Universidade do Minho
Escola de Ciências

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Study of *Hypericum perforatum* defense mechanisms against *Colletotrichum gloeosporioides*: Relevance of phenolic metabolism and hypersensitive response.

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Study of *Hypericum perforatum* defense mechanisms against *Colletotrichum gloeosporioides*: Relevance of phenolic metabolism and hypersensitive response.

Doutoramento em Ciências
Área de Conhecimento em Biologia

Trabalho efectuado sob a orientação do
Prof. Dr. Alberto Dias
e do
Prof. Dr. Rui Tavares

Setembro de 2009

Universidade do Minho, ___/___/_____

Assinatura: _____

The work presented in this PhD thesis was carried out under the supervision of Prof. Dr. Alberto Carlos Pires Dias and Prof. Dr. Rui Tavares, both from Departamento de Biologia, Escola de Ciências da Universidade do Minho. The work was performed at Centro de Biologia, Escola de Ciências da Universidade do Minho, in Braga, Portugal, between October 2003 and February 2009.

Funding was provided by the FCT (Fundação para a Ciência e Tecnologia), Lisboa, Portugal, as a PhD grant ref. SFRH/BD/13318/2003.

Gostaria de agradecer ao Prof. Doutor Alberto Dias, pela orientação e apoio ao longo desses anos, não só na realização do meu doutoramento mas também do estágio de licenciatura (os anos que ele já me atura...), estando sempre presente não só para nortear mas também para *batalhar* pelos seus alunos, característica essa que, não fazendo parte das sua obrigações, nunca se negou a realizar, abnegadamente. Por esse e muitos outros motivos que levariam tempo a enumerar, cabe também agradecer, ainda mais, *ao Alberto*, amigo que estimo e incluo num grupo muito restrito de pessoas que merecem a minha total consideração e confiança nos seus princípios.

Agradeço também ao Prof. Doutor Rui Tavares, por aceitar a co-orientação do meu doutoramento (como também já o fizera durante o estágio), concedendo-me livre acesso a metodologias e condições laboratoriais indispensáveis para a realização e conclusão de inúmeras etapas apresentadas nesta tese.

De uma forma geral, aos restantes professores do Departamento de Biologia, em especial os *das plantas* que, de alguma forma, contribuíram para esse trabalho, fica aqui o meu agradecimento.

Ao Herlânder, pelo grande profissionalismo, paciência e *orientação de bancada* incondicional, que me foram indispensáveis na fase inicial do doutoramento. Trabalho à parte, pelo óptimo ambiente que soube criar e manter à sua volta mesmo quando este, pontualmente, faltava no resto do laboratório.

Ao Minos, Rui e Manu, amigos de tão longa data que já não mais distingo de irmãos. Obrigado pela vossa presença e boa disposição mas, mais que isso, pelo vosso carácter irrepreensível, qualidade cada vez mais rara de se encontrar. Por isso mesmo, poucos (muito poucos) são aqueles por quem, como vocês, seria capaz de *pôr a mão no fogo*. Chegar aqui seria muito mais difícil sem o vosso apoio, amizade e paciência, principalmente daqueles que *aguentaram o barco* durante as minhas paragens para escrita, incentivando-me sempre sem fazer quaisquer cobranças, ainda que de direito.

Ao Paulo, pela amizade, boa disposição e, mais que isso, pela forma abnegada como se apresentou disponível para ajudar na execução de alguns trabalhos laboratoriais. Tenho a certeza que esse agradecimento vale também por um departamento inteiro com o qual colaboraste, inúmeras vezes fora do âmbito das tuas *funções*, algumas delas em prejuízo próprio. Força. *Polish style*!

My appreciation also to Franklin, who came to Braga as a group colleague but soon became good a friend of mine. My inestimable gratitude for all your help and backup in the lab, especially in the *time-consuming/boring/nerve-breaking* maintenance of suspension cell cultures, the most crucial material I needed for the work presented here. Equally important, thanks for contributing in a daily basis, along with others, to a most needed good environment in the lab. Muito obrigado, pá!

I would also like to express my gratitude to Erich, Katja, Lucie and Brigitte, for accepting me as your guest in Max-Planck and making it so easy for me to adapt to a new environment. Besides the professional support (and patience), essential for overcoming many of the technical problems I was facing in Braga, your friendship, shown during all my stay in Cologne, shall not be forgotten, no matter how far you may be (especially in Katja's case). By the way, the Greek recipes and choreography shall not be forgotten also... My appreciation also to Elke, Bekir, Mario and Rainer, for you support and valuable opinions.

À Ana pela boa disposição constante e por mostrar rapidamente independência no laboratório, não tendo dado trabalho nenhum, permitindo-me assim ter mais tempo para terminar esse trabalho.

Aos outros bolseiros *das plantas* (Marta, Mafalda, Ana Patrícia, Alice, Rosa, Sara, Vítor, Sérgio, Briolanja...) mas também do restante DB (JP, Raul, Sofia, Isabel, Rita, Jorge, Clara...) que, entre outros, ajudaram no trabalho e/ou tornaram o departamento num local agradável ao longo desses anos.

Aos funcionários do departamento que, ao trabalhar nos *bastidores*, são peças indispensáveis para o bom funcionamento dos laboratórios. Assim, um obrigado especial para o Amaro, Manuela, Paula, Liliana, para a recém-chegada Cristina mas também a quem já não está no DB, como o Carlos e D. Rosa.

A quem chegou há menos tempo e não tive a oportunidade de conhecer melhor (Teresa, Marta, André, Fábio...) mas que criaram um bom ambiente no curto período em que ainda estive no lab.

Meu agradecimento (e pedido de desculpas) a quem possa ter esquecido de referir aqui.

E porque não agradecer a quem, não atrapalhando, ajudou indirectamente a que pudesse chegar aqui com o trabalho concluído.

Fora do âmbito profissional (porque o trabalho *não* faz o homem, apenas o completa), mas também indispensáveis para que chegasse até aqui com alguma sanidade mental, o meu sincero agradecimento à *malta da Póvoa* (Tiago, Pontes, Luís e Ruben) mas também o meu pedido de desculpas pelo distanciamento ao longo destes últimos tempos. Voltaremos em breve à *programação normal*...

O meu agradecimento aos familiares que, de múltiplas formas, ajudaram-me nesse capítulo da vida que fica, finalmente, concluído. Em especial à Cláudia, Cristina e Manuel, por compreenderem o distanciamento desses últimos tempos, sempre *na torcida*, a incentivarem-me a levar esse projecto até ao fim.

Tudo que sou, tudo o que faço e tudo em que acredito não seriam sem o irrepreensível exemplo de vida, a presença constante, a amizade e o apoio abnegado dos meus pais, pedras basilares na formação do que sou. Obrigado, simplesmente por tudo.

À minha Aninha, a pedra basilar que faltava e que trouxe o equilibro na minha construção, obrigado pelo apoio e compreensão incondicionais em todas as minhas decisões, em todos os meus riscos. Obrigado, de quem te ama, pela alegria, pelo motivo, pelo sentido, por nós.

Por último, e por pouco ortodoxo que seja, agradeço a mim mesmo, pela paciência e perseverança que descobri ter para fechar esse *capítulo* após encontrado novo rumo, nova vida e objectivos. Sem saber se serão mudanças para melhor, faço-as à mesma, revendo-me nas palavras de Paul Anka:

"Regrets, I've had a few,
But then again, too few to mention.
I did what I had to do
And saw it through without exemption.

I planned each charted course,
Each careful step along the byway
But more, much more than this,
I did it my way."

"My Way" (1968), Paul Anka

Mecanismos de defesa de *Hypericum perforatum* contra *Colletotrichum gloeosporioides*: Relevância da resposta hipersensível e do metabolismo fenólico.

H. perforatum (HP) é uma planta medicinal encontrada em todo o mundo, sendo nativa da Europa, norte de África, Oriente Médio e grande parte da Ásia. Introduzida também noutras regiões do globo devido ao seu elevado valor terapêutico, essa planta é descrita desde tempos imemoriais para o tratamento de diversas maleitas. Actualmente, sua principal aplicação consiste no tratamento de depressões leves e moderadas sendo que, em diversos países, a prescrição de produtos à base de Hipericão é superior a dos medicamentos antidepressivos mais comuns. Devido à grande procura daí resultante, a pressão exercida junto aos produtores agrícolas tem aumentado consideravelmente. O cultivo em larga escala, como solução para o abastecimento de um mercado em crescimento, tem sofrido vários revezes. Dentre os mais relevantes encontra-se a contaminação pelo fungo *Colletotrichum gloeosporioides*, causador de antracnose em inúmeras espécies vegetais de elevado interesse económico. A infecção por *C. gloeosporioides* (CG) tem levado a perdas significativas, tanto na quantidade, como na qualidade dos produtos derivados de HP. Embora não existam variedades totalmente resistentes ao fungo, algumas apresentam menor susceptibilidade à antracnose *in vivo*.

Considerando esses aspectos, nosso objectivo consistiu em estudar alguns dos mecanismos de defesa de *H. perforatum* contra *C. gloeosporioides*. Foram avaliadas, nomeadamente, a resposta hipersensível (HR) e o metabolismo fenólico. Adicionalmente, foi avaliada a influência de duas hormonas associadas a mecanismos de defesa sistémica: metil-jasmonato (MeJ) e ácido salicílico (SA).

Todos os estudos descritos foram realizados com recurso a culturas de células em suspensão provenientes de duas variedades de HP, distintas na sua susceptibilidade ao fungo CG *in vivo*. Culturas de células de HPS (variedade susceptível) e *Helos* (menos susceptível) foram avaliadas quanto ao consumo de nutrientes, crescimento e viabilidade, tanto em condições normais de manutenção como após tratamento com os eliciadores descritos acima. O tratamento com uma preparação de CG levou a um aumento significativo no consumo de açúcar bem como a uma diminuição no crescimento e viabilidade celular. Esses efeitos foram corroborados pelos resultados obtidos nos ensaios de TUNEL, durante a avaliação da HR. Foi verificado, em ambas suspensões de HP, o desenvolvimento de um *burst* oxidativo duplo, típico de interacções incompatíveis. Verificou-se igualmente a acumulação de espécies reactivas de oxigénio (ROS), tanto internamente como a nível extracelular, após eliciação com CG. A eliciação foi também responsável pela redução na actividade de enzimas antioxidantes (SOD e CAT), favorecendo a acumulação de ROS. Contraditoriamente, observou-se um aumento do potencial antioxidante dos extractos metanólicos de HP, derivado do aumento na acumulação de xantonas. Esse aumento não foi, no entanto, capaz de contrariar a alteração na homeostasia das ROS, culminando assim num aumento da peroxidação lipídica e degradação do DNA, fenómenos tipicamente associados à HR. O aumento na acumulação de xantonas foi a principal alteração verificada no metabolismo fenólico, após eliciação com CG,

podendo esta resposta estar associada ao aumento no consumo de açúcar, usado como fonte de carbono para a sua síntese. O aumento na produção de xantonas pode ter sido igualmente responsável pelo declínio na acumulação de flavonóides e lenhina visto que estas vias biossintéticas recorrem ao mesmo *pool* de precursores, canalizados para a síntese de xantonas.

Ao contrário do que foi verificado com CG, o tratamento de HP com MeJ ou SA levou a pequenas ou nenhumas alterações, na maior parte dos parâmetros avaliados. Não se verificaram alterações significativas no crescimento, sobrevivência e consumo de nutrientes, excepto um ligeiro aumento no consumo de açúcar, após tratamento com MeJ. Tal como descrito para as xantonas, este aumento poderá estar associado aos acrescidos níveis de lenhina, observados no tratamento com MeJ. Ambas as fitohormonas foram responsáveis por um ligeiro aumento na produção de xantonas (e consequente redução na síntese de flavonóides). De igual modo, o uso destas fitohomonas levou a um ligeiro aumento na acumulação de ROS embora não se tenha verificado HR, provavelmente devido ao aumento da capacidade antioxidante de HP, entretanto observada. Nem sempre se verificam diferenças notórias entre as respostas associadas ao SA e ao MeJ já que as vias de defesa sistémica a que estão associadas apresentam muitos pontos de intercâmbio, com diversas variáveis intervindo nas mesmas. Embora não se tenham verificado alterações muito significativas nas respostas de HP, decorrentes da aplicação isolada das fitohormonas, o seu uso levou a um aumento nas capacidades de resposta de HP quando estas eram aplicadas previamente à exposição ao fungo (CG). Apesar de superior quantitativamente, essa resposta foi, regra geral, qualitativamente semelhante à verificada pelo tratamento isolado com CG. Verificaram-se assim uma maior redução na viabilidade e um incremento no consumo de açúcar, bem como uma maior acumulação de xantonas (notório em HPS). Sendo assim, embora não se tenham verificado efeitos visíveis significativos decorrentes do tratamento isolado de HP com as fitohormonas, as mesmas demonstraram ter alterado o metabolismo de HP de forma a preparar a planta para uma resposta mais rápida e intensa, numa posterior interacção com CG.

