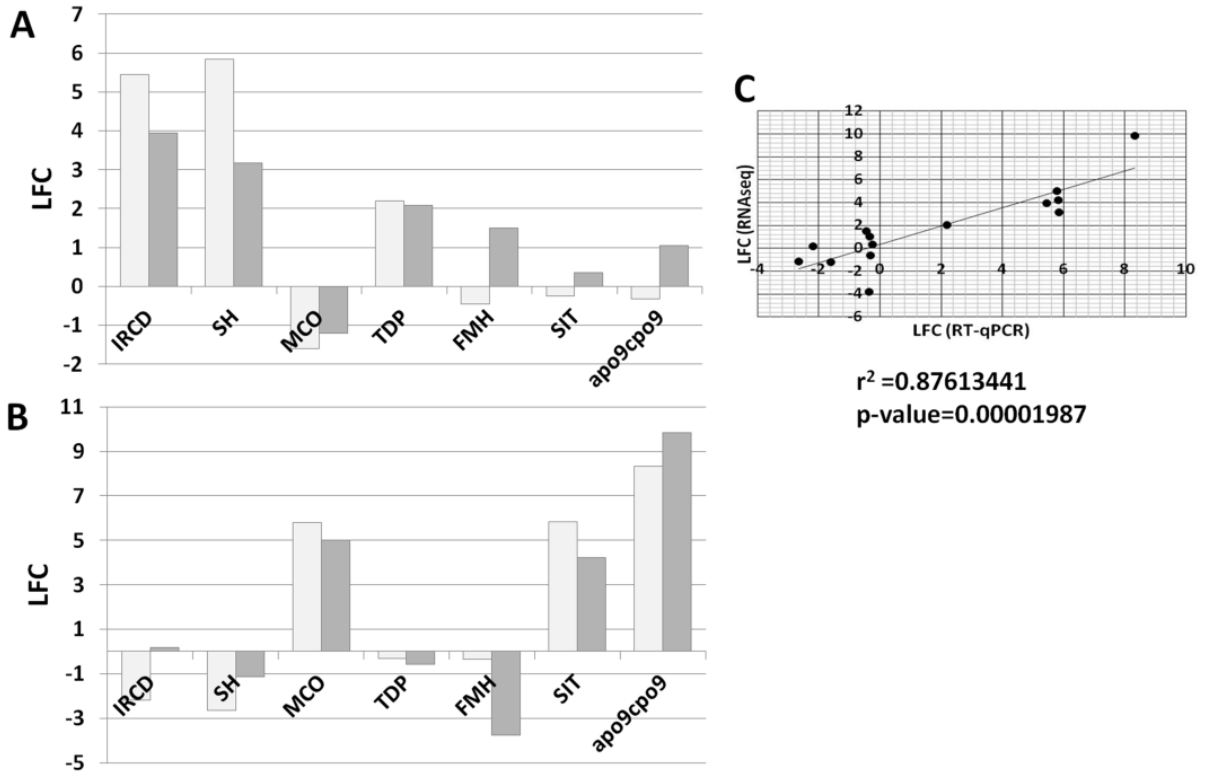

Figure 18. Differential expression quantification through RNA-seq and RT-qPCR. *In vitro* relative gene expression in FWS/FW **(**A) and FW/F (B) contrasting conditions calculated from RT-qPCR (light gray bars) and RNAseq data (dark gray bars). Normalization was done using β-tubulin for RT-qPCR data and count of reads per million (cpm) for RNAseq data. (C) Pearson correlation when comparing the LFC obtained with each technique.

In general, when evaluating the expression *in planta*, a marked regulation of most genes was observed at 7 dpi, and the changes on gene expression were even more defined in stressed plants (Figure 19). *L. theobromae* genes with up-regulated expression *in planta* were those enconding an intradiol ring cleavage dioxygenase (IRCD, comp4276_c0_seq1, selected from cluster 1), salicylate hydroxylase (SH, comp12473_c0_seq1, selected from cluster 1) pectate lyase (PL, comp16237_c0_seq1, selected from cluster 3), xylosidase glycoside hydrolase (XGH, comp5761_c0_seq2, selected from cluster 3) and a fumarylacetoacetate hydrolase (FMH, comp14342_c0_seq1, selected from cluster 1). IRCD was up-regulated at all infection times evaluated, in both heat-stressed and non-stressed plants (Figure 18). At 7 dpi on stressed plants, SH was up-regulated when compared to infected and non-stressed grapevines (Figure 19). In contrast, sugar inositol transporter (SIT, comp8181_c0_seq1), choline dehydrogenase (CHD, comp5526_c0_seq1), homogentisate dioxygenase (HGD, comp8784_c0_seq1), 4-hydroxyphenylpyruvate dioxygenase (HPPD, comp18638_c0_seq1) and glycoside hydrolase family 3 (GH3, comp13725_c0_seq1) did not show differential expression. A putative amylase from glycoside

hydrolase family 35 (AML, comp7101_c0_seq1) and multicopper oxidase (MCO, comp7300_c0_seq1, from cluster 3) were highly down-regulated, specially under HS (Figure 19).

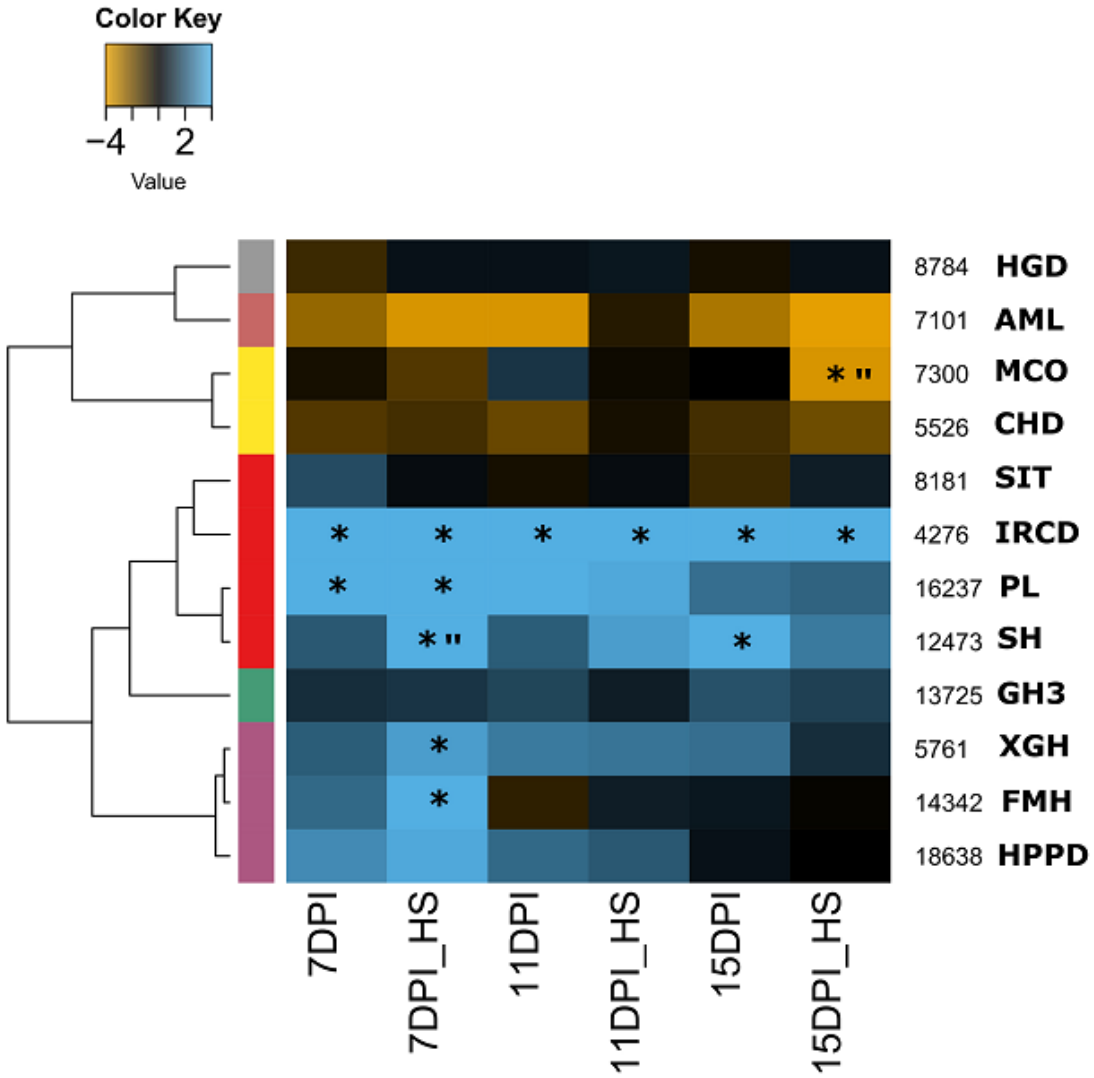

Figure 19. *L. theobromae* gene expression during grapevine infection. Fungal gene expression was evaluated at 7, 11 and 15 days post-infection in unstressed or heat-stressed grapevine (HS). Normalization was done using β-tubulin and the relative gene expression is indicated as logFC (*in planta*/*in vitro*) using HTqPCR package in R. One asterisk indicates significant differences with general linear model statistics (p-values < 0.05) for *in planta*/*in vitro* and quotation marks for heat-stressed/non stressed contrasting conditions. The dendrogram indicates the relationship between gene expression profiles through calculates of Canberra distances and Pearson correlation. The color key indicates logFC values ranging from golden for up-regulated genes, to cyan for down-regulated genes. IRCD: intradiol-ring cleavage dioxygenase (comp4276_c0_seq1); SH: salicylate hydroxylase (comp12473_c0_seq1); FMH: fumarylacetoacetate hydrolase (comp14342_c0_seq1); SIT:sugar inositol transporter (comp8181_c0_seq1), MCO: multicopper oxidase (comp7300_c0_seq1), PL: pectate lyase (comp16237_c0_seq1), AML: amylase (comp7101_c0_seq1), HGD: homogentisate dioxygenase (comp8784_c0_seq1), CHD: choline dehydrogenase (comp5526_c0_seq1), GH3: glycoside hydrolase family 3 (comp13725_c0_seq1), XGH: xylosidase glycoside hydrolase (comp5761_c0_seq2), HPPD: 4- Hydroxyphenylpyruvate dioxygenase (comp18638_c0_seq1).

3.5 Discussion

3.5.1 *L. theobromae* transcriptome features and comparison with related fungal pathogens

The closest fungi taxonomically related to *L. theobromae* with sequenced genomes are *M. phaseolina,* which has 14,249 genes (1,863 encoding secreted proteins (SP)) (Islam *et al*., 2012)*, D. seriata* with 9,398 genes (910 SP) and *N. parvum*, with 10,470 genes (1,097 SP) (Morales-Cruz *et al*., 2015). The number of unique predicted proteins derived from unique isoforms (15,981), and those with predicted secretion signal (850) obtained through *de novo* transcriptome assembly, suggests that the number of genes in *L. theobromae* is similar to its closest relatives.

Most of the assembled contigs covered the expected transcript length (average: 1,310 bp and N50: 2,472 bp, details in Table 6), considering the 1,531 and 1,574 bp average gene length in *N. parvum* and *D. seriata*, respectively (Blanco-Ulate *et al*., 2013b; Morales-Cruz *et al*., 2015).

From the unique predicted proteins for all the transcripts, 399 had hits to PHI-base (Mundy and Manning, 2011). In *M. phaseolina*, 537 PHI-base hits were identified (Winnenburg *et al*., 2006), while 1,120 were identified in *D. seriata* and 1,384 in *N. parvum* (Morales-Cruz *et al*., 2015). The differences found may be explained based on the different methods employed, since the relatives were fully sequenced and here RNAseq was done only in *in vitro* conditions. Further RNAseq analysis *in planta* will be useful to identify higher number of pathogenicity genes in this fungus. The putative pathogenicity transcripts (showing PHI-base hits) in *L. theobromae,* indicate main involvement in protein metabolic process, hydrolization of glycosyl bonds and oxidoreduction process (Figure 12). In coincidence, these GO categories showed most marked regulation when the fungus growing on GW was exposed to HS (Figure 15), suggesting that HS response and pathogenicity require a common transcriptional regulation. From the 399 PHI-base hits, 48 were differentially expressed, 22 were predicted to be secreted, and 8 of them shared both characteristics, indicating that the *L. theobromae* transcriptome is useful to identify putative pathogenicity factors, which could help to further validation of function through knockout or gene expression studies.

Gene families showed expansion in fungi causing grapevine vascular diseases producing similar symptoms as *L. theobromae* (Morales-Cruz *et al*., 2015). Genes belonging to those families were found in the *L. theobromae* transcriptome, that have 65 (20 DEG) dioxygenase (PF00775), 11 (2 DEG) pectate lyase (PL, PF03211), 259 (49 DEG) major facilitator superfamily (MFS, PF07690), 37 (5 DEG)

carboxylesterase (PF00135) and 22 (5 DEG) glucose-methanol-choline oxidoreductase (GMC, C-terminal: PF05199 and N-terminal: PF00732).

3.5.2 Transcriptional regulation of genes with putative role in pathogenicity

Based on the GO enrichment by clusters containing co-regulated genes, a whole fungal response could be inferred (Figure 15). In HS in the absence of GW, genes involved in the translation process were induced, suggesting that a change in protein profile is required to cope with stress. The requirement of heat-shock proteins (HSPs) in fungal HS response to keep proteins folded and active is well documented (Albretch *et al*., 2010; Verghese *et al*., 2012). In fact, several HSP encoding transcripts with different expression levels were identified, indicating the need of the fungal cells to redirect their physiology towards a survival or adaptive condition.

Based on functional enrichment, the fungus responds to HS differently in the presence of GW than in its absence. The specific enrichment of acyl-transferase among up-regulated genes when dealing with HS indicates that this activity could help the fungus to survive periods of stress. Acyl-transferases are involved in the synthesis of polyketides, a large class of secondary metabolites (Xie *et al*., 2009) that could provide the fungus with biochemical protection from stress. In contrast, in the presence of GW is not required, suggesting that some compounds, provided by GW fulfill that requirement. Genes in GO categories of integral component of membranes, mainly involved in transmembrane transport, were enriched specially in HS response in the presence of GW (Figure 16). Most of these genes belong to the major facilitator superfamily (MFS) and have homologues on PHI base, suggesting an important role in fungal pathogenicity. Genes that belong to GO categories involved in nucleotide (GO:0050660), and post-translation modifications (Figure 17) also are indicative of major change in the metabolism of the fungus to sustain growth under stress and in the presence of wood. On the other hand, GO term enriched GTPase activity, contains genes well known for having a role in translation and signaling events in response to stress (Bahn and Jung, 2013), evidencing the importance of a rapid adaptation to HS. Additionally, small GTPases are involved in virulence, due to their key role in secretory pathways (Bahn and Jung, 2013).

The repression of genes associated with electron transport (cytochrome p450 monooxygenase (comp5016_c0_seq3); dsba oxidoreductase protein (comp5774_c1_seq3), acyl- dehydrogenase family

protein (comp7418_c0_seq1) and three hypothetical proteins (comp264619_c0_seq1, comp303165_c0_seq1 and comp1_c0_seq1), in response to HS and/or GW (Figure 16), might contribute to the need of reducing the potential risk of mutation caused by higher production of ROS in mitochondria as an effect of thermal stress (Davidson and Schiestl, 2001). The down-regulation of a superoxide dismutase protein (comp6665_c0_seq1) is an unexpected result, because ROS detoxification has been proposed for superoxide dismutase and, increasing ROS levels in *Saccharomyces cerevisiae* were observed when responding to HS (Morano *et al*., 2012). In the HS response in the absence of GW a 5-oxoprolinase (comp4986_c0_seq1) was specifically induced. This enzyme participate in glutathione mediated ROS detoxification (Pócsi *et al*., 2004), indicating that an alternative to superoxide dismutase/peroxidase could be used to deal with the ROS produced in response to HS.

A model for the *L. theobromae* HS response in the presence of GW was constructed (Figure 20) based on the functional annotation, co-regulation and functional enrichment of genes, that were identified through the *in vitro* global transcriptional response. Based on this model, several putative pathogenicity genes were selected and its gene expression was evaluated during fungal-grapevine interaction.