A interacção incompatível verificada entre HP e CG culminou numa HR, bem como na síntese de xantonas, possivelmente para actuarem como fitoalexinas contra o patogénio. Ambos os mecanismos de defesa são aparentemente ineficazes no combate à infecção por CG, considerando o que se verifica *in vivo*. Tendo em conta que CG pode apresentar tanto um modelo de nutrição biotrófica como necrotrófica, o desenvolvimento de HR pode mesmo favorecer a natureza necrotrófica de CG, ao providenciar ao fungo locais ideais para a colonização do hospedeiro. Além da ineficácia da HR, os compostos fenólicos produzidos por HP após a eliciação não se mostraram capazes de impedir o crescimento de CG *in vitro*. Embora não tenham sido efectuados estudos em plantas, os mecanismos de defesa analisados neste trabalho culminaram em respostas mais intensas em culturas da variedade *Helos*, podendo estas diferenças explicar a sua menor susceptibilidade à antracnose *in vivo*.

Hypericum perforatum defense mechanisms against *Colletotrichum gloeosporioides*: Studies on the relevance of the hypersensitive response and phenolic metabolism.

H. perforatum is a medicinal plant widely distributed across the world, being native in Europe, Northern Africa, Middle East and most of Asia. This plant was also introduced in other regions of the world due to its high therapeutic value, described since ancient times for the treatment of several ailments. Nowadays, the medicinal impact of *H. perforatum* is mainly focused in the treatment of mild to moderate depressions and, in some countries, *Hypericum*-based products are prescribed more often than the most common antidepressants. The increasing medicinal and economic relevance of this plant is responsible for a mounting pressure over raw-material producers. While *H. perforatum* plantations have been established in order to properly supply the growing market, some drawbacks in large-scale production started to appear. The most prominent is contamination by *Colletotrichum gloeosporioides*, a fungus responsible for the development of anthracnose disease in several economically important crops across the globe. *C. gloeosporioides* infection is known to reduce *H. perforatum* yields, as well as the quality of its derived products. Although no *H. perforatum* accessions have so far proven to be fully tolerant to anthracnose, some of them are known to be less-susceptible to this disease *in vivo*.

Considering these aspects, it was our aim to study some of *H. perforatum* defense mechanisms against *C. gloeosporioides*. Namely, the hypersensitive response (HR) and phenolic metabolism were evaluated. Additionally, the influence of methyl-jasmonate and salicylic acid, two phytohormones related to plant defense signaling, was also assessed.

The experiments were carried out in *H. perforatum* suspension cell cultures obtained from two accessions, distinct in their susceptibility to *C. gloeosporioides in vivo*. Cell cultures from HPS (a susceptible accession) and *Helos* (a less-susceptible accession) were characterized in their major nutrients consumption and survival parameters upon normal growth conditions or after treatment with the elicitors described. Treatment with a *C. gloeosporioides* elicitor preparation (CG) was responsible for a significant increase in sugar consumption but also a considerable decrease in cell viability and culture growth. This decrease was in accordance with the results obtained during HR evaluation by TUNEL labeling. *H. perforatum* cultures developed a double oxidative burst, typical of incompatible interactions. Reactive oxygen species (ROS) accumulated both internally and extracellularly after CG treatment. Enzymatic ROS-scavenging (namely, SOD and CAT) activities were suppressed, favoring ROS buildup, while a contradictory increase in non-enzymatic scavenging mechanisms was observed, due to a *boost* in xanthone synthesis. Still, this raise was not enough to prevent the change in ROS homeostasis, which culminated in increased lipid peroxidation, DNA cleavage and, therefore, HR. The *boost* in xanthone accumulation was the most prominent change in phenylpropanoid metabolism upon CG elicitation and should be associated to the enhanced consumption of sugar, a possible source of carbon for the xanthone synthesis. The

increase in xanthones produced could be responsible for the decline in flavonoids and lignin accumulation since these biosynthetic pathways share a common *pool* of precursors, diverted to xanthone synthesis.

Treatment with the phytohormones, on the other hand, led to minor or absent changes in most parameters evaluated. No survival or nutrient consumption parameters were significantly influenced by them, except for a small increase in sugar consumption, observed upon MeJ treatment. As previously referred for the accumulation of xanthones, this increase could be associated to the raise in lignin accumulation observed. Both MeJ and SA were also responsible for a minor increase in xanthone production (and concomitant decrease in flavonoids synthesis). Furthermore, these phytohormones led to a minor increase in ROS accumulation although no HR occurred, perhaps due to the enhancement in scavenging means, also observed. A clear dichotomy between the two systemic defense signaling pathways is not always possible and extensive cross-talk is known to occur, with many variables influencing it. Despite the absence of noticeable changes, when these phytohormones were used prior to CG elicitation, cells displayed a tendency for increased responses, following patterns similar to those found in CG-elicited cultures. A sharper decrease in survival parameters and enhanced sugar consumption were observed, as well as increased xanthone synthesis, especially in HPS cultures. Therefore, while the phytohormones alone didn't provide marked effects, the cell's metabolism could have been primed and tuned for improved defense responses as displayed later, upon CG elicitation.

The incompatible interaction observed between *H. perforatum* and *C. gloeosporioides* culminated in HR as well as the synthesis of xanthones, probably acting as phytoalexins against the pathogen. Considering what is usually observed *in vivo*, both defense mechanisms seem to be ineffective against the fungus. Since *C. gloeosporioides* can display either biotrophic or necrotrophic nutrition models, HR may actually favor its necrotrophic nature by providing new entry points for infection. Additionally, the phenolic compounds induced upon elicitation were not able to stall *C. gloeosporioides* growth *in vitro*. Despite their inefficiency, these defense mechanisms were stronger in *Helos* cultures and could account for the reduced susceptibility of this accession, as observed *in vivo*. Further studies *in vivo* should be performed before drawing definite conclusions about this plant-pathogen interaction.

2,4-D	2,4-dichlorophenoxy acetic acid
$A_{###}$	absorbance at ### nm
Amp	ampicilin
AOPP	L-a-aminooxy-β-phenylpropionic acid
atm	atmosphere
BA	bensylaminopurine
BLAST	Basic Local Alignment Research Tool
BSA	bovine serum albumine
bp	base pairs
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
CG	*Colletotrichum gloeosporiodes*
Ci	Curie
CMV	citomegalovirus
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPPH	1,1-diphenyl-2-picrylhydrazyl
DTT	dithiothreitol
DW	dry wheight
EC_{50}	half maximal effective concentration
EDTA	ethylenediamine-tetraacetic acid
EtBr	ethidium bromide
g	grams
g	gravity acceleration
Gen	gentamicin
h	hour
HR	hypersensitive response
HP	*Hypericum perforatum*
HPLC-DAD	High performance liquid chromatograpy diode array detector
HPLC-DAD - MS/MS	High performance liquid chromatograpy mass spectrometry/mass spectrometry
HPS	HP *sensitive* accession
IAA	isoamyl alcohol or indole-3-acetic acid
IPTG	isopropyl-ß-D-thiogalactopyranoside
ISR	induced systemic resistance
JA	jasmonic acid
Kan	kanamycin
Kin	Kinetin
L	Liter
M	molar
m	meter
min	minute
MDA	malondialdehyde
MOPS	3-(N-morpholino)propane-sulfonic acid
OPDA	12-oxophytodienoic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PCR	polymerase chain reaction
PDA	potato dextrose agar
p.f.u.	plaque-forming units
PKS	Polyketide synthase
PR	pathogenesis-related
PVPP	polyvinylpolypyrrolidinone
RNA	Ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	second
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
Tet	tetraciclin
TMV	Tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	polyoxyethylene-p-isooctylphenol
U	unit of enzymatic activity
u.p.	ultra pure
UV	ultra-violet light
V	volt
VIS	visible light
v; vol	volume
X-gal	5-bromo-4-chloro-indolyl-ß-D-galactopyranoside
XTT	3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate

“Self-defense is Nature’s eldest law.”

J. Dryden (1631-1700)

Chapter 1

Introduction

Chapter 1.1

Hypericum perforatum L.

1.1. *Hypericum perforatum* L.

1.1.1. Taxonomy and morphological characterization of *Hypericum perforatum* L.

The genus *Hypericum* comprises nearly 400 species and is the most popular from the nearly 50 genus (and 1200 species) belonging to the *Guttiferae* (*Clusiaceae*) family. In fact, the relevance of *Hypericum* is such that, for some taxonomists, species from this genus are classified as a distinct family, the *Hypericaceae* [Erdelmeier *et al.*, 2000]. One of the most important *Hypericum* species, found all around the world, is *Hypericum perforatum* L. (Fig. 1.1). *H. perforatum* is an herbaceous, perennial shrub that can grow up to 1.5 m of height, reaching maturity within two years. During the first year, the growth is directed mainly to establishment of the root system [Tisdale *et al.*, 1959]. Moreover, the growth of *H. perforatum* occurs in two distinct phases: during fall and winter it is predominantly basal and, from spring to summer, the growth of one or multiple erect, woody stems can be observed, branching out towards the top of the plant [Gordon *et al.*, 1991]. When fully developed, *H. perforatum* has an underground rhizomatous stem and deep taproot that can reach 1.5 m in depth. This plant also displays many lateral roots that can reach 1 m in depth [URL 1]. The leaves are opposite, sessile, oblong and their sizes may reach up to 3 cm long and 1.6 cm wide. One of the distinctive

morphological characteristics from *H. perforatum* leaves is the presence of translucent and dark secretory glands (Fig. 1.2), which nature and functions will be discussed below. The flowers are numerous, paniculate cymes with 5 yellow petals, 5 - 8 mm long and also display the secretory glands observed in the leaves. Stamens in the flower are also numerous and form clusters divided into three groups [URL 2]. The seeds are held in three chambered capsules of 7 - 8 mm long, ovoid and with dark brown colour [URL 3]. This structure holds thousands of small, pitted, cylindrical seeds [URL 2].

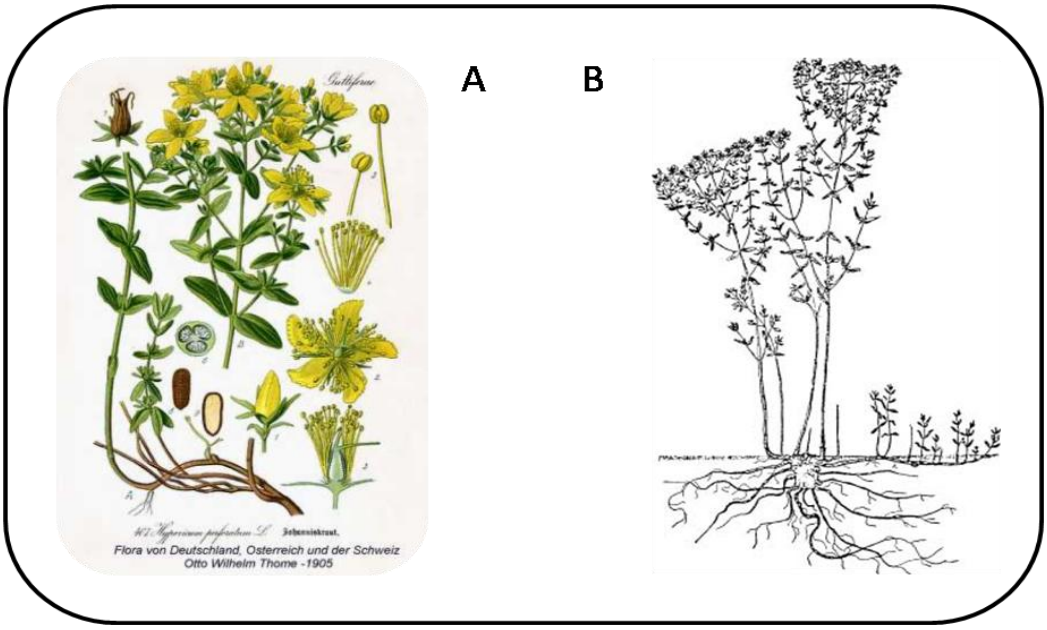


Figure 1.1: Drawings showing general morphological features of *Hypericum perforatum* L. plants. **(A)** URL 4; **(B)** URL 5.

Some of the most remarkable morphological features from *H. perforatum* are their secretory glands (Fig. 1.2). Two distinct types of secretory glands exist: Translucent glands and dark glands [Maggi *et al.*, 2004]. While the translucent glands are present only in leaves, petals and sepals [Ciccarelli *et al.*, 2001], the dark glands are more widely distributed in the plant, being found also in stems and in relatively greater amounts in the stamen [Zobayed *et al.*, 2006].

The translucent glands are responsible for the term "perforatum". They are recognized as transparent dots scattered across the leaves in spaces delimited by the veins, but not associated with them. In the sepals and petals, the translucent glands are oblong and predominantly distributed along the margins [Ciccarelli *et al.*, 2001]. These glands are schizogenous oil cavities [Onelli *et al.*, 2002], extending from abaxial to adaxial epidermis, with no opening for the exterior since the large central space is surrounded by a uniseriate epithelium [Curtis *et al.*, 1990]. Histochemical tests of the translucent glands, carried out by Ciccarelli (2001), revealed the presence of alkaloids, lipids, resins,

essential oils and tannins, in accordance with results from other chemical studies [Bombardelli *et al.*, 1995, Butterweck *et al.*, 1997]. Moreover, recent studies suggest that these glands are also the main site for accumulation of hyperforin, an important phloroglucinol from *H. perforatum* [Soelberg *et al.*, 2007]. Some of these secondary metabolites, produced and accumulated in the translucent glands and secretory canals [Ciccarelli *et al.*, 2001], play a significant role in plant defense to biotic and/or abiotic stresses [Harborne, 1994].

Other secondary metabolites, such as the naphtodianthrones hypericin and pseudohypericin, are only present in the black nodules [Ciccarelli *et al.*, 2001; Butterweck *et al.*, 1997]. These dark glands were probably first described by J.R. Green in 1884 and are also referred as "nodules" [Curtis *et al.*, 1990; Fornasiero *et al.*, 1998] or "black nodules" [Maggi *et al.*, 2004]. They differentiate from a cluster of cells that progressively enlarge and darken, to form a cellular nodule instead of a cavity [Onelli *et al.*, 2002]. The dark-colored glands are the most important secretory structure in *H. perforatum*, being responsible for the final steps in the biosynthesis of hypericin and pseudohypericin, two naphtodianthrones of major importance in *H. perforatum* [Zobayed *et al.*, 2006]. The dark nodules are composed by an outer flat cell layer that may have a specific physiological activity, distinct from the inner nodule cells. It has been postulated that the outer flat cells operate as light filters, protecting the nodule cells from the material accumulated within, such as hypericin [Onelli *et al.*, 2002]. It is known that hypericin is photoactivated [Erdelmeier *et al.*, 2000], becoming toxic upon light exposition [Vandenbogaerde *et al.*, 1997]. Therefore, it is possible that the flavonoids, anthocyanins and other tannin-like substances in the outer flat cells are accumulated for this light-protection mechanism [Onelli *et al.*, 2002].

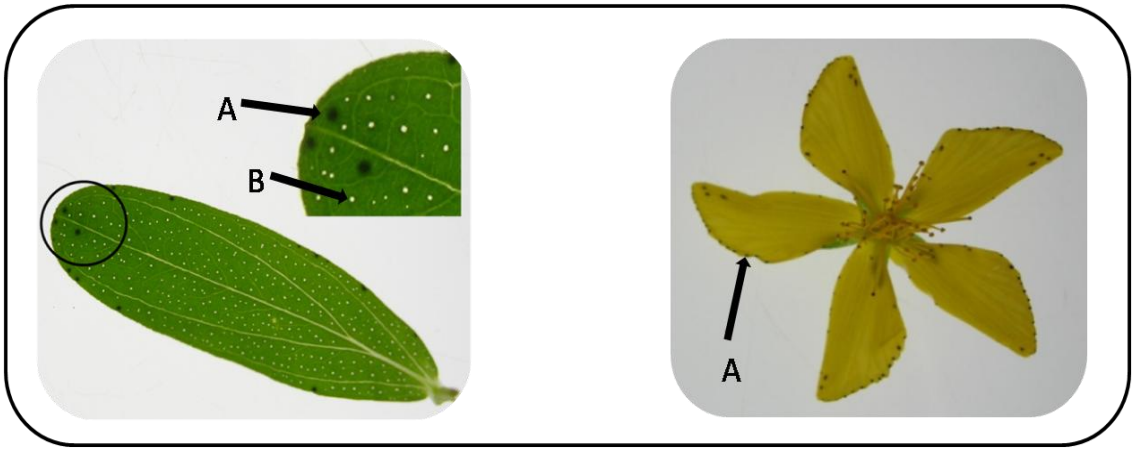


Figure 1.2: Detailed view of the typical **(A)** dark and **(B)** translucent glands, present in leaves and flowers from *Hypericum perforatum* L.

Reproduction of *H. perforatum* is carried out by two ways: vegetatively and through the production of seeds in the flowers. The vegetative reproduction usually occurs after mechanical damage or disturbance of the plant. Upon damage, suckers arise from underground rhizomes and latter separate from the parental plant, concluding this reproduction process [Tisdale *et al.*, 1959]. The flower production in the northern hemisphere occurs from June to September and, since *H. perforatum* is a facultative apomict, the production of seeds may occur without pollination [Barcaccia *et al.*, 2006]. Typically, one plant can produce 30.000 seeds per year, which remain viable for up to 10 years [Tisdale *et al.*, 1959] although in this situation germination and maturity is only reached by a small percentage of individuals [Cech, 1998].

1.1.2. Geographic distribution and ethnobotanic aspects of *H. perforatum* L.

Geographic distribution

H. perforatum L. is native and widely distributed in Europe, northern Africa and the Middle East. Moreover, this species can be found in most regions of Asia, namely western Asia and Siberia, but also in China and some regions of tropical Asia, like India [URL 6]. In other regions, *Hypericum* species were first introduced by European colonists because of their potential uses as medicinal plants [Harris *et al.*, 1997]. In South America and West Indies *H. perforatum* became naturalized but is considered as an invasive species. Moreover, in some other regions like North America, the south of Africa and Oceania, *Hypericum* species are nowadays considered highly dangerous and prominent invasive weeds due to the lack of pathogens and/or successful competing native plants. The importance and economic impact of *H. perforatum* in these regions will be further discussed below. Recently, the presence of *H. perforatum* as an invasive species has also been reported in the coastal regions of Alaska [URL 7].

Figure 1.3: *H. perforatum* as a native plant found across the Portuguese territory.

Considering the Portuguese territory, *H. perforatum* is the most widely distributed species from the genus *Hypericum*, although many other species (Table 1.1) can also be found, scattered across the country [Nogueira *et al.*, 2000 and 2008]. In the Mediterranean basin, namely in Portugal, this species displays narrow (var. *angustifolium*) or small (var. *microphyllum*) leaves, distinct from those found in central and northern Europe, which display large (var. *perforatum*) leaves [Dias, 2000; Hashida *et al.*, 2008].

Table 1.1: Species from the genus *Hypericum* found in the Portuguese territory [Nogueira *et al.*, 2000 and 2008].

H. perforatum L.	*H. elodes* L.	*H. pulchrum* L.	*H. linarifolium* Vahl.
H. androsaemum L.	*H. undulatum* Schousb.	*H. pubescens* Boiss.	*H. montanum* L.
H. foliosum Aiton	*H. tomentosum* L.	*H. perfoliatum* L.	*H. humifusum* L.
H. grandifolium Choisy	*H. dentatum* Loisel.	*H. palustre* Salisb.	*H. canariense* L.
H. hircinum Aiton	*H. calycinum* L.	*H. glandulosum* Aiton	

Origins of common and scientific names of *H. perforatum* L.

Several theories concerning the origin of both common and scientific names of *H. perforatum* exist. One of the most well-known theories says that the scientific name "Hypericum", adopted by Linné to describe the whole genus of St. John's wort [Erdelmeier *et al.*, 2000], is based in the ancient Greek culture, meaning "above (=*hyper*) the images (=*eikon*)". This theory is supported by the fact that, in the past, *Hypericum* flowers were gathered and placed above religious images or statues, in order to *keep away the evil spirits*, especially during the festivities in honor of the sun (summer solstice), when *H. perforatum* is in full bloom [Cardona *et al.*, 1983]. Variants of this theory point that the term *eikon* means "spirit" or "magic", since it was thought that *H. perforatum* had mysterious, exorcist properties [Bombardelli *et al.*, 1995; Dias, 2000], protecting those who kept the plant at home against madness, nightmares and possessions by devils. Finally, the term "perforatum" refers to the transparent glands, mainly visible in the plant leaves, as already explained [Ciccarelli *et al.*, 2001; Erdelmeier *et al.*, 2000].

The common name "St. John's wort" finds its origins in Middle Ages but the explanation for the name is not clear. According to one theory, the plant sprang from the blood of John the Baptist when he was beheaded. A more widely spread (and less *impressive*) theory says that, in the Middle Ages, *H. perforatum* plants seemed to start flowering on June 24, the day when John the Baptist's birthday was celebrated [Erdelmeier *et al.*, 2000]. Apart from "St. John's wort", other common names of *H. perforatum* are displayed in Table 1.2.

Table 1.2: Common names of *H. perforatum* used worldwide [URL 3].

Region	Common names
USA, UK	St. John's wort, Klamathweed, Goatweed, Goatsbeard, Gammock
Germany	Johanniskraut, Tüpfel-Johanniskraut, Gemeines Johanniskraut, Echtes Johanniskraut, Blutkraut, Tüpfel-Hartheu
Italy	Iperico
Portugal, Brazil	Hipericão, Milfurada, Erva de S. João
France	Millepertuis, Millepertuis perforé, Casse-diable
South America, Spain	Hipérico, Todabuena, Corazoncillo, Castellas

1.1.3. Economic relevance of *H. perforatum* L: From ethnopharmacological uses to modern medicine.

Traditional uses of *H. perforatum* L.

H. perforatum has a long, worldwide tradition as a medicinal plant and is included in the traditional pharmacopeia of many countries [Erdelmeier *et al.*, 2000], being the most cited species from the genus *Hypericum* in these publications [Dias, 2000]. In Europe, this species is one of the oldest medicinal plants, being first documented by the ancient Greeks. The therapeutic properties of *H. perforatum* were first described by Hippocrates (ca. 460-377 B.C.) but other herbalists continued his work. The most prominent were Theophrastus (ca. 372-287 B.C.), Dioscorides (ca. 40-90 A.D.) with his work "*De Materia Medica*", Galen (ca. 130-200 A.D.) and Pliny (ca. II A.D.), in his work "XXV Book of *Historiarum Mundi*", also referred the medicinal uses of *H. perforatum* [Bilia *et al.*, 2002]. Most traditional uses were related to mysticism due to the blood-red colour of the extracts obtained from this species. In fact, the red oil, obtained from soaking flowers and leaves in vegetable oil, was thought to have a regenerating action on the blood. Apart from these uses, *H. perforatum* was also referred in the folk medicine for the treatment of diarrhea, dysentery, jaundice, menorrhagia, hysteria, nervous affections, hemoptysis, hemorrhoids and bronchial infections [Erdelmeier *et al.*, 2000]. The Swiss physician Paracelsus (1493-1541) considered this species as *a good plant against dementia* [Dias, 2000], describing it also as *arnica of the nerves* [Bilia *et al.*, 2002] while Mattioli, in his work *Discorsi* (1557), referred diuretic and antimalarial properties from *H. perforatum*. Moreover, the oily preparations from St. John's wort are recommended by folk medicine against dyspeptic conditions and for the external treatment of myalgies, wounds, burns, bruises or swellings [Erdelmeier *et al.*, 2000].

Nonetheless, most of these traditional uses don't have scientific support or still need definite studies for the confirmation of the claimed therapeutic bioactivities. Apart from *H. perforatum*, many other species from the genus *Hypericum* have a long tradition of use in folk medicine for the treatment of several diseases. Some of those species and their uses are presented in Table 1.3. Other uses of *H. perforatum* are better documented in scientific literature, as discussed below.

Table 1.3: Some species from the genus *Hypericum*, used in folk medicine [Dias, 2000].