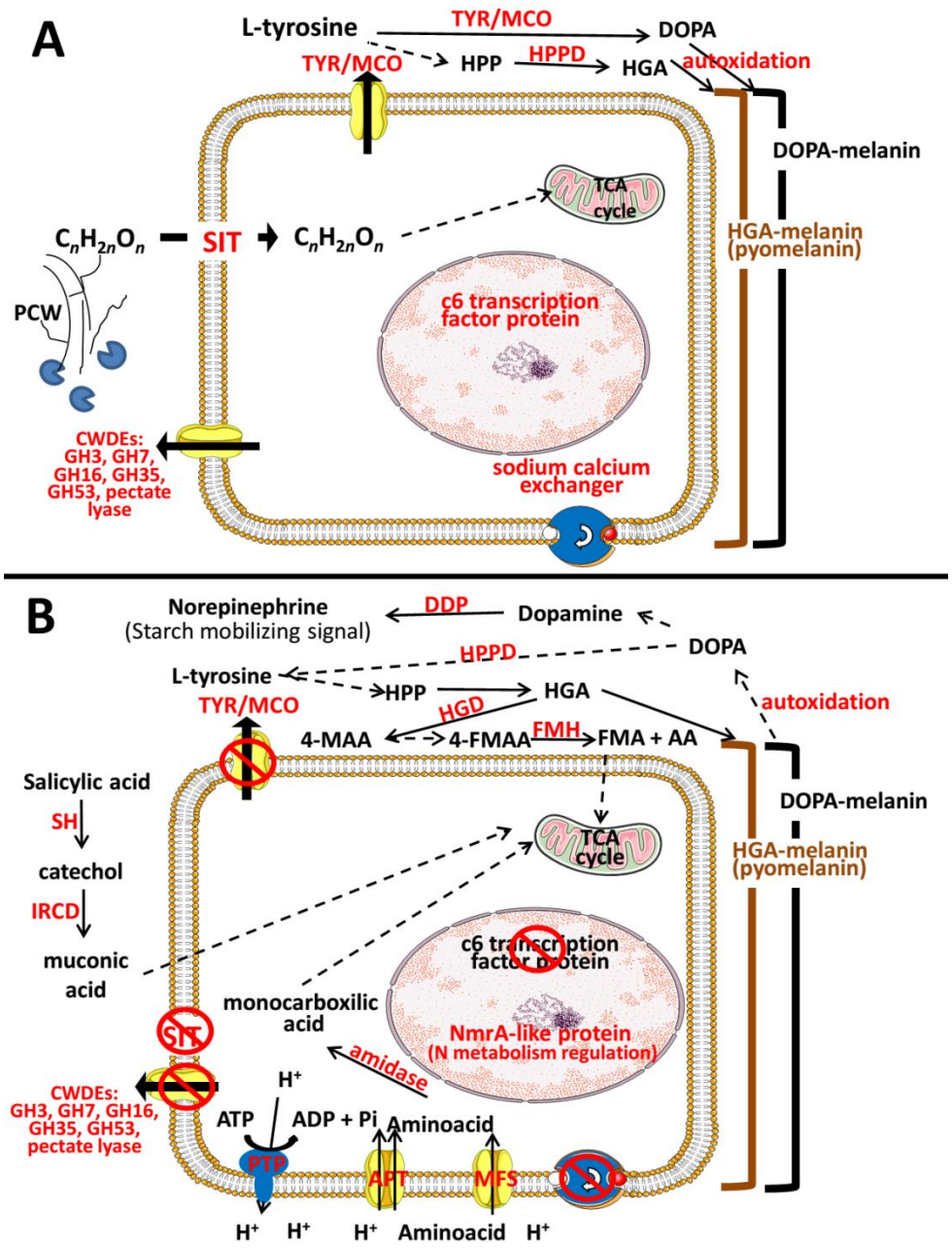

Figure 20. Hypothetical model of in *vitro L. theobromae* heat stress (HS) response, in the presence of grapevine wood (GW). The model of whole cell response was constructed using genes from clusters 1, 3 and 7 (in Figure 14) showing both co-regulation and GO term enrichment. The illustration shows the main functions and processes being carried out by the fungus when growing on GW (A), and the changes that are proposed to be occurring after HS (B). The putative cellular location (intra or extracellular) is indicated in the scheme. A continuous arrow line indicates an enzymatic reaction that is supported on the differentially expression of its encoding gene (shown in red). A dashed arrow line indicates a suggested enzymatic reaction. The substrate/product of reaction is indicated on boxes. TYR: tyrosinase, MCO: multicopper oxidase, DOPA: L-3,4-dihydroxyphenylalanine, HPP: 4-hydroxyphenylpyruvate, HPPD: 4-hydroxyphenylpyruvate dioxygenase, HGD: homogentisate dioxygenase, HGA: homogentisic acid, 4-MAA: 4-maleylacetoacetate, 4-FMAA: 4-fumarylacetoacetate, FM: fumarate, AA: acetoacetate, FMH:fumarylacetoacetate hydrolase, SH: salicylate hydroxylase, IRCD: intradiol ring cleavage dioxygenase, DDP: domon domain containing protein, PCWDEs: Plant cell wall degrading enzymes; PCW: Plant cell wall.

3.5.3 Genes with putative role in grapevine wood degradation and pathogenicity

Most of the up-regulated genes in GW but down-regulated in HS are predicted to be involved in the degradation or modification of plant cell wall (PCW) components, based on their functional annotation (hydrolysis of O-glycosyl compounds, GO: 0004553, Figure 15). A gene coding for a putative secreted enzyme that belongs to the glycosyl hydrolase (GH) family 35, has conserved domains which suggest its role as amylase (comp7101_c0_seq1). Its expression was induced by GW and showed no significant differences in the contrasting condition FWS/FW, indicating that degradation of starch remains active independently of HS. In grapevine wood, starch is stored in ray parenchyma cells, next to xylem vessels and it is used as carbon reserve by the plant (Keller, 2010). *E. lata,* a vascular pathogen of grapevine that has similar colonization strategy as *L. theobromae*, has been shown to degrade plant starch (Rolshausen *et al*., 2008) suggesting that this polymer may play a pivotal role as energy reserve for both the vascular pathogen and the plant metabolisms. Since the relationship among starch degradation and pathogenicity seems important for disease progression, the putative amylase (comp7101_c0_seq1) expression was evaluated *in planta.* Under stress conditions, it showed a marked down-regulation at any time of infection and treatment (Figure 19), indicating that its activity is not required for the establishment of the infection, at least in the evaluated conditions. This enzyme was annotated as a putative amylase, because of one of its domain is a GH35; since this can be considered as an incomplete annotation, the enzyme encoded by comp7101_c0_seq1 sequence might have another function.

Among the genes coding for proteins predicted to be secreted, three are associated with pectin degradation: PL (comp16237_c0_seq1), GMC (comp7222_c0_seq1) and CHD (comp5526_c0_seq1). These genes encode for PCWDEs belonging to an expanded family in grapevine vascular pathogens (Morales-Cruz *et al*., 2015). The first one is involved in pectate cleavage (Morais do Amaral *et al*., 2012), while the second and third one corresponds to a gene family involved in lignin breakdown (Benoit *et al*., 2012). PL and CHD expression during fungal colonization was quite contrasting, while PL was significantly up-regulated, especially under HS at 7 dpi, CHD was down-regulated at any stage (Figure 19). This suggests that lignin break-down was not required for fungal infection, but pectin degradation seems to be important for fungal pathogenicity. Pectin is the main component of middle lamella (Keller, 2010) and therefore have a fundamental role blocking intercellular fungal growth. Recently, it was clearly determined the importance of pectin degrading enzymes for *Fusarium graminearum* intercellular

colonization of maize parenchyma tissue (Zhang *et al*., 2016). We propose that PL allows *L. theobromae* to colonize grapevine tissues intercellularly.

Genes encoding an endopolygalacturonase (comp4965_c2_seq1) and a pectinesterase (comp13568_c0_seq1) were also up-regulated in response to GW and have been associated with PCW degradation (Morais do Amaral *et al*., 2012). Furthermore, expression of secreted enzymes, xylosidase glycoside hydrolase (XGH comp5761_c0_seq2), glycoside hydrolase family 3 (GH3 comp13725_c0_seq1) and endo1,4-betagalactanase (comp15147_c0_seq1), which are involved in cellulose and hemicellulose degradation (Morais do Amaral *et al*., 2012), were found up-regulated in response to GW. Rolshausen *et al* (2008) also reported *in vitro* hemicellulose degradation and increased enzymatic activity in the presence of wood in *E. lata*. Two genes coding for enzymes that belong to GH1 with glucosidase/galactosidase activity (comp2943_c0_seq1 and comp7123_c0_seq1) have homologous genes with recognized intracellular roles in cellobiose and lactose catabolism (Benoit *et al*., 2012).. The expression of both XGH and GH3 were up-regulated *in planta* (Figure 19). XGH showed differential up-regulation at 7 dpi on infected and stressed plants (Figure 19), suggesting that its function on hemicelluloses degradation is required, thus, deeper access to the host cell wall seems to be favored with HS.

On other hand, an ortholog of the glyoxal oxidase (GOX) gene (comp11184_c0_seq1) showed up-regulation in the presence of GW. GOX was previously associated with PCW degradation of wood in *Phanerochaete carnosa*, specifically degrading lignin (MacDonald *et al*., 2016). Furthermore, a gene (comp12572_c0_seq1) that belongs to GH16 encodes an enzyme involved in PCW modification (Eklöf *et al*., 2010) and showed up-regulation only in the presence of GW. Overall, the co-expression of these genes indicates polysaccharide degradation to use the GW as carbon-source. Its concerted enzymatic activity could help *L. theobromae* to colonize the host and cause the development of canker in wood. The necrosis observed close to the pith in heat-stressed plants (Figure 21), likely indicates a deeper colonization of stem by *L. theobromae*. Our data suggest that the change in grapevine phenology occurs in response to HS and this change promotes a faster fungal colonization. Although not all the PCWDEs evaluated were up-regulated *in planta,* and even the putative amylase was down-regulated, it is possible that a specific role of up-regulated genes encoding PL and XGH favors fungal colonization under HS.

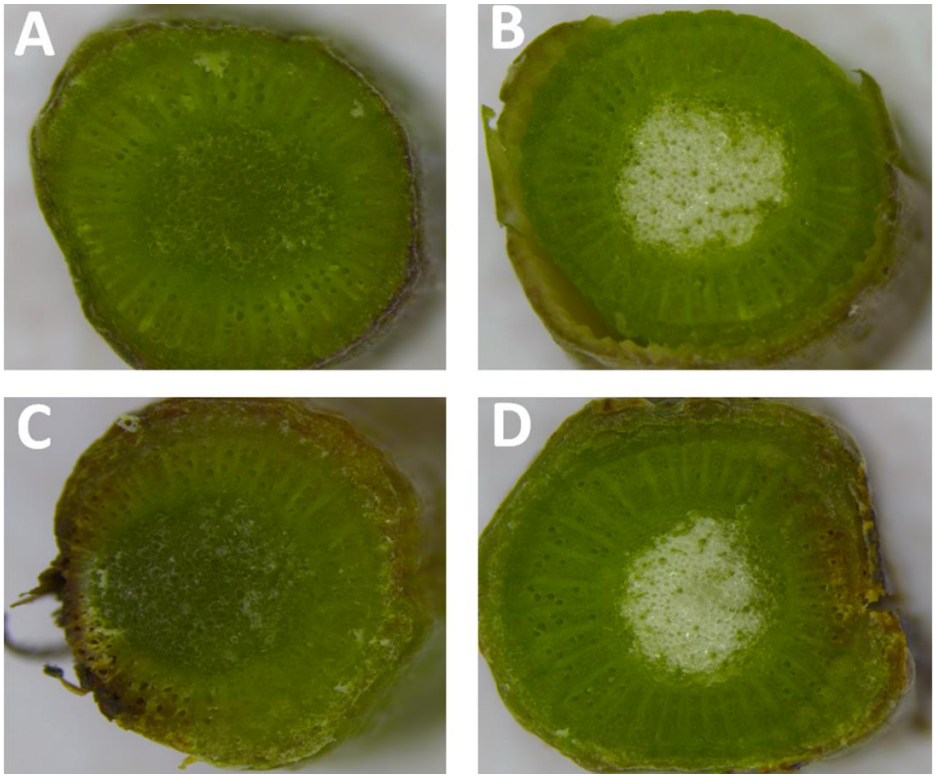

Figure 21. The effect of heat stress (HS) on grapevines uninfected or infected with *L. theobromae*. Stereomicroscope images were taken at 15 days post-infection, corresponding to 10 days of HS treatment (day-night cycles, with temperatures ranging from 42 to 20 °C). Uninfected and unstressed grapevine show healthy green tissue (A). Uninfected and heat-stressed grapevine show a change to a white color of the pith and phloem impairment (B). Infected but unstressed grapevine show black, yellow and brown color tissues close to the site of inoculation (C). Infected and stressed grapevine show white pith and brown/yellow color in primary xylem (D).

3.5.4 Genes with putative role in phenolic, melanin, protein metabolism and pathogenicity

Several genes encoding enzymes involved in different melanin synthesis pathways reported in fungi were identified: 3,4-dihydroxyphenylalanine (DOPA)-melanin, DHN (1,8-dihydroxynaphthalene)-melanin and pyomelanin (Eisenmann *et al*., 2007; Frases *et al*., 2007; Schmaler-Ripcke *et al*., 2009; Chiang *et al*., 2011; Upadhyay *et al*., 2011; Liu *et al*., 2014). Remarkably, the gene for a secreted enzyme associated with DOPA-melanin synthesis (MCO, comp7300_c0_seq1) showed high induction in response to GW, while it did not show regulation upon HS. This gene was found as belonging to a family expanded in *N. parvum* and *D.seriata*, stressing its putative role in pathogenicity (Morales-Cruz *et al*., 2015). A gene encoding enzyme putatively involved in DHN-melanin synthesis (short-chain dehydrogenase reductase, comp4978_c0_seq2) showed *in vitro* co-regulation with MCO. On the other hand, the enzyme involved in homogentisic acid synthesis, HPPD (comp18638_c0_seq1) was up-regulated (LFC= 2.18 and FDR=0.07) in the presence of GW. This gene is associated with pyomelanin synthesis, a fungal adaptation of the L-

tyrosine metabolic pathway, where the intermediary homogentisic acid is polymerized to form melanin (Frases *et al*., 2007; Schmaler-Ripcke *et al*., 2009; Boyce *et al*., 2015). After HS, genes that encode enzymes that degrade homogentisic acid, HGD (comp8784_c0_seq1) and FMH (comp14342_c0_seq1), were up-regulated suggesting that pyomelanin degradation is activated. The gene encoding maleylacetoacetate isomerase, involved in the transformation of 4-maleylacetoacetate to 4-fumarylacetoacetate in the L-tyrosine metabolic pathway (Vasanthakumar *et al*., 2015), was identified (comp3928_c0_seq2 and comp196313_c0_seq1) but it was not differentially expressed in the analyzed contrasting conditions. The observation of L-tyrosine metabolic pathway regulation in response to HS is strengthened by the expression pattern (up-regulated by GW and down-regulated by HS) of the C6 transcription factor (comp6826_c0_seq1). This gene was previously found in the *Aspergillus fumigatus* genome, arranged in a cluster with the genes involved in gentisate catabolism (Greene *et al*., 2014), which is an alternative pathway to tyrosine metabolism in fungi. A mutant in the C6-Zn transcription factor *ProA* in *Epichloë festucae* was unable to establish a symbiotic interaction with its host *Lolium perenne*, proving the importance of this gene for the maintenance of the mutualistic relationship (Tanaka *et al*., 2013). *L. theobromae* has an endophytic behavior (Johnson *et al*., 1992; Burgess *et al*. 2006), and it has been suggested that abiotic stress promotes its switch to a pathogenic behavior (Slippers and Wingfield, 2007; Hwang *et al*., 2003). The C6 transcription factor regulating genes belonging to secondary metabolism pathways in response to HS, suggest the promotion of a pathogenic-like behavior in *L. theobromae* as an effect of HS.