Species	Traditional use	Country (Region)	Reference
H. brasiliense	Antiseptic	Brazil	Rocha *et al.*, 1995
H. laxiusculum	Astringent		Salgues, 1961
H. connatum	Anti-inflammatory, Astringent		
H. ericoides	Used against Renal Calculi and circulatory problems	Spain	Cardona *et al.*, 1983
H. canariensis	Anti-inflammatory, Diuretic, Vermifugal	Spain (Canarias)	Mederos *et al.*, 1996
H. nummularium	Vulnerary	Pyreneans	Nétien *et al.*, 1964
H. erectum	Hemostatic, Astringent, Vulnerary	China/Japan	Yasaki *et al.*, 1990
H. patulum	Hemostatic, Astringent, Anti-tumoral	China	Ishiguro *et al.*, 1993
H. chinense	Antiseptic		Aramaki *et al.*, 1995
H. henryi	Anti-hepatic		Wu *et al.*, 1998
H. japonicum	Antiseptic, Anti-hepatic, Anti-tumoral		Ishiguro *et al.*, 1994
H. sampsonii	Anti-tumoral	Taiwan	Chen *et al.*, 1985
H. salsugineum	Antiseptic	Turkey	Sakar *et al.*, 1988
H. origanifolium			
H. lanuginosum			
H. triquetrifolium	Sedative		Apaydin *et al.*, 1998
H. roeperanum	Treatment of female sterility	Central Africa	Rath *et al.*, 1996
H. papuanum	Antiseptic	Papua New Guinea	Leach *et al.*, 1988
H. hookerianum	Diuretic, Vulnerary, Antiseptic	India	Mukherjee *et al.*, 2000

Current pharmacological applications and market relevance of *H. perforatum* L.

Nowadays, the main clinical uses of *H. perforatum* are related to the treatment of mild to moderate depressions. The "Monograph of the German Commission E" refers the use of aqueous or alcoholic extracts of *H. perforatum* for the treatment of psychovegetative disorders, moderate depression, nervous disturbances and anxiety [Erdelmeier *et al.*, 2000]. Although the mechanism of action is still not completely understood [Poutaraud *et al.*, 2007], the efficacy of *H. perforatum* extracts in mild or moderate depressions has been demonstrated in numerous double-blind, placebo-controlled randomized trials and confirmed by many meta-analyses [Capasso *et al.*, 2008]. Moreover, several trials comparing *H. perforatum* extracts with commercially available antidepressants showed similar therapeutic properties and fewer (apparently) side-effects [Knuppel *et al.*, 2004]. Nonetheless, some side effects have been described and interactions (in some cases life-threatening) affecting the metabolism of several clinically important drugs have also been found [Capasso *et al.*, 2008]. Other properties of *H. perforatum* are under clinical investigation, such as antibacterial, antiviral, antineoplasic

and antipsoriatic activities [Erdelmeier *et al.*, 2000; Miskovsky, 2002; Dell'Aica *et al.*, 2007]. Although many therapeutic properties have been found in *H. perforatum* whole extracts, it has been difficult to find the specific compounds, directly responsible for each bioactivity of this plant. It is now thought that many of these therapeutic properties are the result of the synergistic action of many groups of compounds present in *H. perforatum*.

The success and economic relevance of *H. perforatum* as a medicinal plant in Europe is higher than in other regions of the world. Per instance, prescription of *Hypericum*-based products in Germany during the last decade was approximately 20 times higher than fluoxetine hydrochloride (Prozac®), one of the most prescribed antidepressants [Greeson *et al.*, 2001] with a therapeutic effect similar to some *H. perforatum* extracts [Behnke *et al.*, 2008]. Despite the predominance of the European market, sales in the US reached US$ 10 million in 2005, putting *H. perforatum* among the top 10 selling herbs during that year [Capasso *et al.*, 2008]. This leading position, as a prescribed or over-the-counter remedy for the treatment of mild to moderate depression, is responsible for the titles "Prozac of the XXI century" [Nogueira *et al.*, 2000] or "vegetable Prozac" [Rutten, 2007], given to *H. perforatum*.

1.1.4. *Hypericum*: The invasive species' point of view.

When plants are introduced in new regions, as *H. perforatum* L. was in Oceania, North America and other areas, they are often liberated from their natural enemies [Mitchell *et al.*, 2003; Maron *et al.*, 2004]. In the absence of these pathogens, the invasive species suffers less damage, which means that less energy has to be spent in recovering the damaged tissues. Active defense upon pathogen attack, another energetically costly event, is also minimized. Moreover, after some generations, energy expenses in passive defense mechanisms may also be lower since environmental pressure decreases by the absence of one natural selection promoter, the pathogen. Therefore, invasive species gain competitive advantages over natives, becoming dominant in recipient communities since they can allocate the metabolic flux, as well as more energy, towards growth and reproduction [Maron *et al.*, 2004].

In the case of *H. perforatum*, it is known that this species is a vigorous competitor, even on its native regions, establishing in waste grounds, roadsides, pasture or open woodland [Buckley *et al.* 2003]. *H. perforatum* can also be found on recently disturbed sites, like logging camps, mining and construction areas or regions recently consumed by fire [Tisdale *et al.*, 1959], displacing and inhibiting

the settlement and establishment of native flora [Briese *et al.*, 1995]. The success of *H. perforatum* establishment is due to the high tolerance to a variety of soils, from dry, rocky, shallow soils, to deep fertile ones. Moreover, *H. perforatum* can tolerate drought and disturbance conditions by storing reserves in its root crown [Buckley *et al.*, 2003]. In the particular case of regions where this species was naturalized as an invasive weed, and in the absence of pathogens, the plant growth and dispersal can build up to infestation densities in a relatively short time span of 10 to 20 years [Buckley *et al.*, 2003].

H. perforatum competition for fertile lands is responsible for great economic losses in agriculture. As an example, *H. perforatum* was introduced in California in the beginning of the XX century and, by the middle of the century, the species was already spread over two million acres, occupying lands previously used for agriculture. Besides agricultural crops, livestock was also affected by *H. perforatum* since the plant was also competing for the pasture. Moreover, toxicity associated with some of *H. perforatum* major compounds, such as hypericin, were responsible for photosensitivity, severe dermatitis, loss of weight and, in rare cases, death of grazing livestock (Fig. 1.4) [Tisdale *et al.*, 1959; Mitich, 1994].

Figure 1.4: *H. perforatum* is an invasive species in many countries, displaying a strong impact on **(A)** grazing lands and in **(B)** animals, due to the toxicity of some constituents [URL 8].

Several approaches for the control of *H. perforatum* populations have been made. Mechanical treatments, such as mowing or hand pulling, are not effective since the roots of this species are fragile and fragmentation leads to vegetative reproduction, as previously referred (chapter 1.1.1). Another drawback of mechanical methods is the increased dispersal of seeds [Tisdale *et al.*, 1959]. Fire is equally ineffective since it's not a specific method for *H. perforatum* control and most of the native

vegetation is lost. Moreover, fire treatment leads to empty lands, ideal for the vegetative growth of new individuals, arising from deeper (and therefore protected from fire) roots of *H. perforatum*.

Chemical treatments can cause reduction in the population of *H. perforatum*. Some of the best studied herbicides against *H. perforatum* are *Gliphosate*, *Fluoroxypyr* and a combination of *Triclopyr* and *Picloram* [Campbell *et al.*, 1984]. Promising killing rates have been achieved using *Fluoroxypyr*, which is partially selective for *H. perforatum* since it does not affect grasses or clover, making this herbicide a good option for the treatment of pasture grounds [Maron *et al.*, 2004]. Although promising, the application of chemicals in effective quantities has several environmental drawbacks and is not effective in all *H. perforatum* infested lands. For this reason, other methods for infestation control are under study, namely, biological approaches, discussed below.

1.1.5. Pathogens affecting *Hypericum*: Agents in weed control.

Several organisms are known to be pathogens for *H. perforatum* (Table 1.4). Most (if not all) of them have a broad range of plant hosts but their pathogenic action in *H. perforatum* is under study, especially in countries where this species is considered an infesting weed. Therefore, one of the most dedicated countries is Australia, which has a long history (starting from the early 1930's) and knowledge in research of *Hypericum* pathogenic interactions.

Table 1.4: Natural enemies described for *Hypericum perforatum* L.

Pathogen	Taxonomy – Phylum (common name)	References
Colletotrichum gloeosporioides	Ascomycota (Fungus)	Debrunner *et al.*, 2000
		Schwarczinger *et al.*, 1998
		Filoda, 2004
Diploceras hypericinum		Putnam, 2000
Sphaeropsis tumefaciens		Kerckhove *et al.*, 2002
Fusarium solani		Gaetán *et al.*, 2004
Sclerotium rolfsii	Basidiomycota (Fungus)	Keinath *et al.*, 1999
Melampsora hypericorum		Bruzzese *et al.*, 1992
Aculus hyperici	Arthropoda (Mite)	Jupp *et al.*, 1997b
Chrysolina quadrigemina	Arthropoda (Beetle)	Wilson *et al.*, 1943
Aphis chloris	Arthropoda (Aphid)	Briese *et al.*, 1995
Zeuxidiplosis giardi	Arthropoda (Fly)	Wilson, 1960
Chamaesphecia nigrifrons	Arthropoda (Moth)	Lastuvka *et al.*, 1995

One of these pathogens is the fungus *Colletotrichum gloeosporioides*. This fungus is responsible for anthracnose disease in a broad range of plant species distributed all over the world, as

will be discussed in the next chapter. As for many other plant-pathogen interactions, *H. perforatum* susceptibility to *C. gloeosporioides* disease (Fig. 1.5) varies according to the cultivar of the plant. Other fungi are also known to be pathogenic to *H. perforatum*, such as *Melampsora hypericorum* or *Diploceras hypericinum*.

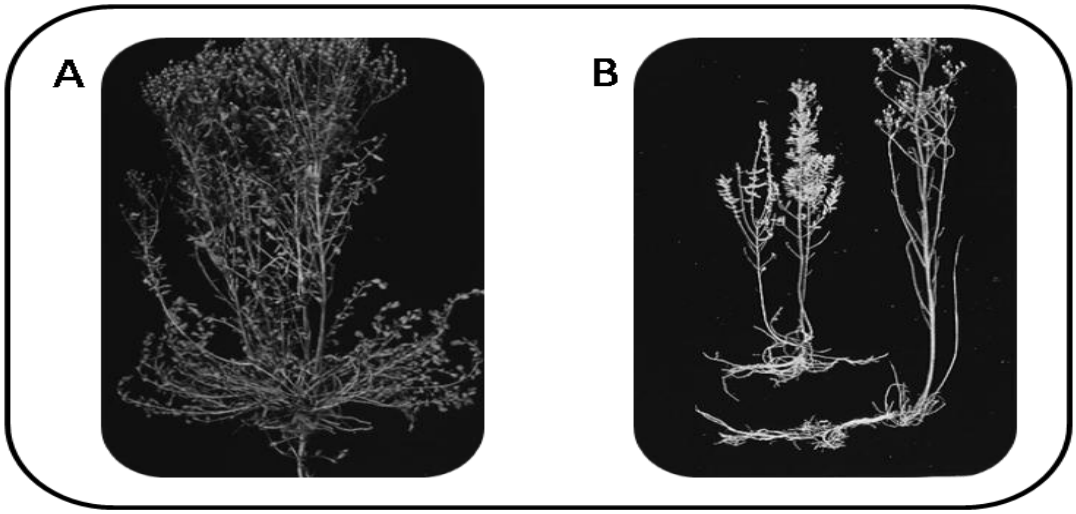


Figure 1.5: *H. perforatum* infection by *C. gloeosporioides*. **(A)** Control plants; **(B)** *C. gloeosporioides*-infected plants [McLaren *et al.*, 1997].

One of the most intensely studied pathogens of *H. perforatum* is the leaf-feeding beetle *Chrysolina quadrigemina* (Fig. 1.6). This beetle is becoming a relatively successful control agent, able to sustain *H. perforatum* spread in Australia. *C. quadrigemina* slows down growth rates in open regions by developing an intense but periodic damage, characteristic of this pathogen. Although resistant to the defense mechanisms of *H. perforatum*, this species has proven to be ineffective in some locations like shaded areas [Buckley *et al.*, 2003], high altitude and cold regions [Campbell *et al.*, 1984].

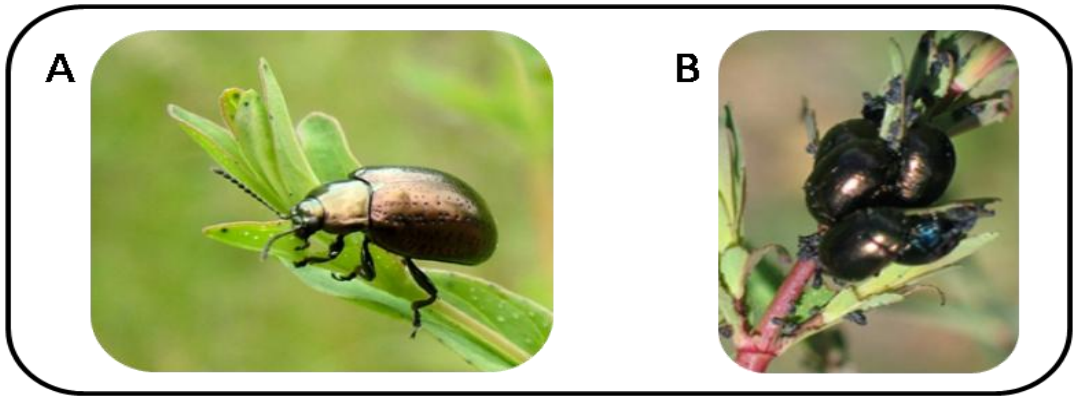


Figure 1.6: *Chrysolina quadrigemina*, a natural enemy of *H. perforatum*. **(A)** URL 9 and **(B)** URL 10.

Another prominent pathogen being studied in Australia is the mite *Aculus hyperici* (Fig. 1.7). This species is well established in south-eastern Australia and stunts *H. perforatum* growth, causing a significant reduction in plant vigour and seed production [Mahr *et al.*, 1997]. Nonetheless, different plant susceptibilities have been found, being responsible for the partial failure of this mite as a control agent of *H. perforatum* alone [Jupp *et al.*, 1997]. Despite this drawback, new studies suggest that this mite may be successfully applied together with other pathogens or with competitive native plants, in the control of *Hypericum* populations [Cullen *et al.*, 1997].

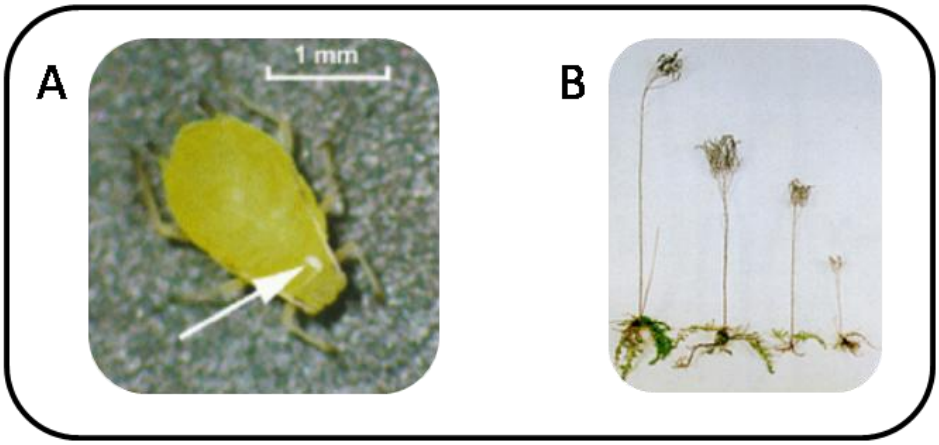


Figure 1.7: The mite *Aculus hyperici*. **(A)** The mite on the top of an aphid, one of the most common means of dispersal for this pathogen. **(B)** Effect of the mite on *H. perforatum* plants [Mahr *et al.*, 1997].

Chapter 1.2

Colletotrichum gloeosporioides

1.2. *Colletotrichum gloeosporioides*

1.2.1. Diseases associated with *Colletotrichum gloesoporioides*.

The genus *Colletotrichum* represents a vast group of Ascomycetes, an economically important group of fungi spread worldwide which can cause anthracnose disease on several significant crops and ornamental plants in tropical, subtropical and temperate regions around the world [Bailey *et al.*, 1992]. It is estimated that nearly 500 plant species are their hosts, with most plants said to be infected by *Colletotrichum gloeosporioides* [Farr *et al.*, 2006]. Considering the present work, *H. perforatum* is also known to be affected by anthracnose [Debrunner *et al.*, 2000; Schwarczinger *et al.*, 1998]. Although fungi from the genus *Colletotrichum* are considered a plant pathogen, some rare reports of human pathogenic interaction can be found, usually associated with corneal ulcers (keratitis) after eye injuries [Fernandez *et al.*, 2002] Additionally, a rare case of subcutaneous infection caused by *C. gloeosporioides* has also been described [Guarro *et al.*, 1998].

1.2.2. Anthracnose dispersal and disease symptoms.

The dispersal methods of *Colletotrichum*, like for most fungi species, are mediated by rain splash [Ntahimpera *et al.*, 1997], wind [Brennan *et al.*, 1985] or through the seeds and fruits carried by animals. Moreover, following dispersal of the spores, viability in the soil is known to stand for at least a year [Freeman *et al.*, 2002] until favorable conditions for germination occurs. The symptoms of this disease are not always similar. There is enormous variation, depending on the plant species infected [Freeman *et al.*, 1998]. Nonetheless the most typical symptoms include early chlorotic spots that may latter develop into necrotic lesions (Fig. 1.8). As the pathogen colonization advances, necrotic lesions coalesce and the infected plant tissue (or the host) eventually dies [Palmateer *et al.*, 2007; Schwarczinger *et al.*, 1998]. Depending on the plant species, anthracnose disease symptoms can be found in nearly all parts of the plant. Strawberry (*Fragaria* × *ananassa* Duch.) is a good example of the broad range of plant structures that can develop anthracnose. The disease is known to be responsible for crown rot and root necrosis (usually leading to wilting and death of the host), fruit rot and irregular, black leaf spots [Xiao *et al.*, 2004]. Additionally, in some plant species the incidence of infection in the same tissue varies according to the age. Young corn leaves, for example, are more commonly affected than mature leaves. This may be related to differences in the surface constitution. Young leaves have an increased wax cover, necessary for spore adhesion, as will be explained latter in this chapter. Symptoms in *H. perforatum*, like in most plants, include brown, sunken necrotic lesions and reddish color of infected plants. At a later stage of the pathogenic interaction, the infected plant tissues dry and finally die [Debrunner *et al.*, 2000]. Moreover, if the infection occurs in the basal parts of the plant, the whole host dies [Schwarczinger *et al.*, 1998]. Methods for controlling anthracnose disease include application of fungicides, proper spacing between plants and periodic pruning to maximize ventilation and minimize shading. Removal of dead biomass from the ground is also useful in minimizing high levels of *Colletotrichum* available to trigger the disease.

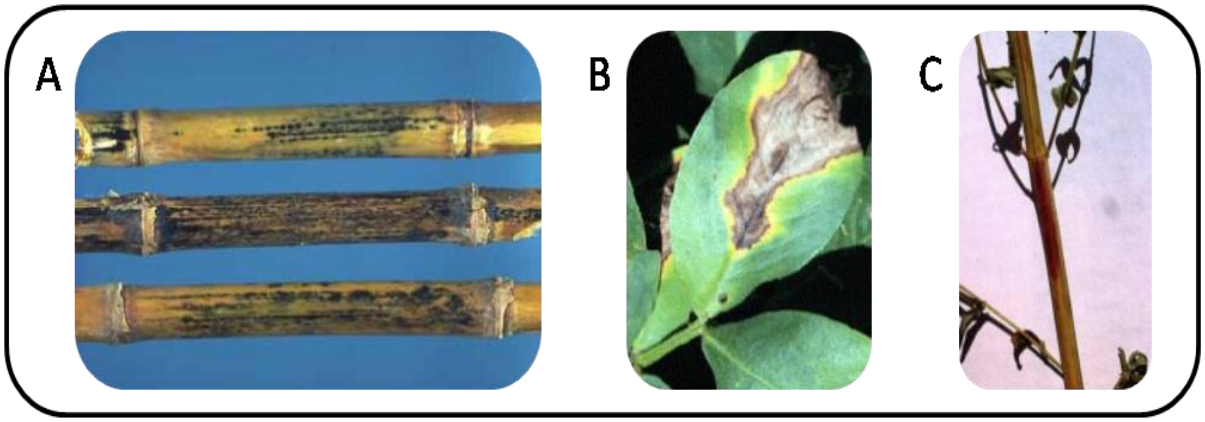


Figure 1.8: Most common anthracnose symptoms in **(A)** stalks and **(B)** leaves on a broad range of hosts, including **(C)** *H. perforatum* [URLs 11 and 12].

1.2.3. Economic relevance of anthracnose

The strong influence of *Colletotrichum* species on economy is due to its pathogenicity to key agricultural plant species all over the world (Fig. 1.9). In the United States, this disease was not considered a relevant problem in corn (*Zea mays*) production, before the 1970s. However, in the early 1970s, severe epidemics affected production in several regions of the country. Some of those regions, like Indiana, were so strongly affected that, 2 years after the beginning of the epidemic, sweet corn canning industry from west-central Indiana was essentially eliminated by shortage of raw-material [Bergstrom *et al.*, 1999]. Another example comes from the fruit from saw palmetto (*Serenoa repens*), which is an important raw-material in pharmaceutical and dietary supplement industries, with global sales over $2 billion per year. In 1997, the fruit harvest in Florida was decimated by premature fruit drop due to anthracnose [Carrington *et al.*, 2001]. One of the main characteristics of *C. gloeosporioides* is the ability to cause latent or quiescent infections by synchronizing its own growth and morphogenesis with the host's development. This capacity turns *C. gloeosporioides* in one of the most important postharvest pathogens. Postharvest diseases can decrease not only the crop yield but also organoleptic characters, especially in fruits. This situation can lead to the complete loss of harvested fruits, when aesthetic features do not correspond with the high-quality market demands [Arauz, 2000]. The impact of anthracnose in agriculture is much higher if we consider long-term crop cultures. Some crops, like apple, mango or citrus require high, long-term investments in growing and maintenance of trees. Depending on the plant tissues affected, the complete tree can be lost before profitable production is

achieved. Moreover, a common process used by farmers to accelerate climateric fruit ripening is to perform an ethylene wash of the fruits [Timmer *et al.*, 1998]. This process not only artificially triggers ethylene-mediated fruit ripening, but also triggers morphogenesis of fungal appressoria [Uhm *et al.*, 2003], as discussed in chapter 1.2.5.3. The economical impact of *C. gloeosporioides* as a plant pathogen is increasing as new anthracnose disease reports come out, for new plant species (Table 1.5). Moreover, due to the growing flux of agriculture goods travelling worldwide, the raising menace of long range epidemics is supported by the potential of *C. gloeosporioides* for cross-infection in a broad range of host plants.

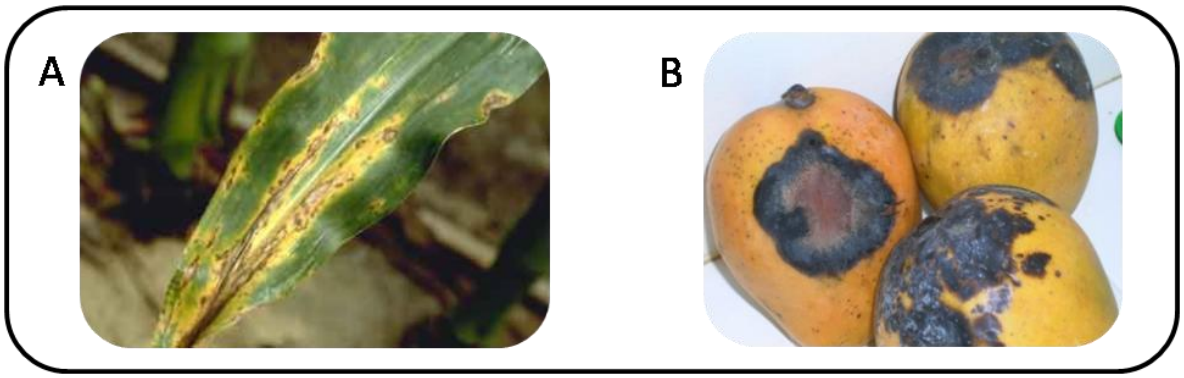


Figure 1.9: Anthracnose affects several economically important crops such as **(A)** corn and **(B)** mango [URL 11].

Table 1.5 – Worldwide reports on the first identification of *C. gloeosporioides* as a pathogen of several plant species.