Several yeast pathogens of mammals showed a phenotypic dimorphic switch (from filamentous to bud-cells and vice versa) when changing from room temperature to 37 °C, also accompanied with a change in melanin metabolism (Nunes *et al*., 2005; Youngchim *et al*., 2013; Pasricha *et al*., 2013). In general, a decrease in DOPA-melanin (tyrosinase downregulation) and/or an increase in pyomelanin production or L-tyrosine metabolism (4-hydroxyphenylpyruvate dioxygenase up-regulation) were observed consistently in previous studies (Nunes *et al*., 2005; Youngchim *et al*., 2013; Pasricha *et al*., 2013; Yang *et al*., 2013). It seems that in mammalian fungal pathogens, the change in the type of melanin produced is important to trigger the morphogenetic switch and to become pathogenic. We propose that a general mechanism for becoming pathogenic under HS conditions is conserved among several fungal taxa, whereby melanin metabolism could play a role in pathogenicity in plants, similarly to fungal mammalian pathogens. Our results also demonstrated that FMH was up-regulated *in planta* mainly at 7 dpi under HS (Figure 19). A fumarylacetoacetase family was exclusively expanded in Ascomycota vascular pathogens (Morales-Cruz *et al*., 2015), suggesting an evolutionary adapted function of these enzymes to favor the colonization of this niche. FMH is involved in the final step of the tyrosine metabolic pathway, converting

fumarylacetoacetate to fumarate and acetoacetate, which could be incorporated in the tricarboxilyc acid cycle to obtain energy. Alternatively, the co-regulated genes, malate synthase (MS, comp6659_c0_seq1) and isocitrate lyase phosphorylmutase (ICP, comp8689_c0_seq1), suggest that acetate could be employed for anabolic processing through the glyoxylate cycle (Lorenz and Fink, 2001) .

On other hand, it has been suggested that FMH regulates pyomelanin production (Frases *et al*., 2007; Schmaler-Ripcke *et al*., 2009). The homogentisate, an intermediary in L-tyrosine metabolic pathway, is used by some fungi as a precursor for pigment production (Frases *et al*., 2007; Schmaler-Ripcke *et al*., 2009). The precursors of lignin are derived from tyrosine or phenylalanine through the phenylpropanoid defensive pathway in plants (Fraser and Chapple, 2011). This pathway is greatly induced in response to biotic and abiotic stress; therefore the fungal elimination of such precursors for DOPA-melanin or through tyrosine catabolic pathway could help the fungus to evade the plant compartmentalization mediated through lignin. When evaluated *in planta*, fungal MCO expression was down-regulated, specially under HS (Figure 19). In contrast, HPPD and FMH were up-regulated at 7 dpi on stressed plants (Figure 19), indicating that the fungal gene regulation under stress *in planta* is similar to that obtained *in vitro* (Figure 19), supporting the hypothesis that DOPA-melanin could act as a tyrosine storage that then is degraded through tyrosine metabolism pathway. If this is the case, the increase of grapevine phenylpropanoid pathways precursors in response to HS, could be used by *L. theobromae* for its metabolism to promote colonization (Figure 20).

In this work, SH (comp12473_c0_seq1) was up-regulated upon HS in the presence of GW during *in vitro* growth (Figure 18). The product of SH activity, catechol, induces the ROS-mediated plant defensive response (vanWees and Glazebrooks, 2003; Ruddrappa *et al*., 2007). IRCD (comp4276_c0_seq1), a gene coding for an enzyme that catalyze the degradation of aromatic rings (Broderik, 1999) was co-regulated with SH. IRCD could act degrading catechol and therefore helping in salicylic acid (SA) degradation Leatham *et al*., 1983. A plausible hypothesis is that the polymerization of catechol to melanin or its catabolism, could prevent this harmful compound from interacting with ROS activating enzymes. Despite the fact that benzoic acid (BZA) is used as a potent fungicide (Pagnussat *et al*., 2013), *L. theobromae* growth was not inhibited in the presence of 15-30 mM of BZA (Figure 22). No other carbon source was added to the medium VMM, suggesting that the fungus catabolizes BZA to sustain its growth. A marked increase in aerial mycelium and dark color was observed, indicating fungal melanin production (Choi and Goodwin, 2011; Wu *et al*., 2012,Yan *et al.*, 2013) and revealing a relationship between phenolic degradation and melanin production.

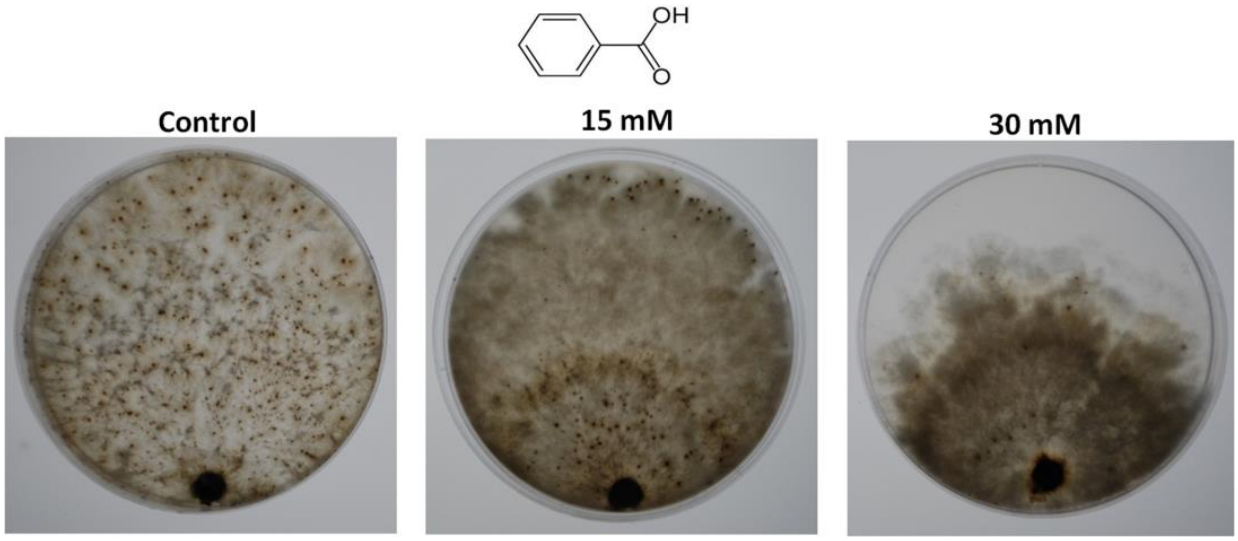

Figure 22. *L. theobromae* growth on Benzoic Acid (BZA). The evaluated medium was Vogel´s minimal medium (VMM) amended with 0, 15 and 30 mM. Photos were taken at 8 days post inoculation (dpi). With increasing concentrations of BZA, higher density colonies with more aerial and darker mycelium were observed.

Furthermore, we found that IRCD and SH expression *in vitro* were induced in response to HS when GW was present (Figure 18). The reason for this response is not clear, since the fungus had no contact with a living plant. However, it suggests a conserved and advantageous response of the fungus to HS, since the enzyme is produced before the plant SA burst starts. To test this hypothesis, the fungal expression in infected grapevine was evaluated and both genes showed significant up-regulation at each time course evaluated (Figure 19), therefore SH and IRCD could be required for grapevine colonization by the fungus. In particular, fungal SH showed a defined up-regulation when compared heat stressed to none stressed plants at 7dpi (Figure 18). It was already reported that SA is produced in grapevine to alleviate the photosynthesis impairment produced under HS (Wang *et al*., 2010), suggesting that the fungus, instead of using carbohydrate-derived carbon, switches its metabolism to degrade one of the major and readily available plant hormones, i.e. SA (schematic model in Figure 23). Indeed, in *Valsa mali*-apple interaction, the fungal benzoate pathway was enriched, indicating an important function in pathogenicity in a lignified host tissue Ke *et al*., 2014. SH was previously associated with *Ustilago maydis* pathogenicity promotion in maize, suggesting that SA is used as either a carbon source or to repress the plant defense response signal (Rabe *et al*., 2013). *Moniliophthora perniciosa,* a pathogen of cacao, contains a gene coding for SH in its genome, which could explain the high tolerance of this pathogen to SA (Mondego *et al*., 2008). Furthermore, SH was one of the most expressed genes during the endosymbiont phase of *E. festucae* in grass, suggesting a role for evading the host SA-mediated response (Ambrose *et al*., 2015).

We propose that SH and IRCD activities, help *L. theobromae* to disrupt the SA defensive system involved in Systemic Acquired Resistance, using at the same time SA as carbon source (Figure 23).

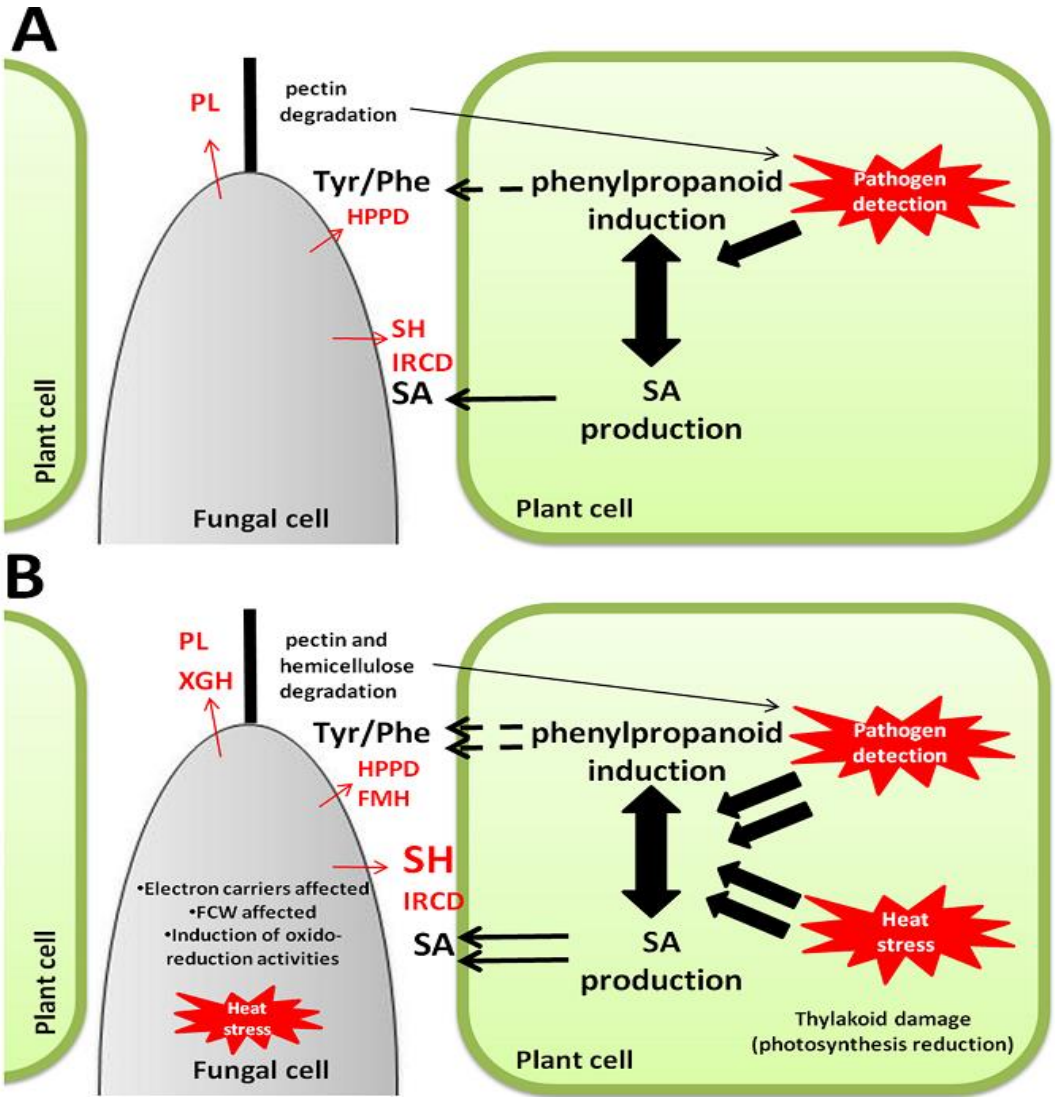

Figure 23. Proposed model of fungal-plant interaction under heat stress (HS). The model was done using gathered information of *in vitro L. theobromae* HS global transcriptional response, gene expression *in planta* (Figure 19) and documented HS response in plant. (A) When fungus infects unstressed plant, selectively degrades pectin (up-regulation of PL compared with the other PCWDEs), this allows it to growth in intercellular spaces without causing major damage to PCW. The presence of the pathogen could trigger the host defensive mechanism mediated through SA, or by the activation of the phenylpropanoid pathway. However, the host defense could be impaired for the activity of SH and IRCD (degrade SA), or by HPPD (phenylpropanoid precursors). (B) Heat stress activates the same metabolic pathways increasing SA and the levels of phenylpropanoid precursors, thus the fungus could benefit from the higher availability of compounds using them as carbon sources. The main differential change on stressed plant, compared with unstressed, is the up-regulation of SH, FMH and XGH, whose activities could facilitate fungal colonization. Tyr: tyrosine. Phe: phenylalanine.SA: salicylic acid, IRCD: intradiol-ring cleavage dioxygenase (comp4276_c0_seq1); SH: salicylate hydroxylase (comp12473_c0_seq1); FMH: fumarylacetoacetate hydrolase (comp14342_c0_seq1); SIT:sugar inositol transporter (comp8181_c0_seq1); PL: pectate lyase (comp16237_c0_seq1); XGH: xylosidase glycoside hydrolase (comp5761_c0_seq2), HPPD: 4-Hydroxyphenylpyruvate dioxygenase (comp18638_c0_seq1). FCW: fungal cell wall. PCW: plant cell wall; PCWDEs: plant cell wall degrading enzymes.

A metalloproteinase (comp1851_c0_seq1) showed the most significant up-regulation in response to HS. Metalloproteinases have previously been observed to be up-regulated in the necrotrophic stage of the pathogenic fungus *Moniliophthora roreri-Theobromae cacao* interaction (Meinhardt *et al*., 2014) and belong to an expanded gene family in pathogenic Onygenales (Li and Zhang, 2014) indicating a putative role of this enzyme in pathogenicity.

On other hand, an NmrA-like protein-encoding gene (comp1446_c0_seq2) was down-regulated only in the presence of GW, but up-regulated with HS. NmrA is part of a system controlling nitrogen metabolite repression in fungi, acting as a negative transcriptional regulator involved in the post-translational modification of the transcription factor AreA (Xiao *et al*., 1995; Adrianopoulos *et al*., 2008; 1998, Schöning *et al*., 2008). The up-regulation of this gene upon HS, suggests a requirement for a different nitrogen source by fungal cells under thermal stress. Besides, AreA has been identified as an important regulator of secondary metabolism in *Fusarium fujikuroi* and *F. graminearum* (Schoning *et al*., 2008; Giese *et al*., 2013) and an NmrA-like protein was strongly up-regulated during a compatible stage in the *M. oryzae*-rice interaction Kawahara *et al.*, 2012, suggesting an important relationship between nitrogen metabolism and pathogenicity.

Other genes co-regulated with the NmrA-like protein and involved in nitrogen metabolism were a gene coding for an amino acid polyamine transporter I family (comp6363_c0_seq1) that is involved in amino acid/H+ transmembranal symport (Jack *et al*., 2000; Vastermark *et al*., 2014) and several genes that belong to the MFS (comp23378_c0_seq1, comp7071_c0_seq1 and comp3035_c0_seq2) that are involved in general transport, including amino acid transport (Pao *et al*., 1998; Xu *et al*., 2014). The orthologs of genes encoding enzymes involved in L-tyrosine metabolism, HGD (comp8784_c0_seq1) and FMH (comp14342_c0_seq1), in *Penicillium marneffei*, showed regulation through AreA (Liu *et al*., 2014). The above mentioned gene expression pattern suggests an important change in the general *L. theobromae* nitrogen metabolism as effect of HS.

3.6 Conclusions

The transcriptome of *L. theobromae* was established, providing nucleotidic sequences to the scientific community that will help to understand and eventually control this important pathogen. Based on functional annotation, several genes with putative functions in pathogenicity were identified,

highlighting those encoding PCWDEs and involved in phenolic metabolism. Genes involved in melanin production were co-regulated with key transcription factors that control carbon and nitrogen usage, suggesting that strong metabolic and morphological changes occurs during fungal heat stress adaptation, in a similar manner to those documented for some fungal mammal´s pathogens.

Based on the in vitro L. theobromae global transcriptional regulation under HS, it was possible to identify fungal genes with up-regulation both in planta under optimum conditions and also with distinctive expression under HS, which helped to establish a model of the fungal-plant interaction under stress. These results indicated that HS overall facilitated L. theobromae growth and colonization. We propose that these observations are triggered by the fact that phenylpropanoid precursors increase under heat stress, and that L. theobromae is able to utilize these compounds for its own metabolism Although additional experiments are required to evaluate the robustness of the proposed model, this work provides the foundation to future research in order to understand the complexity of this important grapevine vascular pathogen.