Crop culture	Country	Year of first report	Reference
Hypericum perforatum (St. John's wort)	Switzerland	1995	Debrunner *et al.*, 2000
	Hungary	1997	Schwarczinger *et al.*, 1998
	Poland	1998	Filoda, 2004
Salsola tragus (Russian-thistle)	Hungary	1996	Schwarczinger *et al.*, 1998(b)
	Greece	2005	Berner *et al.*, 2006
Hibiscus rosa-sinensis (Chinese Rose)	Argentina	1999	Rivera *et al.*, 2000
Hylocereus undatus (Pitahaya)	USA (Fl)	2004	Palmateer *et al.*, 2007
Fragaria × ananassa (Strawberry)	Argentina	1999	Mónaco *et al.*, 2000
Gaultheria procumbens (Wintergreen)	Canada (BC)	2001	Elmhirst *et al.*, 2003
Eugenia dysenterica (Cagaita)	Brazil (DF)	2000	Anjos *et al.*, 2001
Syagrus oleracea (Gueroba)	Brazil (DF)	1999	Charchar *et al.*,2002
Taxus mairei (Chinese yew)	Taiwan	2003	Fu *et al*, 2003
Lupinus albus (White Lupin)	Poland	1995	Frencel, 1998
Liriodendron tulipifera (tuliptree)	Argentina	1986	Lori *et al.*,2004
Arceuthobium tsugense (Hemlock dwarf mistletoe)	Canada	1996	Kope *et al.*, 1997
Prunus africana (Red Stinkwood)	Kenya	1996	Mwanza *et al.*, 1999
Trichosanthes kirilowii (Gourd)	China	2000	Li *et al.*, 2007
Manihot esculenta (Cassava)	Nigeria	1994	Fokunang *et al.*, 1997
Persea americana (Avocado)	Mexico	2003	Avila-Quezada *et al.*, 2007
Passiflora edulis (Passion fruit)	Argentina	1997	Wolcan *et al.*, 2000
Psidium guajava (Guava)	Argentina	2001	Carranza *et al.*, 2002

Potential uses of *Colletotrichum* species in agriculture

Curiously, although *Colletotrichum* species have a great range of hosts, being responsible for severe losses in agriculture, some strains are being used as mycoherbicides in biologic control of invasive plants around the world (Fig. 1.10). Highly specific strains of *C. gloeosporioides* are being used in the control of invasive plants, like *Miconia calvescens* in Hawaii [Killgore *et al.*, 1999], *Lygodium microphyllum* and *L. japonicum* in Florida [Jones *et al.*, 2003] and *Pueraria montana* in Philadelphia [Britton *et al.*, 2002]. Furthermore, several strains of *Colletotrichum* species have already been included in commercial patents. A strain of *C. gloeosporioides* has been patented for the control of *Aeschynomene virginica* (northern jointvetch), a weed in rice and soybean crops [Freeman *et al.*, 1998]. A strain of *C. truncatum* was included in a U.S. patent for biological control of *Sesbania exaltata*, another weed in soybean crops [Boyette, 1991 and 1991b]. Another strain, from *C. capsici*, has been reported for control of *Ipomoea lacunosa* [Cartwright *et al.*, 1994].

Figure 1.10: Some strains of *C. gloeosporioides* are being used as biological control agents (mycoherbicide) in several economically important crop cultures [URL 13].

1.2.4. Taxonomy of the genus *Colletotrichum*

The genus *Colletotrichum* belongs to the subphyla Ascomycotina, a group of fungi from the phyla Dikaryomycota [Scheffer, 1997]. The taxonomy of the genus *Colletotrichum* has seen much progress in the last decades. Nonetheless, the systematics of this fungal pathogen still has many flaws and further studies are necessary. The lack of basic knowledge can be demonstrated by the fact that, depending on the taxonomic guidelines used, the number of species in this genus can range from 29 to

over 600 [Sutton, 1992]. As an example, in 1957, von Arx reclassified nearly 600 *Colletotrichum* species as synonyms of *C. gloeosporioides* [Dickman *et al.*, 2003]. His taxonomic guidelines relied mainly on morphological features of the conidia, vegetative and sexual structures, host specificity and colony growth characteristics [Smith *et al.*, 1990]. Despite von Arx efforts, correct identification and classification is difficult since some conidial morphology and colony characteristics vary even within isolates [Chakraborty *et al.*, 1997]. This situation leads to several cases of incorrect pathogen classification [Agostini *et al.*, 1992; Ureña-Padilha *et al.*, 2002]. The morphological variations observed in some structures could be part of the key for the success of this genus as a pathogen for a broad range of plants. One of the most confusing species in the genus *Colletotrichum* has been *C. gloeosporioides* (teleomorph *Glomerella cingulata*). A good example for the complexity of *C. gloeosporioides* identification, recurring solely to classical morphological criteria, can be seen in table 1.6 [Freeman *et al.*, 1998].

Table 1.6: Comparison between isolates of *C. gloeosporioides* from avocado and almond [Freeman *et al.*, 1998].

Character	Almond	Avocado
Morphology in culture	White to gray	White, gray to black
Sexual stage	Absent	Present
Optimal growth temperature	20 to 22°C	26 to 28°C
Average growth rate on PDA at optimal temperature (mm/day)	2.2	6.4
Infected plant part	Immature fruit (dry rot)	Leaves, twigs, immature and mature fruits
Latent infection	Absent	Present
Benomyl sensitivity	Insensitive	Sensitive

Due to the difficulty on identification of species from such an economically important group of Ascomycetes, biochemical and molecular approaches are being used, since the last decade, as complements for the classical identification methods, based on morphological features [Freeman *et al.*, 1996]. These new molecular approaches have proven useful in several cases, pointing out incorrect pathogen identifications [Brown *et al.*, 1996]. The use of polymerase chain reaction (PCR) specific primers for polymorphic ribosomal DNA regions, such as the "internal transcribed spacer" (ITS), have proven to be an important and efficient identification tool [Freeman *et al.*, 2000]. Nonetheless, some species of *Colletotrichum* still cannot be distinguished by this method [Ureña-Padilha *et al.*, 2002]. Other molecular approaches, such as "random amplified polymorphic DNAs" (RAPDs) [Xiao *et al.*, 2004; Martinez-Culebras *et al.*, 2002], "arbitrarily primed polymerase chain reaction" (ap-PCR) analysis [Freeman *et al.*, 1996], or sequencing other polymorphic regions from ribosomal DNA [Johnston *et al.*,

1997] are now arising as powerful tools for correct distinction of closely related *Colletotrichum* species, as well as for correct identification of morphologically distinct *Colletotrichum* species. Biochemical approaches, like sensitivity tests for the fungicide *Benomyl*, are also useful for identification of *Colletotrichum* species. Although screening of fungicide sensitivity is primarily used to estimate the killing potential of chemical compounds, this method has proven to be quite useful in specific and subspecific grouping in *Colletotrichum* [Freeman *et al.*, 1998]. An example of the potential of this method was carried out in Israel [Bernstein *et al.*, 1995] where isolates of unidentified *Colletotrichum* species from several plants were screened using *Benomyl*. During the identification, isolates from *C. gloeosporioides* and *C. acutatum* could be distinguished due to the known higher sensitivity of *C. gloeosporioides* to this fungicide, when compared to *C. acutatum* [Freeman *et al.*, 1998]. Nowadays, the combination of morphological, molecular and biochemical approaches in fungal identification is responsible for a list of 40 distinct species comprising the *Colletotrichum* genus. This list is expected to increase since many "*Colletotrichum* candidate species" have not yet been scrutinized by all available approaches [Kirk *et al.*, 2001; Farr *et al.*, 2006].

1.2.5. *Colletotrichum* as a plant pathogenic fungi: Infection process and mechanisms of penetration.

1.2.5.1. Spore germination

Colonization and pathogenesis in most *Colletotrichum* species require adhesion of spores to the aerial parts of the plant, involving hydrophobic interactions. Moreover, the spore adhesion signaling process in *Colletotrichum* species is thought to occur through surface proteins, as already described for many other pathogenic fungi [Mercure *et al.*, 1994]. Attachment as a prerequisite for spore germination had already been proven for other species of fungi, such as the plant-pathogen *Phyllosticta ampelicida* [Shaw *et al.*, 2000] and the aquatic hyphomicetes *Anguillospora longissima* and *Lunulospora curvula* [Webster *et al.*, 1984; Chaky *et al.*, 2001]. A good exemplificative experiment of the importance, during the attachment, of hydrophobic interactions in successful germination was carried out in Vaillancourt's lab [Chaky *et al.*, 2001]. Germination of *C. graminicola* spores was assayed in two distinct artificial surfaces. A high level of germination (>80%) was observed in hydrophobic polystyrene Petri dishes,

while germination rates in hydrophilic glass slides was very small (<20%). Moreover, after studying this species in depth, the group observed that the two known spore types, characteristic from *C. graminicola*, displayed distinct patterns of response to surface hydrophobicity. While falcate spores could only germinate efficiently in hydrophobic surfaces, oval spores efficiently germinated in artificial hydrophilic surfaces. This difference could be connected to distinct roles of these spores in *C. graminicola* pathogenicity. While falcate spores, produced in acervuli in the plant surface, are responsible for dispersal of the fungi to other plants, oval spores are produced within the plant xylem vessels, being responsible for the systemic spread of the disease in the host [Bergstrom *et al.*, 1999; Chaky *et al.*, 2001]. As information about spore germination builds up, the relevance of spore attachment as a signaling process in germination is increasing in such way that attachment is, nowadays, considered as a pathogenicity factor in plant-microbe interactions [Chaky *et al.*, 2001].

Although hydrophobicity and rigidity of the contact surface are important factors triggering germination of spores, other factors, like the presence of a carbon source, may also promote germination, despite the presence or absence of these rigidity/hydrophobicity signals. Therefore, multiple response pathways are involved in this early (and complex) step of plant-pathogen interaction [Chaky *et al.*, 2001]. The relevance of all those distinct triggering factors may vary for different species of *Colletotrichum*, since some of them require signals that others do not need [Uhm *et al.*, 2003]. For example, *C. gloeosporioides* do not require hydrophobicity signals for appressorium formation in red pepper interaction, while this signal is essential for triggering appressoria formation in *C. trifolii* interactions [Uhm *et al.*, 2003; Warwar *et al.*, 1996].

1.2.5.2. Germ tube formation

Recognition of both physical and chemical signals from the plant surface triggers germination of *Colletotrichum* spores, leading to the formation and elongation of germ tubes. Germ tubes elongate by apical deposition of glycoproteins and polysaccharides like chitin and glucans. Those compounds are assembled in microfibrils and crosslinked in adjacent polysaccharide chains. The forward growth of the germ tube is mainly driven by actin filaments and other cytoskeletal elements. During growth, the germ tube is directed by the contact with the rigid surface (thigmotropism) of the host, enabling the pathogen to recognize proper infection sites. Some pathogens recognize stomatal openings as infection sites while others recognize anticlinal (perpendicular) walls in the host surface [Mendgen *et al.*, 1996].

1.2.5.3. Appressoria

The physical and chemical signals required for the early steps of fungi development are also necessary for the next step, the differentiation of appressoria (from the Latin *apprimere*, meaning "to press against") [Dean, 1997]. For most pathogenic fungi, the development of appressoria is necessary for the overcoming of a strong physical barrier from the plant, composed by the cell wall and the cuticle. This structure is primarily made of cutin, a structural polymer that comprises 50 to 90% of the cuticle composition [Dickman *et al.*, 2003]. Several studies on how the fungi overcome this structure exist and they point for enzymatic digestion and/or physical pressure approaches [Dickman *et al.*, 2003; Dixon *et al.*, 1999]. Nonetheless, many studies suggest that cell wall degrading enzymes, involved in tissue maceration, have no more than a minor role in pathogenicity of plant infecting organisms [Scheffer, 1997b]. Therefore, the role of physical pressure, in which the appressoria is directly implicated, is now thought to be the most important mechanism in penetration of the host cells.

Physical signaling factors

As previously observed for spore germination, formation of appressoria also requires physical signals, in the form of a hard surface [Hoch *et al.*, 1987]. Spores from *C. gloeosporioides* differentiate appressoria upon contact with a hard surface. Although the spores do germinate in soft agar, they do not differentiate appressoria, suggesting that physical contact is required for appropriate morphogenesis [Perfect *et al.*, 1999]. However, other factors like the presence of a carbon source (previously described as "positively interfering" in the germination of spores), are known to repress appressoria formation, regardless of the surface rigidity signs.