Chapter 4. General discussion

Much information remains elusive in Botryosphaeriaceae behavior infecting grapevine. Microscopy studies highlighted the important stages for the fungal establishment. *B. dothidea* grows intercellularly on apple twig tissue, at the beginning of the colonization process (Han *et al*., 2016); this requires fungal degradation of middle lamella to gain access between plant cells. The plant cell wall was not affected at this stage, indicating a specialized function only on the middle lamella, which is a layer rich in pectin and lignin in plants (Agrios, 2005), indicating that the degradative enzymes pectinases and phenol oxidases are required for the intercellular growth. Lignin is derived from polymerization of phenylalanine-derived precursors and acts as glue for neighboring cells (Keller *et al*., 2011). If a similar behavior occurs during the *L. theobromae*-grapevine interaction, these enzymes must play an important role during the first stage of infection. Phenylalanine is converted to tyrosine in a single oxidation step (Underfriend and Cooper, 1951). In *Aspergillus fumigatus* tyrosine is used to produce DOPA-melanin and pyomelanin (Schmaler-Ripcke *et al*., 2009). Tyrosine is also the precursor for the production of lignin in plants (Vogt *et al*., 2010); therefore it is problable that in *L. theobromae,* the role of melanin is to control lignin precursors.

Multicopper oxidases (MCO) are phenol oxidases associated with the production of DOPA-melanin, and in some cases with lignin depolymerization (Kües and Rhül, 2011). MCO gene expression was down regulated during *L. theobromae* grapevine infection, indicating that its activity is not required for fungal establishment. Other phenol oxidases were identified in the *L. theobromae* transcriptome that could have a role on lignin degradation.

On the other hand, the first stage of *L. theobromae* colonization of cashew trees is characterized by the absence of defensive response by the host (Muniz *et al*., 2011). This behavior is common in well-studied endophytes and is mediated through effectors that disrupt defense signaling (Rovenich *et al*., 2014). In this sense, the up-regulation of salicylate hydrolase (SH) and intradiol ring cleavage dioxygenase (IRCD), and the absence of symptoms a few centimeters away from the inoculation site, suggest that intercellular growth is promoted by the activity of these enzymes. An endophytic stage is also supported by the observation under the stereomicroscope of several hyphae far away from the inoculation point.

At later stages, a coincident observation was obtained for Botryosphaeriaceae when the plant cells surrounding the hyphae became malformed without mediating PCW degradation (Han *et al*., 2016). This phenomenon could be explained by the secretion of effectors with the capacity of get into the host

cytoplasm through the pit field and once inside, to exert a host-manipulated necrotic process such as Programmed Cell Death (PCD). In this regard, fumarylacetoacetate is an intermediary in L-tyrosine metabolic pathway that has been associated with the induction of PCD (Han *et al*., 2013). This could be one of the additional roles for the L-tyrosine degradative pathway in fungal pathogenicity, besides the regulation of extracellular levels of tyrosine and phenylalanine (discussed in Chapter 4).

After the marked disruption of plant host cells surrounding *B. dothidea* hyphae infecting apple twigs, the PCW is degraded (Han *et al*., 2016), suggesting that the fungus produces PCWDEs. Several PCWDEs were identified in the transcriptome of *L. theobromae.* The pectate lyase, a PCWDE was up-regulated during the first stages of *L. theobromae* infection in grapevine, and therefore is a promising target to restrict fungal colonization of the plant.

The accumulation of lipid bodies was observed in *B. dothidea* during fruit colonization (Kim *et al*., 2001). These lipid bodies were suggested to serve as reserve storage of lipids for periods of starvation (Kim *et al*., 2004), like those encountered during infection. The same authors mentioned that glyoxysomes enable the fungus to survive nutrient deficiency and host defensive response, through the use of lipids via the glyoxylate cycle until the fungus finds more suitable nutrients. Lipid catabolism is possible through the □-oxidation pathway to produce acetyl-CoA; this could be used to produce ATP through the tricarboxylic acid cycle (TCA) or for anabolic carbohydrate production through the glyoxylate cycle (GC) (Kretschmer *et al*., 2012). In this respect, the genes encoding key enzymes of the GC were identified, including malate synthase, whcih was up regulated under heat stress. This enzyme has been identified as a pathogenicity factor in several microorganisms (Dunn *et al.*, 2009). An in depth study on the function of GC associated genes in pathogenicity would be interesting, since this pathway could be used as a selective target for disease control.

A differential characteristic of fungi growing in wood is their capacity to feed from harsh and complex structures such as PCW (Dashtban *et al*., 2010). A complex mechanism is required to degrade lignocellulose, since it requires first a non-enzymatic stage mediated by low molecular weight compounds (LMWCs) capable of reaching deep targets of the PCW structure (Sigoillot and Berrin, 2012). LMCWs are characterized by the production of hydroxyl radicals through Fenton reaction, which requires hydrogen peroxide and iron (Sigoillot and Berrin, 2012). The enrichment in oxido-reductases with putative extracellular localization in *L. theobromae* in respond to heat stress, suggests that stress could facilitate the first stages of wood degradation, probably by the increase of ROS surrounding PCW, the labilized PCW can then be degraded by PCWDEs.

Chapter 5. General conclusions

- β-tubulin RT-qPCR normalization was useful for *in vitro* and *in planta* gene expression analyses, therefore it is recommended for future *L. theobromae* gene expression analyses. Alternative reference genes were also proposed.
- The identification of *L. theobromae* genes represents a major milestone for future molecular and genetics studies in this fungus contributing to a better understanding of botryosphaeria dieback disease.
- Based on the fungal transcriptional regulation, we propose that the colonization could be promoted by heat stress because of the fungal capacity to degrade phenolics that are produced in the plant as an attempt to contend the fungal infection under abiotic stress.
- The expression of pectate lyase, salicylate hydroxylase and intradiol ring cleavage dioxygenase during fungal infection, support a role in fungal pathogenicity. The design of inhibitory compounds with these enzymes as targets for disruption of fungal establishment would be a promising strategy for the control of botryosphaeria dieback.

Bibliographic references

Adler, D., Nenadic, O., and Zucchini, W. (2003). Rgl: A r-library for 3d visualization with opengl. In *Proceedings of the 35th Symposium of the Interface: Computing Science and Statistics, Salt Lake City* (Vol. 35).

Agrios, G. N. (2005). Plant pathology (5th ed.). Elsevier Academic Press.*Burlington, Ma. USA*.

Albrecht, D., Guthke, R., Brakhage, A. A., and Kniemeyer, O. (2010). Integrative analysis of the heat shock response in Aspergillus fumigatus. *BMC genomics*,*11*(1), 1. doi:10.1186/1471-2164-11-32.

Aldridge, D. C., Galt, S., Giles, D., and Turner, W. B. (1971). Metabolites of Lasiodiplodia theobromae. *Journal of the Chemical Society C: Organic*, 1623-1627.

Allen, C. D., Breshears, D. D., and McDowell, N. G. (2015). On underestimation of global vulnerability to tree mortality and forest die-off from hotter drought in the Anthropocene. *Ecosphere*, *6*(8), 1-55.; doi:10.1890/ES15-00203.1.

Allen, C. D., Macalady, A. K., Chenchouni, H., Bachelet, D., McDowell, N., Vennetier, M., ... and Gonzalez, P. (2010). A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest ecology and management*, *259*(4), 660-684; doi:10.1016/j.foreco.2009.09.001.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403-410; doi:10.1016/S0022-2836(05)80360-2.

Alvarez-Loayza, P., White Jr, J. F., Torres, M. S., Balslev, H., Kristiansen, T., Svenning, J. C., and 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*(1), e16386.; doi:10.1371/journal.pone.0016386.

Alves da Cunha, M. A., Barbosa, A. M., Giese, E. C., and Dekker, R. F. (2003). The effect of carbohydrate carbon sources on the production of constitutive and inducible laccases by Botryosphaeria sp. *Journal of basic microbiology*,*43*(5), 385-392; doi:10.1002/jobm.200310250

Ambrose, K. V., Tian, Z., Wang, Y., Smith, J., Zylstra, G., Huang, B., and Belanger, F. C. (2015). Functional characterization of salicylate hydroxylase from the fungal endophyte Epichloë festucae. *Scientific reports*, *5*; doi:10.1038/srep10939.

Amponsah, N. T., Jones, E. E., Ridgway, H. J., and Jaspers, M. V. (2014). Factors affecting Neofusicoccum luteum infection and disease progression in grapevines. *Australasian Plant Pathology*, *43*(5), 547-556; doi:10.1007/s13313-014-0294-7

Amponsah, N. T., Jones, E. E., Ridgway, H. J., and Jaspers, M. V. (2012). Microscopy of some interactions between Botryosphaeriaceae species and grapevine tissues. *Australasian Plant Pathology*, *41*(6), 665-673; doi:10.1007/s13313-012-0159-x

Andersen, C. L., Jensen, J. L., and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research*, *64*(15), 5245-5250.

Andolfi, A., Mugnai, L., Luque, J., Surico, G., Cimmino, A., and Evidente, A. (2011). Phytotoxins produced by fungi associated with grapevine trunk diseases. *Toxins*, *3*(12), 1569-1605; doi:10.3390/toxins3121569

Andrews, S. (2010). FastQC: A quality control tool for high throughput sequence data. *Reference Source*. Retrieved from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Andrianopoulos, A., Kourambas, S., Sharp, J. A., Davis, M. A., and Hynes, M. J. (1998). Characterization of the Aspergillus nidulans nmrA gene involved in nitrogen metabolite repression. *Journal of bacteriology*, *180*(7), 1973-1977.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., ... and Harris, M. A. (2000). Gene Ontology: tool for the unification of biology.*Nature genetics*, *25*(1), 25-29; doi:10.1038/75556

Ayllon Gutiérrez, R. R. y Hernández Martínez, R. (2011). Producción de enzimas hidrolíticas degradadoras de pared celular vegetal en especies de Botryosphaeria. En XIV Congreso Internacional de Ciencias Agrícolas. 27 de octubre. Mexicali, Baja California, México. Instituto de Ciencias Agrícolas, UABC.

Bahn, Y. S., and Jung, K. W. (2013). Stress signaling pathways for the pathogenicity of Cryptococcus. *Eukaryotic cell*, *12*(12), 1564-1577; doi:10.1128/EC.00218-13.

Barbosa, A. M., Steluti, R. M., Dekker, R. F., Cardoso, M. S., and Da Silva, M. C. (2003). Structural characterization of Botryosphaeran: a (1→ 3; 1→ 6)-β-d-glucan produced by the ascomyceteous fungus, Botryosphaeria sp.*Carbohydrate Research*, *338*(16), 1691-1698; doi:10.1016/S0008-6215(03)00240-4

Benoit, I., Coutinho, P. M., Schols, H. A., Gerlach, J. P., Henrissat, B., and de Vries, R. P. (2012). Degradation of different pectins by fungi: correlations and contrasts between the pectinolytic enzyme sets identified in genomes and the growth on pectins of different origin. *BMC genomics*, *13*(1), 1; doi:10.1186/1471-2164-13-321.

Bertsch, C., Ramírez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., ... and Fontaine, F. (2013). Grapevine trunk diseases: complex and still poorly understood. *Plant Pathology*, *62*(2), 243-265; doi:10.1111/j.1365-3059.2012.02674.x.

Billones-Baaijens, R., Ridgway, H. J., Jones, E. E., and Jaspers, M. V. (2015). Spatial distribution of Neofusicoccum species within a rootstock mother vine indicates potential infection pathways. *European Journal of Plant Pathology*,*141*(2),267-279; doi:10.1007/s10658-014-0540-4

Bioconductor Retrieved from: http://www.bioconductor.org

Blanco-Ulate, B., Rolshausen, P., and Cantu, D. (2013). Draft genome sequence of Neofusicoccum parvum isolate UCR-NP2, a fungal vascular pathogen associated with grapevine cankers. *Genome announcements*, *1*(3), e00339-13; doi:10.1128/genomeA.00339-13

Blanco-Ulate, B., Rolshausen, P. E., and Cantu, D. (2013). Draft genome sequence of the grapevine dieback fungus Eutypa lata UCR-EL1. *Genome announcements*, *1*(3), e00228-13; doi:10.1128/genomeA.00228-13.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, btu170; doi:10.1093/bioinformatics/btu170.

Boyce, K. J., McLauchlan, A., Schreider, L., and Andrianopoulos, A. (2015). Intracellular growth is dependent on tyrosine catabolism in the dimorphic fungal pathogen Penicillium marneffei. *PLoS Pathog*, *11*(3), e1004790; doi:10.1371/journal.ppat.1004790.

Broderick, J. B. (1999). Catechol dioxygenases. *Essays Biochem.; 34*, 173-189.

Broderick, J. B. (1999). Catechol dioxygenases. *Essays in biochemistry*, *34*, 11-11.

Burgess, T. I., Barber, P. A., Mohali, S., Pegg, G., de Beer, W., and Wingfield, M. J. (2006). Three new Lasiodiplodia spp. from the tropics, recognized based on DNA sequence comparisons and morphology. *Mycologia*, *98*(3), 423-435; doi:10.3852/mycologia.98.3.423.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... and Vandesompele, J. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*,*55*(4), 611-622.

Camacho-Sanchez, M., Burraco, P., Gomez-Mestre, I., and Leonard, J. A. (2013). Preservation of RNA and DNA from mammal samples under field conditions. *Molecular ecology resources*, *13*(4), 663-673.

Camps, C., Kappel, C., Lecomte, P., Léon, C., Gomès, E., Coutos-Thévenot, P., and Delrot, S. (2010). A transcriptomic study of grapevine (Vitis vinifera cv. Cabernet-Sauvignon) interaction with the vascular ascomycete fungus Eutypa lata. *Journal of experimental botany*, erq040; doi:10.1093/jxb/erq040

Candolfi-Arballo, O. (2009). Caracterización de anamorfos de botryosphaeriaceae asociados a deterioro progresivo en vid (*Vitis vinífera* L.) aislados de la región vitivinícola de Baja California. Tésis de Maestria. CICESE, Ensenada, Mexico.

Chiang, Y. M., Meyer, K. M., Praseuth, M., Baker, S. E., Bruno, K. S., and Wang, C. C. (2011). Characterization of a polyketide synthase in Aspergillus niger whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-γ-pyrone. *Fungal Genetics and Biology*, *48*(4), 430-437; doi:10.1016/j.fgb.2010.12.001.

Choi, Y. E., and Goodwin, S. B. (2011). MVE1, encoding the velvet gene product homolog in Mycosphaerella graminicola, is associated with aerial mycelium formation, melanin biosynthesis, hyphal swelling, and light signaling. *Applied and environmental microbiology*, *77*(3), 942-953;77:942-953.

Chong, J., Poutaraud, A., and Hugueney, P. (2009). Metabolism and roles of stilbenes in plants. *Plant Science*, *177*(3), 143–155. doi:10.1016/j.plantsci.2009.05.012.

Cobos, R., Barreiro, C., Mateos, R. M., and Coque, J. J. R. (2010). Cytoplasmic- and extracellular-proteome analysis of Diplodia seriata: a phytopathogenic fungus involved in grapevine decline. *Proteome Science*, *8*, 46. doi:10.1186/1477-5956-8-46.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics (Oxford, England), 21*(18), 3674–6. doi:10.1093/bioinformatics/bti610.

Dashtban, M., Schraft, H., Syed, T. A., and Qin, W. (2010). Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology, 1*(1), 36-50.

Davidson, J. F., and Schiestl, R. H. (2001). Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *21*(24), 8483–9. doi:10.1128/MCB.21.24.8483-8489.2001.

Dekker, R. F. H., Barbosa, A. M., Giese, E. C., Godoy, S. D. S., and Covizzi, L. G. (2007). Influence of nutrients on enhancing laccase production by Botryosphaeria rhodina MAMB-05. *International Microbiology : The Official Journal of the Spanish Society for Microbiology*, *10*, 177–185. doi:10.2436/20.1501.01.25.

Deo, A., Carlsson, J., and Lindlöf, A. (2011). How to choose a normalization strategy for mirna quantitative real-time (qPCR) arrays. *Journal of Bioinformatics and Computational Biology*, *09*(06), 795–812. doi:10.1142/S0219720011005793.

Desprez-Loustau, M. (2006). Interactive effects of drought and pathogens in forest trees. *Annals of Forest Sciences*, *63*, 597–612. doi:10.1051/forest :2006044.

Dickman, MB and Patil, S. (1986). A rapid and sensitive plate assay for the detection of cutinase production by plant pathogenic fungi. *Phytopathology*, *76*(5), 473–475.

DiGuistini, S., Wang, Y., Liao, N. Y., Taylor, G., Tanguay, P., Feau, N., … and Breuil, C. (2011). Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont Grosmannia clavigera, a lodgepole pine pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(6), 2504–9. doi:10.1073/pnas.1011289108.