It has been suggested that the surface firmness may "prime" the spores of some fungi, like *C. gloeosporioides*, via Ca^{2+}-calmodulin-mediated signaling pathway [Kim *et al.*, 1998; Chaky *et al.*, 2001]. Although the precise mechanisms involved in this pathway are not fully understood, it is known that calmodulin, a ubiquitous protein responsible for the activation of some enzymes, is involved in fungal cell responses implicated in development. Since calmodulin action is directly connected with availability of Ca^{2+} in the cytosol, all the mechanisms involved in the homeostasis of calcium play a role in appressorium formation [Uhm *et al.*, 2003].

Chemical signaling factors

Plants release a variety of chemical compounds like sugars, phenolics and volatile substances, which can be perceived by the pathogen and used for the development of an infection [Dean, 1997]. Chemical signals, not directly connected with surface hardness, also play an important role in triggering the appressorium formation. Although hydrophobicity and surface hardness could be similar in different plants, the recognition of the chemical signals by a specific pathogen may be decisive for the development of the appressorium and consequent pathogenic interaction. As an example, the fatty alcohol fraction of the surface wax from avocado triggers selective appressorium formation in *C. gloeosporioides* while this triggering cannot be found in other wax-coated plants [Podila *et al.*, 1993]. A scenario for the chemical signaling pathway, postulated for *C. trifolii* is that, after spores recognize the chemistry of the host's surface, endogenous cutinases hydrolyze the cutin polymers, present in the cuticle of the host, into monomers. These long-chain fatty acid monomers will then trigger lipid-induced protein kinases (LIPK), necessary for the appressoria differentiation [Dickman *et al.*, 2003]. Another chemical signal for appressoria formation that is recognized by *C. gloeosporioides* is ethylene, a molecule also known to act as a hormone in climateric fruit ripening [Uhm *et al.*, 2003; Kolattukudy *et al.*, 1995] and on defense signaling mechanisms in plants. The transmission of pre-penetration chemical signals to the fungus is most likely mediated by second messengers like calcium [Ahn *et al.*, 2003], in a pathway similar to the one by which physical signals are also transmitted. The functional redundancy of some triggering signs may provide pathogens with an evolutionary advantage in ever-changing plant-pathogen interactions [Dean, 1997].

1.2.5.4. Differentiation and penetration

After recognition of the triggering signs, appressoria formation and tip swelling occurs, becoming delimited by a septum. At this stage, the spore and the germ tube of *Colletotrichum* species are usually devoid of cytoplasm, which migrates to the appressorium [Dean, 1997]. Maturation of appressorium involves formation of a penetration hypha in the base of the cell and secretion of extracellular matrix materials. These include cell wall degrading enzymes such as pectate lyases, cellulases and cutinases, responsible for the maceration of the physical barriers and other tissues involved in the penetration of the pathogen [Yakoby *et al.*, 2000]. The majority of the extracellular matrix is composed by glycoproteins, responsible for the adhesion of the pathogen to the host [Pain *et al.*,

1996]. Moreover, some of these glycoproteins, like laccases, may be actively involved in fungal defense mechanisms, as well as in morphogenesis and pathogenicity [Ranocha *et al.*, 2002; Anderson *et al.*, 1996]. The high level of proline residues that can be found in the composition of laccases exhibit an increased affinity for polyphenolic compounds [Bergstrom *et al.*, 1999], like those produced by the plant defense mechanisms.

Even though *Colletotrichum* species employ the same assortment of cuticle and cell wall lytic enzymes as other fungi, it has been postulated that they may be able to penetrate the host cuticle and cell wall just by means of turgor pressure [Mendgen *et al.*, 1996]. The turgor pressure exerted by the appressoria is supported by the deposition of new wall layers and melanization [Dean, 1997]. At this stage, the appressoria becomes darker and is considered fully mature. Melanin is a dark, water-insoluble polyketide, which is deposited in a layer of the cell wall, close to the plasma membrane. The only exception is the small pore through which the penetration hypha will develop. Melanin is apparently essential for the generation of turgor pressure, necessary for mechanical penetration, since *Colletotrichum* defective mutants for melanin biosynthesis cannot penetrate plants [Kubo *et al.*, 1985]. Moreover, turgor pressure in appressoria of melanin-deficient mutants is only 30–70% of that measured in normal, fully melanized appressoria. The accumulation of melanin is responsible for a decrease in the porosity of the structure and, therefore, blocks the efflux of cytosolic solutes [Dean *et al.*, 1997]. As a consequence, the melanized appressoria increase and/or maintain the turgor pressure. One solute that is thought to act as an osmolyte, responsible for appressoria turgor pressure, is glycerol [Money *et al.*, 1996].

1.2.5.5. Colonization of the host: From penetration hyphae to biotrophic/necrotrophic interaction

Species from the genus *Colletotrichum* can establish a compatible interaction with their host either by subcuticular or intracellular growth. In subcuticular growth, the pathogen develops beneath the cuticle, forming a network of hyphae. Latter, the pathogen spreads thought the tissue with both inter- and intracellular hyphae, killing the host cells upon contact [Perfect *et al.*, 1999]. Although subcuticular growth occurs in several cases, intracellular colonization is the most common host infection strategy. Typically, the initial stages of *Colletotrichum* infection are similar in both host-interaction methods and include adherence and germination of conidia on the plant surface and production of germ-tubes that latter differentiate to form melanized appressoria [Perfect *et al.*, 1999].

During intracellular growth interaction, and after penetration of the appressoria, a penetration hyphae starts to grow from a thin pore surrounded by walls strong enough to support the turgor pressure, in the middle of the appressoria base. The penetration hypha has a characteristic cell wall, distinct from the one observed in germ tubes. The wall has modified or reduced amounts of chitin. This change in the composition is responsible for an increased resistance to endochitinases secreted by the plant. Moreover, since less cleavage products are created, plant defense signaling will be less effective by the lack of these molecules, known to act as elicitors of plant defense [Mendgen *et al.*, 1996]. While the penetration hypha moves through the plant barriers, host cells respond by the production of papillae. This preinvasion structure response is characterized by the deposition of new cell wall material, in the location of the penetration attempt. At this stage, if the aggressor fails to overcome the reinforced host barrier (proving to be a nonadapted pathogen), termination of anthracnose pathogenesis occurs [Shimada *et al.*, 2006]. On the other hand, if the pressure exerted by the appressoria and the incursion of the penetration hypha to the cell lumen prevails over the strengthened host cell barrier, a pathogenic interaction arises and colonization of the host may take place. After penetration hyphae development and incursion through cuticle and cell wall, one or more primary hyphae grow within the cell lumen, without perturbing the host membrane, developing between the plasma membrane and the cell wall from the host plant. This region is likely to be a key area for avoidance of host defense response, as well as for establishment of biotrophy [Perfect *et al.*, 1999]. These biotrophic hyphae will later spread to the adjacent epidermal cells and inner, hypodermal cells. At a certain stage of host development, the pathogen produces secondary hyphae, characteristic of the necrotrophic interaction. The development of this interaction leads to the formation of macroscopically visible symptoms of anthracnose disease such as dark, necrotic lesions and tissue dead, from where the necrotic hyphae acquire nutrients. Moreover, during necrotrophic interaction, the pathogen differentiates the acervuli and begins sporulation in the surface of the senescent organ [Latunde-Dada, 2001].

The initial feeding on living host cells, prior to subsequent switching to necrotrophy, is the reason why *Colletotrichum* species are considered hemibiotrophic or facultative biotrophs [Perfect *et al.*, 1999]. All the specialized structures and triggering signals previously described constitute *Colletotrichum* infection mechanisms, essential for an effective biotrophic colonization of the host plant. During this stage, the pathogen uses these specialized structures to provide a less confrontational interaction with the host [Dean, 1997]. Moreover, lytic enzymes are secreted in lower amounts while

other extracellular matrix constituents, secreted by the pathogen, play an important role in suppressing defense response mechanisms from the host [Silva *et al.*, 2006].

1.2.6. *Colletotrichum* species: Tools for the study of pathogen-related defense mechanisms in plants.

Species from the genus *Colletotrichum*, such as *C. gloeosporioides*, are often used as models in several studies, from pathogen development and differentiation to the study of plant-pathogen interaction [Farr *et al.*, 1989]. The haploidy of *Colletotrichum* species makes them a good experimental tool for mutational analysis by facilitating the estimation of gene functions, by target disruption approaches [O'Connell *et al.*, 2004]. The relevance of *Colletotrichum* species as model organisms in the study of plant defense responses is growing. This importance is supported by the increase in the screening for *Colletotrichum* species pathogenic for *Arabidopsis thaliana*, one of the most useful and resourceful models in plant biology [Liu *et al.*, 2007].

Another interesting aspect about *Colletotrichum* species is that, during the establishment and colonization of the host plant, members of this genus acquire their nutrients via biotrophy and/or necrotrophy. Therefore, in a single interaction with the host, these pathogens can exhibit two nutrition acquisition models. Initially these nutrients are obtained from the living cells of the host, followed by a necrotic phase where nutrients are obtained from the dead plant cells, killed by the fungus. These two strategies can be used by any species of this genus, at the same time or separately [Farr *et al.*, 1989]. Moreover, species from this genus develop a series of specialized infection related structures such as germ tubes, appressoria, primary hyphae and secondary, necrotrophic hyphae, as previously described. The great variety of structures and nutrient acquisition methods exhibited by species from the genus *Colletotrichum* also make them experimentally attractive organisms for the study of molecular, biochemical and cellular basis of fungal pathogenicity, development and signal transduction [Bailey *et al.*, 1992].

Chapter 1.3

Secondary metabolism

1.3. Secondary metabolism

1.3.1. Introduction

When the first pioneering plant species started the occupation of dry land, nearly 450 million years ago, a new set of challenges, distinct from those existing in watery environments, soon began to appear. This important environmental transformation was accompanied by several physiological adaptations, including the evolutionary emergence of entirely new specialized metabolic pathways [Noel *et al.*, 2005]. While in the beginning of their adaptation to the new, dry environment their challenges were mostly abiotic (temperature, humidity or light intensity), "soon" plants started facing a great, evolving diversity of biotic (viruses, fungi or bacteria) environmental stresses. Moreover, their lack of mobility renders them unable to escape from these potentially damaging agents. This fact has led them to develop many efficient and polyvalent defense mechanisms and, as a consequence, disease is the exception rather than the norm [Bellés *et al.*, 2008]. Most of these protection mechanisms are related to secondary metabolites, defined as compounds produced by plants or other sessile organisms which are not directly essential for basic photosynthetic or respiratory metabolism. The relevance of secondary metabolites from plants dates back from the earliest days of human history. As an example, the

Sumerian civilization described a long ago, in 4.000 B.C, some "therapeutic" properties of *Papaver somniferum*, known at that time as *hul gil* (joy plant). These properties were most likely connected to the high concentration of two secondary metabolites, the alkaloids morphine and codeine, present in this plant species [Theis *et al.*, 2003]. Despite the negative impact of these secondary compounds in human health and society, many other biologically active secondary metabolites have been successfully exploited during the search for new plant-based pharmaceutical products [Noel *et al.*, 2005], cosmetics, fine chemicals or, more recently, nutraceuticals [Bourgaud *et al.*, 2001]. Nowadays, more than 200.000 secondary compounds from plants have been characterized, with their chemical structure resolved [Hartmann, 2007]. The diversity and specificity of secondary compounds is such that many of them are nowadays used in taxonomic identification, as "chemical signatures" of particular species. This remarkable chemical diversity of biologically active compounds present in plants is the result of specialized biosynthetic pathways. These pathways were developed by ongoing evolutionary processes, usually as a response to physical and biotic interactions of plants with their challenging ecosystems [Noel *et al.*, 2005] and, although distinct from primary metabolism (Table 1.7), secondary metabolism is nowadays known to be indispensable for survival.

Table 1.7: Main differences between primary and secondary metabolism [Hartmann, 2007].