Dunn, M. F., Ramírez-Trujillo, J. A., and Hernández-Lucas, I. (2009). Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology (Reading, England)*, *155*(Pt 10), 3166–75. doi:10.1099/mic.0.030858-0.

Dvinge, H., and Bertone, P. (2009). HTqPCR: high-throughput analysis and visualization of quantitative real-time PCR data in R. *Bioinformatics (Oxford, England), 25*(24), 3325–6. doi:10.1093/bioinformatics/btp578.

Eisenman, H. C., Mues, M., Weber, S. E., Frases, S., Chaskes, S., Gerfen, G., and Casadevall, A. (2007). Cryptococcus neoformans laccase catalyses melanin synthesis from both D- and L-DOPA. *Microbiology (Reading, England)*, *153*(Pt 12), 3954–62. doi:10.1099/mic.0.2007/011049-0.

Eklöf, J. M., and Brumer, H. (2010). The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiology*, *153*(2), 456–66. doi:10.1104/pp.110.156844.

Encinas, O., and Daniel, G. (1995). Wood cell wall biodegradation by the blue stain fungus Botryodiplodia theobromae Pat. *Material Und Organismen*, *29*(4), 255–272.

Epstein, L., and Kaur, S. (2008). Botryosphaeria-related dieback and control investigated in noncoastal California grapevines. *California Agriculture, 62*(4), 161–166.

Evidente, A., Punzo, B., Andolfi, A., Cimmino, A., Melck, D., and Luque, J. (2010). Lipophilic phytotoxins produced by Neofusicoccum parvum , a grapevine canker agent. *Phytopathologia Mediterranea, 49*(1), 74-79. doi:10.14601/Phytopathol_Mediterr-5433.

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., …, and Bateman, A. (2010). The Pfam protein families database. *Nucleic Acids Research, 38*, 211–222. doi:10.1093/nar/gkp985.

Fraser, C. M., and Chapple, C. (2011). The phenylpropanoid pathway in Arabidopsis. *The Arabidopsis Book, 9*, e0152. doi:10.1199/tab.0152

Frases, S., Salazar, A., Dadachova, E., and Casadevall, A. (2007). Cryptococcus neoformans can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. *Applied and Environmental Microbiology, 73*(2), 615–21. doi:10.1128/AEM.01947-06

Fussler, L., Kobes, N., Bertrand, F., Maumy, M., Grosman, J., and Savary, S. (2008). A characterization of grapevine trunk diseases in France from data generated by the National Grapevine Wood Diseases Survey. *Phytopathology, 98*(5), 571–9. doi:10.1094/PHYTO-98-5-0571.

Gambino, G., Perrone, I., and Gribaudo, I. (2008). A Rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis : PCA, 19*(6), 520–5. doi:10.1002/pca.1078.

Gardefa, U. M. (2015). Effect of seed-borne fungi associated with seeds of silver oak (*Grevillea robusta*) on seedling growth and development of dieback and stem canker in Southeastern Ethiopia. Thesis of Master of Science in Agriculture at the Haramaya University, Ethiopia, 70 p.

Giese, H., Sondergaard, T. E., and Sørensen, J. L. (2013). The AreA transcription factor in Fusarium graminearum regulates the use of some nonpreferred nitrogen sources and secondary metabolite production. *Fungal Biology*, *117*(11-12), 814–21. doi:10.1016/j.funbio.2013.10.006

Gotz, S., Garcia-Gomez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., …, and Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*(10), 3420–3435. doi:10.1093/nar/gkn176

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., …, and Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–52. doi:10.1038/nbt.1883

Greene, G. H., McGary, K. L., Rokas, A., and Slot, J. C. (2014). Ecology drives the distribution of specialized tyrosine metabolism modules in fungi. *Genome Biology and Evolution, 6*(1), 121–32. doi:10.1093/gbe/evt208

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., …, and Regev, A. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols*, *8*(8), 1494–512. doi:10.1038/nprot.2013.084.

Hammerbacher, A., Schmidt, A., Wadke, N., Wright, L. P., Schneider, B., Bohlmann, J., …, and Paetz, C. (2013). A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Physiology*, *162*(3), 1324–36. doi:10.1104/pp.113.218610

Han, C., Ren, C., Zhi, T., Zhou, Z., Liu, Y., Chen, F., …, and Xie, D. (2013). Disruption of fumarylacetoacetate hydrolase causes spontaneous cell death under short-day conditions in Arabidopsis. *Plant Physiology*, *162*(4), 1956–64. doi:10.1104/pp.113.216804.

Han, Q., Gao, X., Wang, J., Wang, H., and Huang, L. (2016). Cytological and histological studies of the interaction between Botryosphaeria dothidea and apple twigs. *Scientia Horticulturae*, *202*, 142–149. doi:10.1016/j.scienta.2016.03.002.

Hawkins, S., and Boudet, A. (1996). Wound-induced lignin and suberin deposition in a woody angiosperm (Eucalyptus gunnii Hook.): Histochemistry of early changes in young plants. *Protoplasma*, *191*(1-2), 96–104. doi:10.1007/BF01280829.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology*, *8*(2), R19. doi:10.1186/gb-2007-8-2-r19.

Hwang, L., Hocking-Murray, D., Bahrami, A. K., Andersson, M., Rine, J., and Sil, A. (2003). Identifying phase-specific genes in the fungal pathogen Histoplasma capsulatum using a genomic shotgun microarray. *Molecular Biology of the Cell*, *14*(6), 2314–26. doi:10.1091/mbc.E03-01-0027.

Islam, M. S., Haque, M. S., Islam, M. M., Emdad, E. M., Halim, A., Hossen, Q. M. M., … Alam, M. (2012). Tools to kill: Genome of one of the most destructive plant pathogenic fungi Macrophomina phaseolina. *BMC Genomics*, *13*(1), 493. doi:10.1186/1471-2164-13-493.

Jack, D. L., Paulsen, I. T., and Saier, M. H. (2000). The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. *Microbiology (Reading, England)*, *146* (8), 1797–814. doi:10.1099/00221287-146-8-1797.

Jacobson, E. S. (2000). Pathogenic roles for fungal melanins. *Clinical Microbiology Reviews*, *13*(4), 708–17.

Johnson, G. I., Mead, a. J., Cooke, a. W., and Dean, J. R. (1992). Mango stem end rot pathogens - Fruit infection by endophytic colonisation of the inflorescence and pedicel. *Annals of Applied Biology*, *120*(2), 225–234. doi:10.1111/j.1744-7348.1992.tb03420.x

Kabbage, M., Yarden, O., and Dickman, M. B. (2015). Pathogenic attributes of Sclerotinia sclerotiorum: Switching from a biotrophic to necrotrophic lifestyle. *Plant Science*, *233*, 53–60. doi:10.1016/j.plantsci.2014.12.018

Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., and Minami, E. (2012). Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PloS One*, *7*(11), e49423. doi:10.1371/journal.pone.0049423

Ke, X., Yin, Z., Song, N., Dai, Q., Voegele, R. T., Liu, Y., …, and Huang, L. (2014). Transcriptome profiling to identify genes involved in pathogenicity of Valsa mali on apple tree. Fungal Genetics and Biology : FG and B. doi:10.1016/j.fgb.2014.04.004

Keller, M. (2010). The science of grapevines: anatomy and physiology. Elsevier, San Diego, CA.

Keller, S., Macheleidt, J., Scherlach, K., Schmaler-Ripcke, J., Jacobsen, I. D., Heinekamp, T., and Brakhage, A. A. (2011). Pyomelanin formation in *Aspergillus fumigatus* requires *hmgX* and the transcriptional activator *hmgR* but is dispensable for virulence. *PLoS ONE*, *6*(10), e26604. doi:10.1371/journal.pone.0026604

Kim, K. W., Park, E. W., and Ahn, K. (1999). Pre-penetration behavior of *Botryosphaeria dothidea* on apple fruits. *Plant Pathology*, *15*(4), 223–227.

Kim, K. W., Park, E. W., and Kim, K. S. (2004). Glyoxysomal nature of microbodies complexed with lipid globules in *Botryosphaeria dothidea*. *Phytopathology*, *94*(9), 970–7. doi:10.1094/PHYTO.2004.94.9.970

Kim, K. W., Park, E. W., Kim, Y. H., Ahn, K. K., Kim, P. G., and Kim, K. S. (2001 Latency- and defense-related ultrastructural characteristics of apple fruit tissuesi with *Botryosphaeria dothidea*. *Phytopathology*, *91*(2), 165–72. doi:10.1094/PHYTO.2001.91.2.165

Kim, S., Park, J., Park, S.-Y., Mitchell, T. K., and Lee, Y.-H. (2010). Identification and analysis of in planta expressed genes of Magnaporthe oryzae. *BMC Genomics*, *11*(1), 104. doi:10.1186/1471-2164-11-104

Krabel, D., Morgenstern, K., and Herzog, S. (2013). Endophytes in changing environments - do we need new concepts in forest management? *iForest - Biogeosciences and Forestry*, *6*(2), 109–112. doi:10.3832/ifor0932-006.

Kretschmer, M., Wang, J., and Kronstad, J. W. (2012). Peroxisomal and mitochondrial ??-oxidation pathways influence the virulence of the pathogenic fungus Cryptococcus neoformans. *Eukaryotic Cell*, *11*(8), 1042–1054. doi:10.1128/EC.00128-12

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology*, *305*(3), 567–80. doi:10.1006/jmbi.2000.4315.

Kües, U., and Rühl, M. (2011). Multiple multi-copper oxidase gene families in basidiomycetes - what for? *Current Genomics*, *12*(2), 72–94. doi:10.2174/138920211795564377.

Lambert, C., Bisson, J., Waffo-Téguo, P., Papastamoulis, Y., Richard, T., Corio-Costet, M. F., …, and Cluzet, S. (2012). Phenolics and their antifungal role in grapevine wood decay: Focus on the Botryosphaeriaceae family. *Journal of Agricultural and Food Chemistry*, *60*(48), 11859–11868. doi:10.1021/jf303290g.

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–9. doi:10.1038/nmeth.1923.

Leatham, G. F., Crawford, R. L., and Kirk, T. K. (1983). Degradation of phenolic compounds and ring cleavage of catechol by Phanerochaete chrysosporium. *Applied and Environmental Microbiology*, *46*(1), 191–7.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., …, and Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)*, *25*(16), 2078–9. doi:10.1093/bioinformatics/btp352

Li, J., and Zhang, K. Q. (2014). Independent expansion of zincin metalloproteinases in Onygenales fungi may be associated with their pathogenicity. *PloS One*, *9*(2), e90225. doi:10.1371/journal.pone.0090225.

Lima, T., Auchincloss, A. H., Coudert, E., Keller, G., Michoud, K., Rivoire, C., … Bairoch, A. (2009). HAMAP: a database of completely sequenced microbial proteome sets and manually curated microbial protein families in UniProtKB/Swiss-Prot. *Nucleic Acids Research*, *37*(Database issue), D471–8. doi:10.1093/nar/gkn661

Liu, D., Wei, L., Guo, T., and Tan, W. (2014). Detection of DOPA-melanin in the dimorphic fungal pathogen Penicillium marneffei and its effect on macrophage phagocytosis in vitro. *PloS One*, *9*(3), e92610. doi:10.1371/journal.pone.0092610.

Llanos, A., François, J. M., and Parrou, J.-L. (2015). Tracking the best reference genes for RT-qPCR data normalization in filamentous fungi. *BMC Genomics*, *16*(1), 71. doi:10.1186/s12864-015-1224-y.

Lorenz, M. C., and Fink, G. R. (2001). The glyoxylate cycle is required for fungal virulence. *Nature*, *412*(6842), 83–6. doi:10.1038/35083594

Ma, Z., Morgan, D. P., Michailides, T. J., Pathology, P., and Avenue, S. R. (2001). Effects of water stress on Botryosphaeria blight of pistachio caused by Botryosphaeria dothidea. *Plant Disease*, *85*, 745–749.

MacDonald, J., Doering, M., Canam, T., Gong, Y., Guttman, D. S., Campbell, M. M., and Master, E. R. (2011). Transcriptomic responses of the softwood-degrading white-rot fungus Phanerochaete carnosa during growth on coniferous and deciduous wood. *Applied and Environmental Microbiology*, *77*(10), 3211–8. doi:10.1128/AEM.02490-10

Mahoney, N., Lardner, R., Molyneux, R. J., Scott, E. S., Smith, L. R., and Schoch, T. K. (2003). Phenolic and heterocyclic metabolite profiles of the grapevine pathogen Eutypa lata. *Phytochemistry*, *64*(2), 475–84.

Makatini, G. J. (2014). The role of sucker wounds as portals for grapevine trunk pathogen infections. Thesis of Master of Science Agriculture in Plant Pathology at the University of Stellenbosch, South Africa, 121 p.

Mar, J. C., Kimura, Y., Schroder, K., Irvine, K. M., Hayashizaki, Y., Suzuki, H., …, and Quackenbush, J. (2009). Data-driven normalization strategies for high-throughput quantitative RT-PCR. *BMC Bioinformatics*, *10*(1), 110. doi:10.1186/1471-2105-10-110

Marshall, O. J. (2004). PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics (Oxford, England)*, *20*(15), 2471–2. doi:10.1093/bioinformatics/bth254

Martos, S., Andolfi, A., Luque, J., Mugnai, L., Surico, G., and Evidente, A. (2008). Production of phytotoxic metabolites by five species of Botryosphaeriaceae causing decline on grapevines, with special interest in the species Neofusicoccum luteum and N. parvum. *European Journal of Plant Pathology*, *121*(4), 451–461. doi:10.1007/s10658-007-9263-0.

Mathioni, S. M., Beló, A., Rizzo, C. J., Dean, R. A., and Donofrio, N. M. (2011). Transcriptome profiling of the rice blast fungus during invasive plant infection and in vitro stresses. *BMC Genomics*, *12*(1), 49. doi:10.1186/1471-2164-12-49.

Meinhardt, L. W., Costa, G. G. L., Thomazella, D. P. T., Teixeira, P. J. P. L., Carazzolle, M. F., Schuster, S. C., …, and Bailey, B. A. (2014). Genome and secretome analysis of the hemibiotrophic fungal pathogen, Moniliophthora roreri, which causes frosty pod rot disease of cacao: mechanisms of the biotrophic and necrotrophic phases. *BMC Genomics*, *15*(1), 164. doi:10.1186/1471-2164-15-164.

Mondego, J. M. C., Carazzolle, M. F., Costa, G. G. L., Formighieri, E. F., Parizzi, L. P., Rincones, J., …, and Pereira, G. A. G. (2008). A genome survey of Moniliophthora perniciosa gives new insights into Witches' Broom Disease of cacao. *BMC Genomics*, *9*(1), 548. doi:10.1186/1471-2164-9-548

Morais do Amaral, A., Antoniw, J., Rudd, J. J., and Hammond-Kosack, K. E. (2012). Defining the Predicted Protein Secretome of the Fungal Wheat Leaf Pathogen Mycosphaerella graminicola. *PLoS ONE*, *7*(12), e49904. doi:10.1371/journal.pone.0049904

Morales-Cruz, A., Amrine, K. C. H., Blanco-Ulate, B., Lawrence, D. P., Travadon, R., Rolshausen, P. E., …, and Cantu, D. (2015). Distinctive expansion of gene families associated with plant cell wall degradation, secondary metabolism, and nutrient uptake in the genomes of grapevine trunk pathogens. *BMC Genomics*, *16*(1), 469. doi:10.1186/s12864-015-1624-z

Morano, K. A., Grant, C. M., and Moye-rowley, W. S. (2012). The response to heat shock and oxidative stress in Saccharomyces cerevisiae. *Genetics*, *190*, 1157–1195. doi:10.1534/genetics.111.128033

Moricca, S., and Ragazzi, A. (2008). Fungal endophytes in Mediterranean oak forests: a lesson from Discula quercina. *Phytopathology*, *98*(4), 381–386.

Mosquera, G., Giraldo, M. C., Khang, C. H., Coughlan, S., and Valent, B. (2009). Interaction transcriptome analysis identifies Magnaporthe oryzae BAS1-4 as Biotrophy-associated secreted proteins in rice blast disease. *The Plant Cell*, *21*(4), 1273–90. doi:10.1105/tpc.107.055228.

Müllen, J. M., Gilliam, C. H., Hagen, A. K., and Morgan Jones, G. (1991). Canker of dogwood caused by Lasiodiplodia theobromae, a disease influenced by drought stress or cultivar selection. *Plant* Disease, 75, 886-889.

Mundy, D., and Manning, M. (2011). Physiological response of grapevines to vascular pathogens: a review. *New Zealand Plant Protection*, *16*, 7–16.

Muniz, C. R., Freire, F. C. O., Viana, F. M. P., Cardoso, J. E., Cooke, P., Wood, D., and Guedes, M. I. F. (2011). Colonization of cashew plants by Lasiodiplodia theobromae: microscopical features. *Micron (Oxford, England : 1993), 42*(5), 419–28. doi:10.1016/j.micron.2010.12.003

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, *8*(19), 4321–5.

Nunes, L. R., Costa de Oliveira, R., Leite, D. B., da Silva, V. S., dos Reis Marques, E., da Silva Ferreira, M. E., …, and Goldman, G. H. (2005). Transcriptome analysis of Paracoccidioides brasiliensis cells undergoing mycelium-to-yeast transition. *Eukaryotic Cell*, *4*(12), 2115–28. doi:10.1128/EC.4.12.2115-2128.2005

O'Neil, S. T., and Emrich, S. J. (2013). Assessing De Novo transcriptome assembly metrics for consistency and utility. *BMC Genomics*, *14*(1), 465. doi:10.1186/1471-2164-14-465

Obrador-Sanchez, J. A. (2011). Comportamiento de *Botryosphaeria* spp. en presencia de madera de vid: Inicio de la colonización y características microscópicas. Tesis de Maestría. CICESE. 125 p.

Octave, S., Roblin, G., Vachaud, M., and Fleurat-Lessard, P. (2006). Polypeptide metabolites secreted by the fungal pathogen Eutypa lata participate in Vitis vinifera cell structure damage observed in Eutypa dieback. *Functional Plant Biology*, *33*(3), 297. doi:10.1071/FP05230

Oliva, J., Stenlid, J., and Martínez-Vilalta, J. (2014). The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought-induced mortality. *New Phytologist*, *203*(4), 1028–1035. doi:10.1111/nph.12857.

Pagnussatt, F. A., Kupski, L., Darley, F. T., Filoda, P. F., Ponte, É. M. Del, Garda-Buffon, J., and Badiale-Furlong, E. (2013). Fusarium graminearum growth inhibition mechanism using phenolic compounds from Spirulina sp. *Ciência E Tecnologia de Alimentos*, *33*, 75–80. doi:10.1590/S0101-20612013000500012

Pao, S. S., Paulsen, I. T., and Saier, M. H. (1998). Major facilitator superfamily. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(1), 1–34.

Paolinelli-Alfonso, M., Galindo-Sánchez, C. E., and Hernandez-Martinez, R. (2016). Quantitative real-time PCR normalization for gene expression studies in the plant pathogenic fungi Lasiodiplodia theobromae. *Journal of Microbiological Methods*, *127*, 82–88. doi:10.1016/j.mimet.2016.05.021.

Pasricha, S., Payne, M., Canovas, D., Pase, L., Ngaosuwankul, N., Beard, S., …, and Andrianopoulos, A. (2013). Cell-type-specific transcriptional profiles of the dimorphic pathogen Penicillium marneffei reflect distinct reproductive, morphological, and environmental demands. *G3 (Bethesda, Md.)*, *3*(11), 1997–2014. doi:10.1534/g3.113.006809

Pearson, K. (1895) Notes on regression and inheritance in the case of two parents. Proc R Soc Lond.;58,240-242.

Perpetua, N. S., Kubo, Y., Yasuda, N., Takano, Y., and Furusawa, I. (1996). Cloning and characterization of a melanin biosynthetic THR1 reductase gene essential for appressorial penetration of Colletotrichum lagenarium. *Molecular Plant-Microbe Interactions : MPMI*, *9*(5), 323–9.

Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, *8*(10), 785–6. doi:10.1038/nmeth.1701

Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters*, *26*(6), 509–15.

Phillips, A. J. L., Slippers, B., Groenewald, J. Z., and Crous, P. W. (2013). Plant pathogenic and endophytic Botryosphaeriales known from culture. *Studies in Mycology, 76*, 167p.

Pócsi, I., Prade, R. A., and Penninckx, M. J. (2004). Glutathione, altruistic metabolite in fungi. *Advances in Microbial Physiology*, *49*, 1–76. doi:10.1016/S0065-2911(04)49001-8

Pratt, C. (1974). Vegetative Anatomy of Cultivated Grapes--A Review. *Am. J. Enol. Vitic.*, *25*(3), 131–150.

Punithalingam, E. 1976. Botryodiplodia theobromae. *CMI Descriptions of Pathogenic Fungi and Bacteria. 519*:1-2.

R Development Core Team (2008). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Rabe, F., Ajami-Rashidi, Z., Doehlemann, G., Kahmann, R., and Djamei, A. (2013). Degradation of the plant defence hormone salicylic acid by the biotrophic fungus Ustilago maydis. *Molecular Microbiology*, *89*(1), 179–88. doi:10.1111/mmi.12269

Ramírez-Suero, M., Bénard-Gellon, M., Chong, J., Laloue, H., Stempien, E., Abou-Mansour, E., …, and Bertsch, C. (2014). Extracellular compounds produced by fungi associated with Botryosphaeria dieback induce differential defence gene expression patterns and necrosis in Vitis vinifera cv. Chardonnay cells. *Protoplasma*. doi:10.1007/s00709-014-0643-y

Reid, K. E., Olsson, N., Schlosser, J., Peng, F., and Lund, S. T. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology*, *6*, 27. doi:10.1186/1471-2229-6-27

Robinson, M., McCarthy, D., Chen, Y, Smyth, G. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics, 26*(1), 139-140.

Rolshausen, P. E., Greve, L. C., Labavitch, J. M., Mahoney, N. E., Molyneux, R. J., and Gubler, W. D. (2008). Pathogenesis of Eutypa lata in grapevine: identification of virulence factors and biochemical characterization of cordon dieback. *Phytopathology*, *98*(2), 222–9. doi:10.1094/PHYTO-98-2-0222

Rolshausen, P. E., Mahoney, N. E., Molyneux, R. J., and Gubler, W. D. (2006). A Reassessment of the Species Concept in Eutypa lata, the Causal Agent of Eutypa Dieback of Grapevine. *Phytopathology*, *96*(4), 369–77. doi:10.1094/PHYTO-96-0369

Rolshausen, P. E., Urbez-Torres, J. R., Rooney-Latham, S., Eskalen, A., Smith, R. J., and Gubler, W. D. (2010). Evaluation of Pruning Wound Susceptibility and Protection Against Fungi Associated with Grapevine Trunk Diseases. *Am. J. Enol. Vitic.*, *61*(1), 113–119.

Rovenich, H., Boshoven, J. C., and Thomma, B. P. H. J. (2014). Filamentous pathogen effector functions: of pathogens, hosts and microbiomes. *Current Opinion in Plant Biology*, *20*, 96–103. doi:10.1016/j.pbi.2014.05.001

Rudrappa, T., Quinn, W. J., Stanley-Wall, N. R., and Bais, H. P. (2007). A degradation product of the salicylic acid pathway triggers oxidative stress resulting in down-regulation of Bacillus subtilis biofilm formation on Arabidopsis thaliana roots. *Planta*, *226*(2), 283–97. doi:10.1007/s00425-007-0480-8

Sakalidis, M. L., Hardy, G. E. S. J., and Burgess, T. I. (2011). Endophytes as potential pathogens of the baobab species Adansonia gregorii : A focus on the Botryosphaeriaceae. *Fungal Ecology*, *4*, 1–14. doi:10.1016/j.funeco.2010.06.001

Salazar, H. M., and Melgarejo, M. P. (2005). Viticultura, técnicas de cultivo de la vid, calidad de la uva y atributos de los vinos. Editorial mundi-prensa.*Madrid. España*.

Saldanha, R. L., Garcia, J. E., Dekker, R. F., Vilas-Boas, L. A., and Barbosa, A. M. (2007). Genetic diversity among Botryosphaeria isolates and their correlation with cell wall-lytic enzyme production. *Brazilian Journal of Microbiology*, *38*(2), 259-264.

Schmaler-Ripcke, J., Sugareva, V., Gebhardt, P., Winkler, R., Kniemeyer, O., Heinekamp, T., and Brakhage, A. A. (2009). Production of pyomelanin, a second type of melanin, via the tyrosine degradation pathway in Aspergillus fumigatus. *Applied and Environmental Microbiology*, *75*(2), 493–503. doi:10.1128/AEM.02077-08

Schönig, B., Brown, D. W., Oeser, B., and Tudzynski, B. (2008). Cross-species hybridization with Fusarium verticillioides microarrays reveals new insights into Fusarium fujikuroi nitrogen regulation and the role of AreA and NMR. *Eukaryotic Cell*, *7*(10), 1831–1846. doi:10.1128/EC.00130-08

Shapiro, S. S., and Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*(3), 591–611.

SIAP (2014). Información de las Delegaciones de la SAGARPA. Servicio de Información Agroalimentaria y Pesquera, Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación. México. Recuperado el 27 de febrero de 2016 de: www.siap.gob.mx

Sieber, T. N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biology Reviews*, *21*(2-3), 75–89. doi:10.1016/j.fbr.2007.05.004

Sigoillot, J., and Berrin, J. (2012). Fungal strategies for lignin degradation. *Advances in Botanical Research*, *61*, 263–308. doi:10.1016/B978-0-12-416023-1.00008-2

Singh, B., and Sharma, R. A. (2015). Plant terpenes: defense responses, phylogenetic analysis, regulation and clinical applications. *Biotech*, *5*(2), 129–151. doi:10.1007/s13205-014-0220-2

Singh, K., Nizam, S., Sinha, M., and Verma, P. K. (2012). Comparative transcriptome analysis of the necrotrophic fungus Ascochyta rabiei during oxidative stress: insight for fungal survival in the host plant. *PloS One*, *7*(3), e33128. doi:10.1371/journal.pone.0033128.

Singh, L. P., Gill, S. S., and Tuteja, N. (2011). Unraveling the role of fungal symbionts in plant abiotic stress tolerance. *Plant Signaling and Behavior*, *6*(2), 175–91.

Slippers, B., and Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews*, *21*(2-3), 90–106. doi:10.1016/j.fbr.2007.06.002.

Smith, H., Wingfield, M. J., and Petrini, O. (1996). Botryosphaeria dothidea endophytic in Eucalyptus grandis and Eucalyptus nitens in South Africa. *Forest Ecology and Management*, *89*(1-3), 189–195. doi:10.1016/S0378-1127(96)03847-9

Song, M.-H., Lee, J.-W., Kim, M. S., Yoon, J.-K., White, T. C., Floyd, A., …, and Bahn, Y.-S. (2012). A flucytosine-responsive Mbp1/Swi4-like protein, Mbs1, plays pleiotropic roles in antifungal drug resistance, stress response, and virulence of Cryptococcus neoformans. *Eukaryotic Cell*, *11*(1), 53–67. doi:10.1128/EC.05236-11.

Spagnolo, A., Magnin-robert, M., Alayi, T. D., Cilindre, C., Schaeffer-reiss, C., Dorsselaer, A. Van, …, and Fontaine, F. (2014). Differential responses of three grapevine cultivars to Botryosphaeria dieback. *Phytopathology*, *104*(10), 1–48. doi:10.1094/PHYTO-01-14-0007-R.

Tabacchi, R., Fkyerat, A., Poliart, C., and Dubin, G. M. (2000). Phytotoxins from fungi of esca grapevine [Vitis vinifera L.]. In *First International workshop on grapevine trunk diseases - esca and grapevine declines, Siena (Italy), 1-3 Oct 1999*.

Tanaka, A., Cartwright, G. M., Saikia, S., Kayano, Y., Takemoto, D., Kato, M., …, and Scott, B. (2013). ProA, a transcriptional regulator of fungal fruiting body development, regulates leaf hyphal network development in the Epichloë festucae-Lolium perenne symbiosis. *Molecular Microbiology*, *90*(3), 551–568. doi:10.1111/mmi.12385

Taylor, A., Hardy, G. S. J., Wood, P., and Burgess, T. (2005). Identification and pathogenicity of Botiyosphaeria species associated with grapevine decline in Western Australia. *Australasian Plant Pathology*, *34*(2), 187-195.

Teskey, R., Wertin, T., Bauweraerts, I., Ameye, M., McGuire, M. A., and Steppe, K. (2014). Responses of tree species to heat waves and extreme heat events. *Plant, Cell and Environment*. doi:10.1111/pce.12417

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., ... and Heinen, E. (1999). Housekeeping genes as internal standards: use and limits.*Journal of biotechnology*, *75*(2), 291-295.

Timpner, C., Braus-Stromeyer, S. a, Tran, V. T., and Braus, G. H. (2013). The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. *Molecular Plant-Microbe Interactions : MPMI*, *26*(11), 1312–1324. doi:10.1094/MPMI-06-13-0181-R

Tudzynski, P., and Sharon, A. (2002). Fungal pathogenicity genes. *Molecular Plant Pathology*, *12*(5), 507–514. doi:10.1111/j.1364-3703.2010.00680.x

UC Pest Management guidelines. Retrieved from http://www.ipm.ucdavis.edu/PMG/r302100611.html.

Umezurike, G. M. (1979). The cellulolytic enzymes of Botryodiplodia theobromae Pat. Separation and characterization of cellulases and β-glucosidases. *Biochemical Journal*, *177*(1), 9-19.

Udenfriend, S., and Cooper, J. R. (1952). The enzymatic conversion of phenylalanine to tyrosine. *Journal of Biological Chemistry*, *194*(2), 503-511.

Upadhyay, S., Torres, G., and Lin, X. (2013). Laccases involved in 1,8-dihydroxynaphthalene melanin biosynthesis in Aspergillus fumigatus are regulated by developmental factors and copper homeostasis. *Eukaryotic Cell*, *12*(12), 1641–1652. doi:10.1128/EC.00217-13

Úrbez-Torres, J. R., Adams, P., Kamas, J., and Gubler, W. D. (2009). Identification, incidence, and pathogenicity of fungal species associated with grapevine dieback in Texas. *American Journal of Enology and Viticulture*, *60*(4), 497-507.

Úrbez-Torres, J. R., Leavitt, G. M., Guerrero, J. C., Guevara, J., and Gubler, W. D. (2008). Identification and pathogenicity of Lasiodiplodia theobromae and Diplodia seriata, the causal agents of Bot Canker disease of grapevines in Mexico. *Plant Disease*, *92*(4), 519–529. doi:10.1094/PDIS-92-4-0519

Úrbez-Torres, J. R., Leavitt, G. M., Voegel, T. M., and Gubler, W. D. (2006). Identification and distribution of *Botryosphaeria* spp. associated with grapevine cankers in California. *Plant Disease*, *90*(12), 1490–1503. doi:10.1094/PD-90-1490

Urbez-Torres, J. R. (2011). The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea*, *50*(4), 5-45.

Van Niekerk, J. M., Crous, P. W., Groenewald, J. Z. E., Fourie, P. H., and Halleen, F. (2004). DNA phylogeny, morphology and pathogenicity of Botryosphaeria species on grapevines. *Mycologia*, *96*(4), 781–98.

Van Niekerk, J.M., Fourie, P., Halleen, F., Crous, P. W. (2006). Botryosphaeria spp. as grapevine trunk disease pathogens. *Phytopathologia Mediterranea*, *45*(4), 43–54.

Van Niekerk, J., Strever, A. E., Du Toit, G. P., Halleen, F., and Fourie, P. H. (2011). Influence of water stress on Botryosphaeriaceae disease expression in grapevines. *Phytopathologia Mediterranea*, *50*(4), 151-165.

Van Wees, S., and Glazebrook, J. (2003). Loss of non-host resistance of Arabidopsis NahG to Pseudomonas syringae pv. phaseolicola is due to degradation products of salicylic acid. *The Plant Journal*, *33*(4), 733-742.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), research0034.1–research0034.11. doi:10.1186/gb-2002-3-7-research0034

Vandesompele, J. (2009). qPCR guide. Retrieved 5 Feb 2016 from http://www. Eurogentec. com/uploads/qPCR-Guide.pdf.

Vasanthaiah, H. K. N., Katam, R., and Sheikh, M. B. (2008). Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies. *Electronic Journal of Biotechnology*, *11*(3), 1-9. doi:10.2225/vol11-issue3-fulltext-5

Vasanthakumar, A., DeAraujo, A., Mazurek, J., Schilling, M., and Mitchell, R. (2015). Pyomelanin production in Penicillium chrysogenum is stimulated by L-tyrosine. *Microbiology (Reading, England)*, *161*(6), 1211–1218. doi:10.1099/mic.0.000030

Vastermark, A., Wollwage, S., Houle, M. E., Rio, R., and Saier, M. H. (2014). Expansion of the APC superfamily of secondary carriers. *Proteins*, *82*(10), 2797–2811. doi:10.1002/prot.24643

Verghese, J., Abrams, J., Wang, Y., and Morano, K. A. (2012). Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiology and Molecular Biology Reviews : MMBR*, *76*(2), 115–158. doi:10.1128/MMBR.05018-11

Vieira, A., Talhinhas, P., Loureiro, A., Duplessis, S., Fernandez, D., Silva, M. do C., …, and Azinheira, H. G. (2011). Validation of RT-qPCR reference genes for in planta expression studies in Hemileia vastatrix, the causal agent of coffee leaf rust. *Fungal Biology*, *115*(9), 891–901. doi:10.1016/j.funbio.2011.07.002

Vogel, H. J. (1956). A convenient growth medium for *Neurospora crassa*. *Microbial Genetics Bulletin,13*, 42–43.

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Molecular Plant*, *3*(1), 2–20. doi:10.1093/mp/ssp106

Waldboth, M., and Oberhuber, W. (2009). Synergistic effect of drought and chestnut blight (Cryphonectria parasitica) on growth decline of European chestnut (Castanea sativa). *Forest Pathology*, *39*(1), 43–55. doi:10.1111/j.1439-0329.2008.00562.x

Walter, W., Sánchez-Cabo, F., and Ricote, M. (2015). GOplot: an R package for visually combining expression data with functional analysis. *Bioinformatics (Oxford, England)*, *31*(17), 2912–2914. doi:10.1093/bioinformatics/btv300

Wang, L.-J., Fan, L., Loescher, W., Duan, W., Liu, G.-J., Cheng, J.-S., …, and Li, S.-H. (2010). Salicylic acid alleviates decreases in photosynthesis under heat stress and accelerates recovery in grapevine leaves. *BMC Plant Biology*, *10*(1), 34. doi:10.1186/1471-2229-10-34

Winnenburg, R., Baldwin, T. K., Urban, M., Rawlings, C., Köhler, J., and Hammond-Kosack, K. E. (2006). PHI-base: a new database for pathogen host interactions. *Nucleic acids research*, *34*(suppl 1), D459-D464. doi:10.1093/nar/gkj047

Wu, D., Oide, S., Zhang, N., Choi, M. Y., and Turgeon, B. G. (2012). ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen Cochliobolus heterostrophus. *PLoS Pathogens*, *8*(2), e1002542. doi:10.1371/journal.ppat.1002542

Xiao, X., Fu, Y. H., and Marzluf, G. A. (1995). The negative-acting NMR regulatory protein of Neurospora crassa binds to and inhibits the DNA-binding activity of the positive-acting nitrogen regulatory protein NIT2. *Biochemistry*, *34*(27), 8861–8868.

Xie, X., Meehan, M. J., Xu, W., Dorrestein, P. C., and Tang, Y. (2009). Acyltransferase mediated polyketide release from a fungal megasynthase. *Journal of the American Chemical Society*, *131*(24), 8388–8389. doi:10.1021/ja903203g

Xu, X., Chen, J., Xu, H., and Li, D. (2014). Role of a major facilitator superfamily transporter in adaptation capacity of Penicillium funiculosum under extreme acidic stress. *Fungal Genetics and Biology : FG and B*, *69*, 75–83. doi:10.1016/j.fgb.2014.06.002

Yan, X., Que, Y., Wang, H., Wang, C., Li, Y., Yue, X., … Wang, Z. (2013). The MET13 methylenetetrahydrofolate reductase gene is essential for infection-related morphogenesis in the rice blast fungus Magnaporthe oryzae. *PloS One*, *8*(10), e76914. doi:10.1371/journal.pone.0076914

Yang, E., Wang, G., Woo, P. C. Y., Lau, S. K. P., Chow, W.-N., Chong, K. T. K., … Cai, J. J. (2013). Unraveling the molecular basis of temperature-dependent genetic regulation in Penicillium marneffei. Eukaryotic Cell, 12(9), 1214–1224. doi:10.1128/EC.00159-13

Youngchim, S., Nosanchuk, J. D., Pornsuwan, S., Kajiwara, S., and Vanittanakom, N. (2013). The role of L-DOPA on melanization and mycelial production in Malassezia furfur. PloS One, 8(6), e63764. doi:10.1371/journal.pone.0063764

Yim, A. K.-Y., Wong, J. W.-H., Ku, Y.-S., Qin, H., Chan, T.-F., and Lam, H.-M. (2015). Using RNA-Seq Data to Evaluate Reference Genes Suitable for Gene Expression Studies in Soybean. PloS One, 10(9), e0136343. doi:10.1371/journal.pone.0136343

Zdobnov, E. M., and Apweiler, R. (2001). InterProScan - an integration platform for the signature-recognition methods in InterPro. *Bioinformatics*, *17*(9), 847–848.

Zhang, Y., He, J., Jia, L.-J., Yuan, T.-L., Zhang, D., Guo, Y., …, and Tang, W.-H. (2016). Cellular tracking and gene profiling of *Fusarium graminearum* during maize stalk rot disease development elucidates its strategies in confronting phosphorus limitation in the host apoplast. *PLoS Pathogens*, *12*(3), e1005485. doi:10.1371/journal.ppat.1005485

Supplementary material

Supplementary 1

List of primers employed in RT-qPCR

Primer Name	Predicted protein enconding gene	Sequence (5'-3')	Primer Lenght (bp)	Tm	Amplicon length
comp7300_FW	multicopper oxidase type 1 (comp7300_c0_seq1)	ATGCAAGACAACGGCACTTC	20	65.6	139
comp7300_RV		ATTGCGTGGCTTGAAACCTG	20	67.5	
comp8181_FW	sugar inositol transporter (comp8181_c0_seq1)	ATCAAGAGCTGGAAGATGACGA	22	61.73	98
comp8181_RV		CTCCTTTATTGCGTACTGGATCA	23	60.7	
comp13164_FW	apo9 cpo9 protein (comp13164_c0_seq1)	GGATGCGAGGTTAACTTCCC	20	60.95	108
comp13164_RV		TCCCAACCGCATTATCCTTC	20	60.3	
comp4276_FW	intradiol ring-cleavage dioxygenase (comp4276_c0_seq1)	CCTACAGCACCAACACTCAG	20	60.8	128
comp4276_RV		AGAAGATGCCGTCAGAAACC	20	60.52	
comp8066_FW	thioesterase family protein (comp8066_c0_seq1)	GCCAGCTTGACGATTAACTAC	21	59.55	126
comp8066_RV		CATCCACCAACGTCTCCAAC	20	61.43	
comp14342_FW	Fumarylacetoacetase (comp14342_c0_seq1)	TTTGGAGGACGGAGATGAGA	20	60.45	114
comp14342_RV		GAACGGGAATTTGACAGCAG	20	59.95	
comp12473_FW	salicylate hydroxylase (comp12473_c0_seq1)	TCCAGCAACACCAATATCGG	20	60.59	119
comp12473_RV		TCTCCTCCTTGAAATTGTCTCGT	23	61.85	
comp8784_FW	homogentisate 1,2-dioxygenase (comp8784_c0_seq1)	ATACCAAGGACACTTCGAGCTG	22	64.7	75
comp8784_RV		TGAAAATCGCGAGCATTGGC	20	70.6	
comp7101_FW	glycoside hydrolase family 35 (comp7101_c0_seq1)	AGCGTCGTTGCTGATGTAAG	22	66.9	100
comp7101_RV		TGTCGATGGCATTGAGTTCC	20	66.8	
comp5526_FW	choline dehydrogenase protein (comp5526_c0_seq1)	AGATGGTTCGACGGGATAATCG	22	68.1	73
comp5526_RV		TGTTGGTGTATGGTGCGAAG	20	65	
comp16237_FW	pectate lyase (comp16237_c0_seq1)	GAGCAATCCATCAACCGCTTAC	22	66.9	166
comp16237_RV		ACACTTACACTGCCGTTGAG	20	60.9	
comp13725_FW	glycoside hydrolase family 3 (comp13725_c0_seq1)	AAAAAGTCGGCCAGCTGTTC	20	65.7	129
comp13725_RV		TGTTGAAGTGCGTCATGAGC	20	65.6	
comp5761_FW	xylosidase glicosylhydrolase (comp5761_c0_seq2)	AAAACGGACTTTGGCGAACG	20	68.4	120
comp5761_RV		CGCCTCATACACCATCTTGTTG	22	66.7	
comp18638_FW	4-hydroxyphenylpyruvate dioxygenase (comp18638_c0_seq1)	TATGGAGAAGCCGCTTGTTG	20	65.5	80
comp18638_RV		TCGCATTCCGTGACCAATTC	20	68.5	
TUB_FW	β-tubuline (comp6710_c0_seq1)	GAACGTCTACTTCAACGAGGT	21	60.1	114
TUB_RV		GAGGATAGCACGAGGAACGT	20	60.5	

Supplementary 2

RNA extraction from infected grapevine shoots

Total RNA from *L. theobromae*-infected grapevine was isolated using a cetyltrimethylammonium bromide (CTAB)-based extraction method (Gambino et al., 2008). Briefly, a day previous to RNA extraction, the transversal sections (around 5 mm of length and 100 mg of weight) of infected grapevines was removed from NAP solution and placed in 2.O mL tubes, containing one ¼ inch ceramic bead and 50 mg of 0.5 mm and 100 mg of 1 mm glass beads (Biospec). Tubes were beaten on a Mini-Beadbeater (Biospec Products) for 30 s. Immediately, tubes were chilled on ice, and pre-heated lysis solution was added (2% v/V CTAB, 2% v/V PVP, Tris-HCl 100 mM pH 8.0, NaCl 2 M, EDTA 25 mM, and 2% of β -mercaptoethanol added just before use) before subjecting the tubes to a second beating cycle for 30 s. Lysed samples were incubated at 65 °C for 5 min and 500 µl of CIA (24:1 v:v) was added and mixed twice in a Vortex. Tubes were centrifuged at 4,000 rpm for 30 min at 4 °C. The washing with CIA was repeated and then the aqueous phase was transferred to a new 1.5 mL tube and mixed with 1 v of 75% ethanol v:v. The total mix was passed through a silica column (Epochlab) and centrifuged at maximal velocity for 1 min at room temperature. Thereafter, the column was washed with RPE buffer (Qiagen) and twice with ethanol 75% v:v. Finally, total RNA was eluted in 48 µL of nuclease-free water (Qiagen) by centrifugation at maximal velocity.

Supplementary 3

General classification based on biological process category in gene ontology

The functional annotation was done through Blast2GO using all the sequences assembled of transcripts (all the conditions together). The gene ontology structure employs several levels to classify the relations among GOterms (Ashburner *et al*., 2000) using higher numbers for more specific categories. The three trunk classification was molecular function, biological process or cellular component (Ashburner *et al*., 2000).

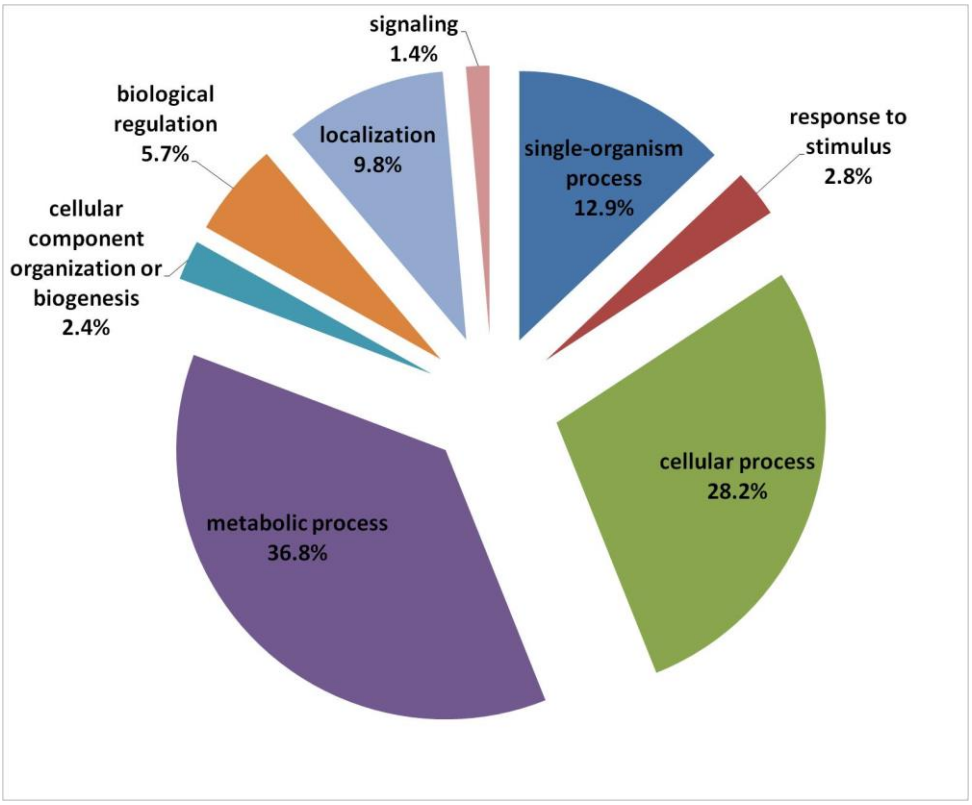

Figure 24. Biological process in second level of gene ontology classification. Most of the transcripts are involved in metabolic processes (2,283) and cellular processes (1,750). Also a good number are classified as single-organism processes (801) and localization (607). And with a significant number also are represented process like biological regulation (351), response to stimulus (176) and signaling (87).

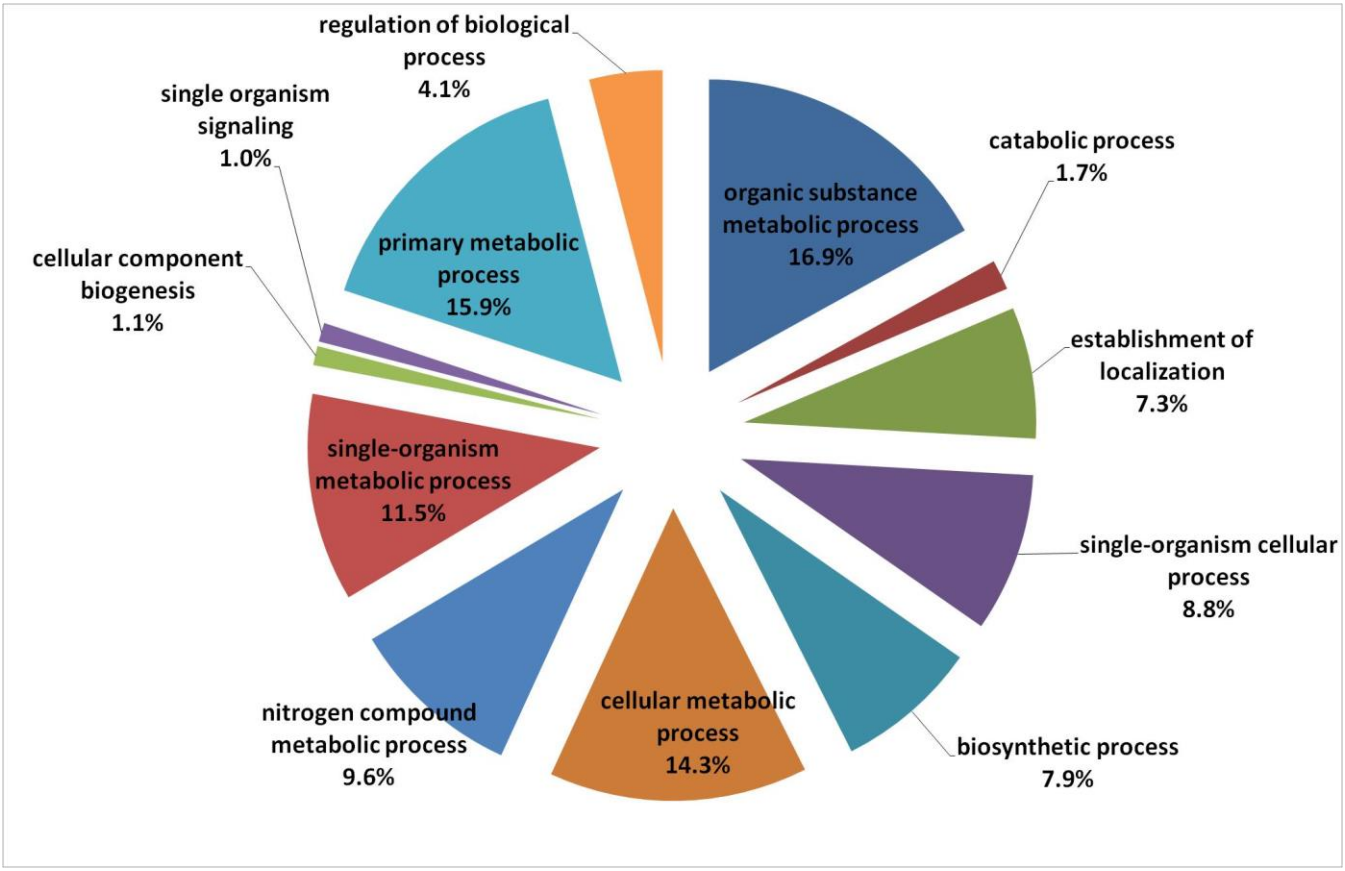

Figure 25. Biological process in third level of gene ontology classification. Most of the transcripts were distributed in primary metabolic processes (1316), organic substances metabolic process (1405), nitrogen compound metabolic processes (796), establishment of localization (603), single organism cellular (729) and metabolic (954) processes.

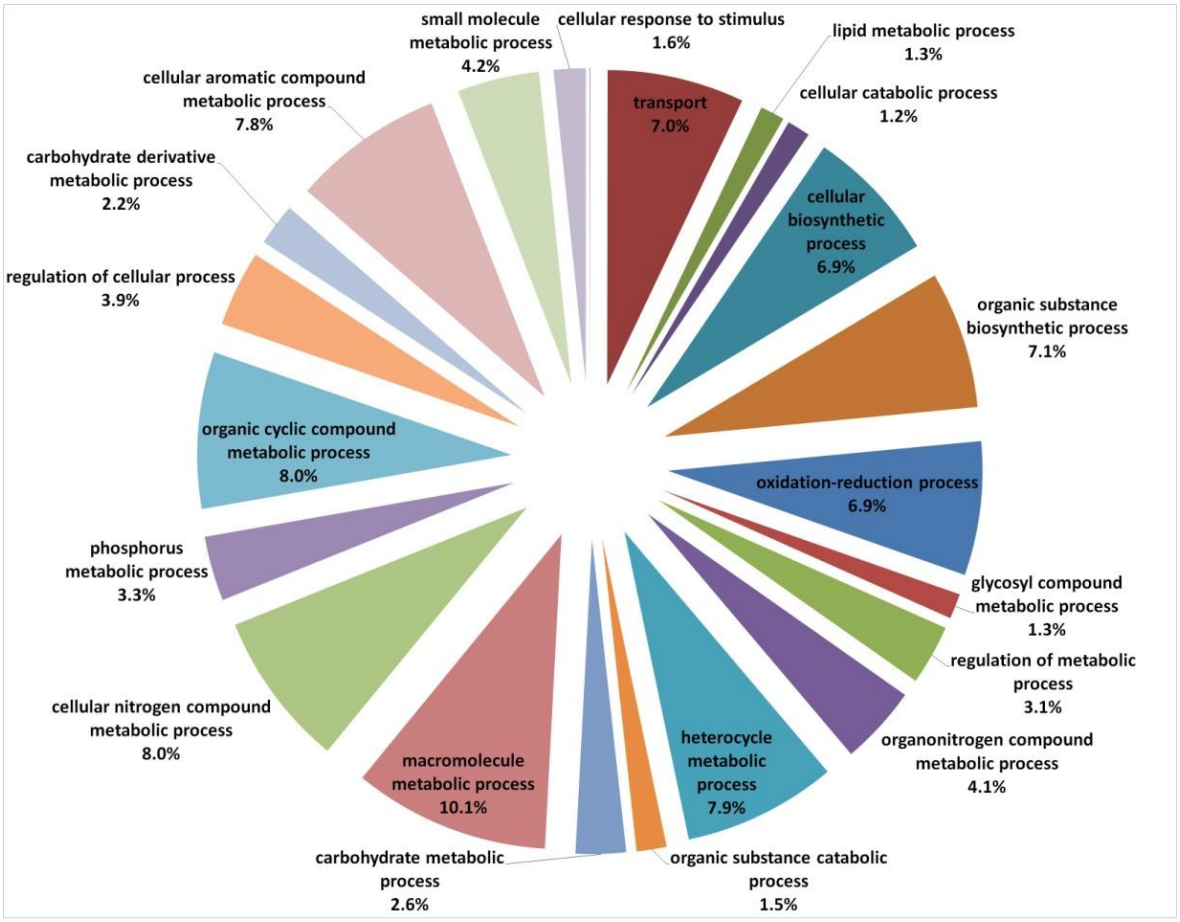

Figure 26. Biological process in fourth level of gene ontology classification. Most of the transcripts were involved in nitrogen metabolism (684), aromatic (665), organic cycles (689) and heterocyclic (676) compounds metabolism. Also there are 589 transcripts involved in oxidation-reduction process.

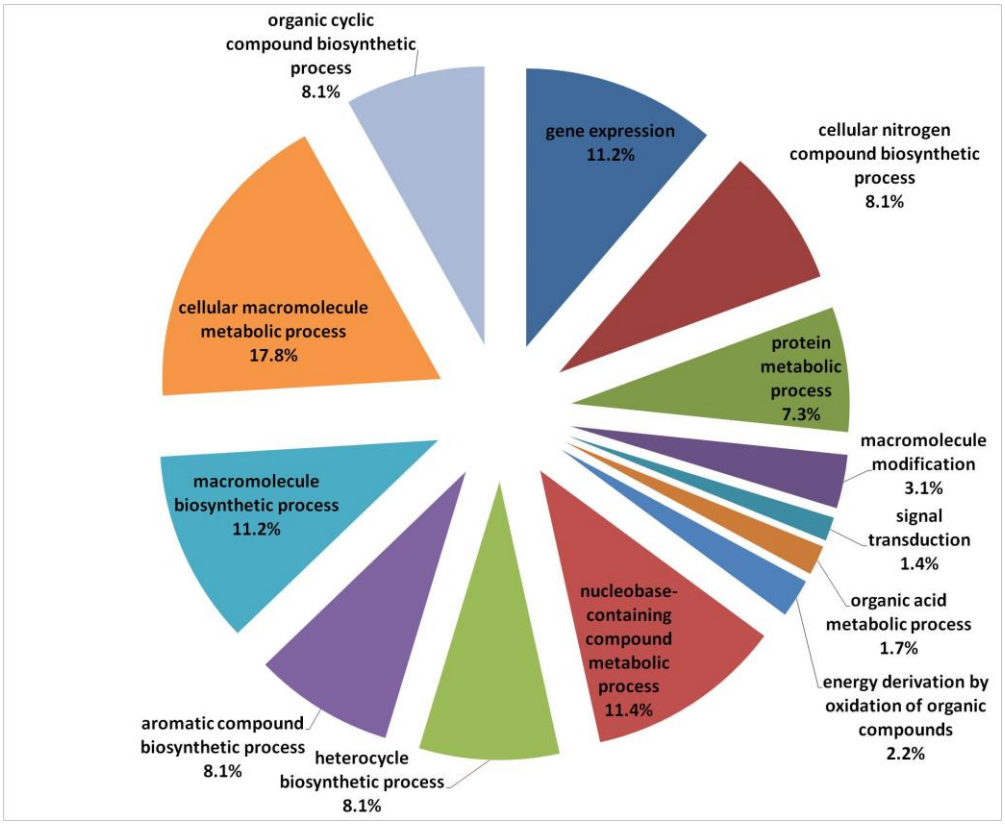

Figure 27. **Biological process in fifth level of gene ontology classification.** Most of the transcripts were involved in cellular macromolecule metabolic process (103), nucleobase-containing compound metabolic process (66), gene expression (65) and macromolecule biosynthetic process (65).

Supplementary 4

Basic analysis of data used in gene expression calculates

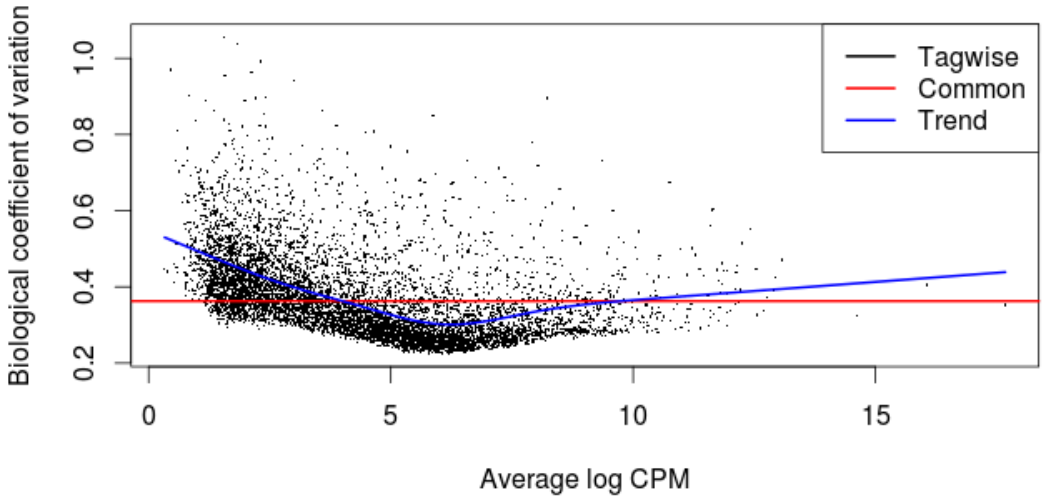

Figure 28. **Biological coefficient of variation (BCV).** Normalized data obtained using the plotBCV function in edger package version 3.4.2 (Robinson et al., 2010). The square root of common BCV is denoted by the red line. CPM: counts per million.

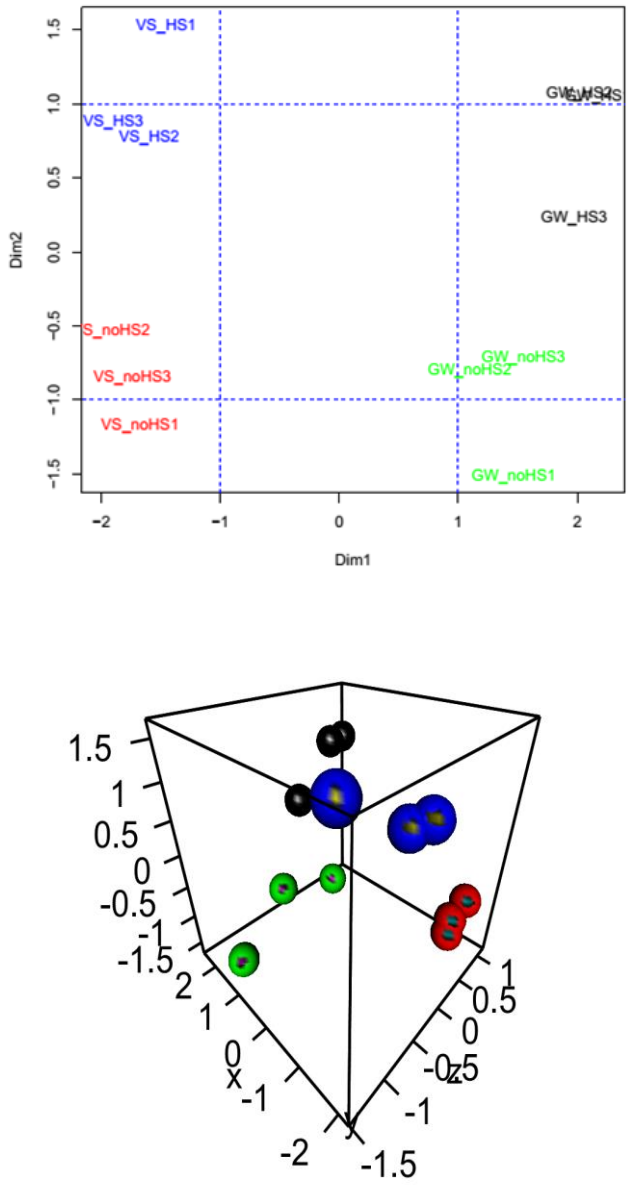

Figure 29. Two-dimensional scaling plot. Normalized data obtained using plotMDS function in edgeR edgeR package version 3.4.2 (Robinson et al., 2010) (A) and a three-dimensional scaling plot (B) obtained through plot3d function from the library rgl version 0.93.996 (Adler et al. 2014, http://CRAN.R-project.org/package=rgl).

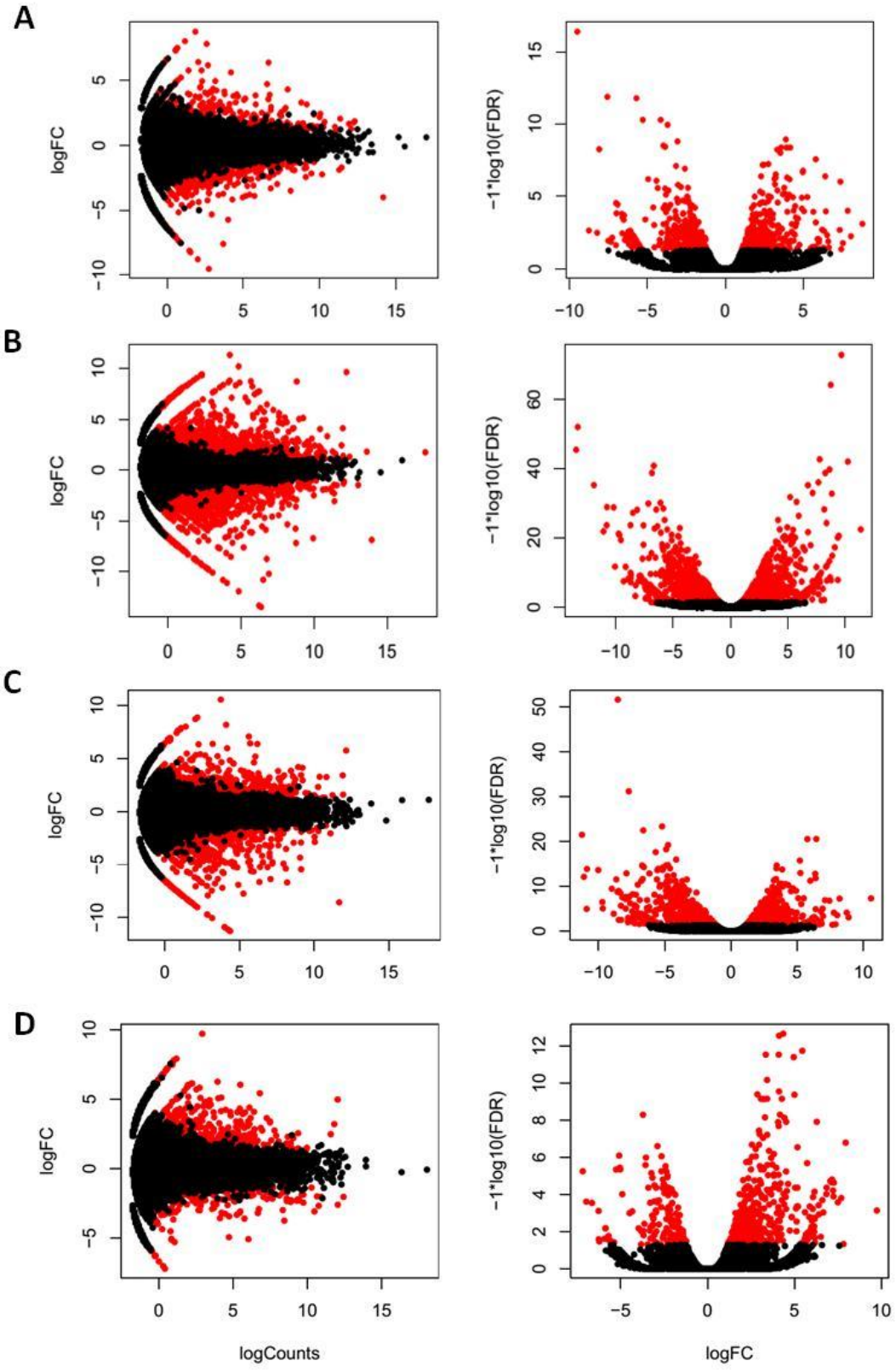

Figure 30. Dispersion of normalized data. Analyzed through Smear plot (left panel) and Volcano plot (right panel) for contrasting conditions FWS/FW (A), FWS/F (B), FW/F (C) and FS/F (D). Red dots indicate differentially expressed genes. FC: Fold change. FDR: False Discovery Rate. Graphs were obtained with edgeR package version 3.4.2 (Robinson et al., 2010).