Primary metabolism	Secondary metabolism
Genes with high stringency controlling essential functions	Genes with high plasticity that are under selection pressure of the evolving environment
Covers growth and development	Covers interactions with the environment
Indispensable	Dispensable for growth but indispensable for survival in environment
Universal	Unique
Uniform	Diverse
Conservative	Adaptative

1.3.2. Overview on secondary metabolism history: From *expendable* to *essential*

The research of plant secondary metabolites began nearly 200 years ago, when Friedrich Wilhelm Sertürner first isolated from opium poppy (*Papaver somniferum*), in 1806, the *principium somniferum*, nowadays known as morphine. The work of Friedrich was the first to demonstrate that the therapeutic uses could be directly related to one or a few compounds, present in a given plant. As other compounds were being isolated and characterized, the research of natural products began to play an important role in the development of pharmaceutical research, as well as in other areas of organic

chemistry. The increasing knowledge about natural products was afterwards responsible, in the second half of the 19th century, for the first generally accepted definition of secondary and primary metabolisms. In 1873, Julius Sachs, considered one of the "founding fathers" of plant physiology, defined secondary metabolites as "compounds formed during metabolism which are no longer used in the formation of new cells". Apart from being considered "waste or detoxification products", Sachs did not attribute any other functional relevance to these compounds for the survival of plants. Although incomplete, that was the first definition towards the "correct", modern characterization of secondary metabolites [Hartmann, 2007]. Moreover, the terms "primary" and "secondary" metabolism were introduced by Kossel a few years later, in 1891, when he first differentiated secondary metabolites, as opposed from primary ones [Bourgaud *et al.*, 2001].

Until the first half of the 20th century the pathways responsible for the production of secondary compounds were predicted mainly by analogy with organic chemistry reactions. One of the main tools at the time was chemical degradation, in order to produce and compare the chemical structures of the resulting fragments [Staunton *et al.*, 2001]. Until that time, secondary metabolites were still regarded as waste products from plants. Nonetheless, this view began to change by the introduction, in the early 1950's, of radioactivity-labeling techniques for tracing the metabolic flux of compounds (defined as the amount of converted metabolite per unit of time [Matsuda *et al.*, 2005]). This technical breakthrough significantly supported the characterization of several biosynthetic pathways, by means of accurate biochemical evidence and was responsible for the description, during the next two decades, of the basic outlines for almost all major secondary metabolite pathways [Hartmann, 2007]. Moreover, tracing by radioactivity-labeling was also extremely valuable in unveiling the transportation patterns of several compounds throughout the plant tissues, as well as in pointing out the tissues committed to their synthesis and/or accumulation. The accumulated knowledge made the scientific community realize, nearly one decade later, that secondary metabolites were no inert, end products but a dynamic component of plant metabolism. Moreover, at that time the first theories about the importance of secondary metabolites in plant defense (Fraenkel, 1959) and coevolution with herbivores and insects (Raven, 1964) were also arising. Despite the great advantages of radioactivity-labeling, this technique was not enough to provide a deeper understanding and characterization of all the individual steps composing a metabolic pathway. Therefore, the plant was still seen as "a black box" to whom labeled precursors were fed and, a few days or weeks later, labeled products would arise [Hartmann, 2007]. Later in the 1970's the development and optimization of other techniques, like the application of new

sorbents for selective protein separation through column chromatography, added new tools for the purification of proteins. Together with the establishment of more easily handable plant systems (*in vitro* cultures), optimized methods for the separation of proteins were responsible for new breakthroughs in secondary metabolism study, by improving both quality and variety of enzymology tools available.

In the mid 1980's it was generally accepted that secondary products were synthesized *de novo* from simple precursors of primary metabolism through a sequence of reactions catalyzed by specific enzymes. There was "no room" in this definition for spontaneous reactions or unspecific side-activities of enzymes involved in both primary and secondary metabolic pathways. Nonetheless, during the mid 1980's, the first steps in applying molecular tools for the study of secondary metabolism were taken. Genes from phenylpropanoid pathway enzymes like 4-coumarate:CoA ligase (4CL), phenylalanine ammonia-lyase (PAL) or chalcone synthase (CHS) were successfully isolated and functionally expressed. The development of these new molecular tools was the basis for the recent improvements in metabolic engineering of plants. In one hand, molecular tools were useful in providing methods for cell-specific localization of secondary pathways, by immunolocalization of pathway-specific enzymes. On the other hand, these tools allowed gene-transfer (maybe even whole pathways, in the near future) between organisms, for both the study of biosynthetic pathways of secondary metabolites, as well as for the improvement of crops, for instance, against adverse environmental conditions [Hartmann, 2007].

The molecular and enzymology technical achievements developed in the mid 1980's were responsible for many improvements in secondary metabolism research. It is now clear that many metabolic pathways, such as phenylpropanoid, alkaloid or terpenoid pathways, are clustered in metabolons. These multienzyme complexes, a common feature in secondary metabolism, are responsible for the organized metabolic channeling, developed by plants for an optimized biosynthesis of several chemical compounds. These technical achievements were also responsible for the knowledge that enzymes from the secondary metabolism are often found in low concentrations and usually lack feedback regulations, typical from primary metabolism. Despite the low amounts of some enzymes present in plants, the increasing availability of genes encoding secondary metabolism enzymes allow the expression of recombinant enzymes and the recovery of substantial amounts of protein for detailed kinetic and structural studies [Hartmann, 2007].

Nowadays it is also accepted that some enzymes, related only to the secondary metabolism, have evolved in order to create an increased chemical diversity of compounds. For example, some sesquiterpene synthases from conifers are known to generate a few major products, accompanied by

up to 50 chemically distinct minor ones. The high plasticity and diversity guarantees flexible adaptation of plants to the demands of their continuously-changing environment. Therefore, while primary metabolism is known to be responsible for the essential processes of growth and development, secondary metabolism is crucial for the survival of the plants in its environment. By being "dispensable" for growth, secondary metabolism components can be continuously modified and adapted to also dynamic ecological niches [Hartmann, 2007].

From the growing knowledge obtained, especially in the last decades, in scientific research fields like enzymology, molecular biology or ecology, one major conclusion on how secondary metabolism is important for plants can be pointed out: Rather than meaning "less important metabolism", the term "secondary" is now mainly regarded as a "different functional metabolism", much like the "secondary" structure of proteins is different, but equally important as their "primary" or "tertiary" structures [Hartmann, 2007].

1.3.3. Localization and functions of secondary metabolites in plants

The ability of secondary metabolism pathways to generate a great diversity of compounds could only be achieved by means of optimized metabolic channeling, sometimes occurring in specific sub-cellular and/or tissue locations [Winkel, 2004]. In one hand, metabolic channeling brings co-operating enzyme active sites into close proximity and thereby decreases the transit time for intermediates along the biosynthetic pathway [Jørgensen *et al.*, 2005]. On the other hand, it is known that secondary metabolites occur in a wide range of plant tissue types and, for many of those compounds, specialized localization may be necessary for obtaining high local substrate concentrations, indispensable for efficient metabolic rates. Compartmentalization of specific pathways also allows better regulation and coordination of competition between branch pathways for shared enzymes or intermediates. Besides these advantages, both metabolic channeling and compartmentalization of pathways and/or final secondary metabolites are mainly observed in cases where the metabolite, or an intermediate, displays toxic or reactive effects [Winkel, 2004]. In fact, many secondary metabolites are produced by the plant in order to serve as defensive chemicals against pathogens and, due to the usually high bioactivity and autotoxicity risk, they are often secured in specialized secretory structures where concentrations can be kept high enough to allow a successful defensive response, without damaging the surrounding plant

tissues. These structures are often localized externally in the plant, enhancing the effectiveness of the defensive secondary metabolites accumulated within, as they serve as the first line of defense against possible pathogens [Theis *et al.*, 2003]. The role of metabolic channeling during the synthesis of toxic intermediates is clear: To secure swift conversion of labile and/or toxic intermediates into more stable and less toxic constituents by sequestration and by preventing their diffusion into the surrounding cell matrix, where chemical decomposition would take place [Jørgensen *et al.*, 2005]. Interestingly, some secondary metabolites produced by plants for their defense, like some pyrrolizidine alkaloids, can be used by specialized herbivores that, during the evolutionary process, managed to avoid the toxic effects of those compounds, modifying their structure and accumulating them in order to attain protection against their own enemies [Kutchan, 1995; Theis *et al.*, 2003]. Despite the general recognition of secondary metabolites as defense-related compounds, their great variety is translated into a broad range of functions (Table 1.8). Some secondary metabolites also display allelopathic effects, inhibiting the growth of competing plants, while others are used as surfactants, light absorbing agents [Vining, 1990], metal transporting agents (by improving metal solubilization and subsequent uptake [Dixon *et al.*, 1995]), sexual hormones, differentiation effectors and symbiotic agents between organisms [Demain *et al.*, 2000]. In most of these cases, specialized accumulation structures are less common since most of these compounds are readily released to other tissues and/or to the environment as soon as they are synthesized. One of the most studied uses of secondary metabolites, apart from direct plant defense, is related to symbiotic functions. That includes secondary metabolites that are produced by the plants in order to attract pollinators and, in addition, other compounds that are released to attract insects, not for pollination, but for their predatory/parasitic effects on herbivores feeding on the plant. Moreover, another possible (but still controversial) function of some secondary metabolites has been postulated. Some volatile compounds that are produced by the plant upon herbivore feeding are thought to be responsible for communication between neighboring plants, enabling the surrounding plants to "know" about the presence of a nearby pathogen, in a mechanism similar to that of predatory/parasitic organisms. Although some models have been demonstrated, plant-to-plant communication by volatile signals is considered a rare situation [Theis *et al.*, 2003].

Table 1.8: Brief list of functions associated to some secondary metabolites.

Pollination	Allelopathy	Symbiosis
Seed dispersal	Antibiotic	Defense (anti-feeding)
Plant-plant communication	Photoprotection	Metal transport
Oviposition	Surfactant	Phytohormones

Apart from the "typical" secondary pathway metabolites, other compounds, originally synthesized for "secondary" functions, were recruited by plants during the course of their evolution, for developing several primary functions, like signaling or hormonal activities, as described in table 1.9. Moreover, compounds like lignin or canavanine (an arginine antimetabolite that substitutes arginine for the production of the structurally aberrant canavanyl proteins [Rosenthal *et al.*, 1989]) are known to act in both primary and secondary metabolism of plants [Hartmann, 2007].

Table 1.9: Compounds of secondary origin that attained primary functions.

Compounds	Compound class	Function
Gibberellins	Diterpenoid	Phytohormones
Abcisic acid	Sesquiterpenoid	
Brassinosteroids	Triterpenoid	
Carotenoids, xanthophylls	Tetraterpenoids	Photoprotection
Some flavonoids	Flavonoids	Developmental regulators
Salicylate	Benzoate	Stress signal
Lignin	Lignins	Cell wall strengthening and chemical defense
Canavanine	Amino-acids	Seed nitrogen storage and chemical defense

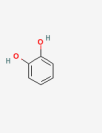
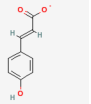
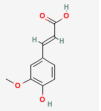
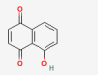
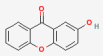
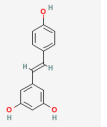
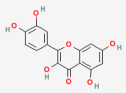
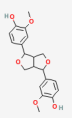
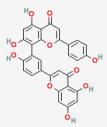
1.3.4. Phenylpropanoid pathway and phenolics: A major class of secondary metabolites in plants

Secondary compounds can be classified through their chemical structure, solubility or by their metabolic pathways. A broad, simple classification usually differentiates secondary compounds in three major groups, according to their biosynthetic pathways: terpenoids (lipid-based compounds), alkaloids (nitrogen-based compounds) and phenolics (aromatic ring-based compounds) [Bourgaud *et al.*, 2001]. Considering the scope of this work, and the relevance of phenolic compounds in *H. perforatum* defense against *C. gloeosporioides*, the biosynthetic pathways of two major, ubiquitously distributed phenolics (flavonoids and lignin) will be discussed in more detail. A specific class of phenolic compounds found in *H. perforatum* (xanthones) will be presented later in this thesis.

Most of the thousands of phenolics known are from plant origins. These compounds are characterized by their C_6 aromatic ring(s) bearing at least one hydroxyl group, as described in table 1.10 [Strack, 1997]. Some phenolics, like lignins, are present in virtually all plant species, performing roles of great importance such as water transport, mechanical support and physical barrier against possible pathogens. Other phenolics can be more or less specific to a given plant species. Nonetheless, most of these specific phenolic compounds play a myriad of common functions, displaying active roles as

pollinator attractants, pathogen deterrents, UV-light protectors, allelopathic compounds, antioxidants or signal molecules [Strack, 1997; Mayer *et al.*, 2001].

Table 1.10: Major classes of phenolic compounds found in plants [Strack, 1997; URL 14].

Carbon skeleton	Compound class	Sample Structures
C_6	Simple phenols	 Catechol
C_6-C_1	Hydroxibenzoates	 4-Hydroxybenzoate
C_6-C_2	Acetophenones, phenylacetates	 4-Hydroxyacetophenone
C_6-C_3	Hydroxicinnamates, phenylpropenes, coumarins, chromones	 Caffeate  Coumarate  Ferrulate
C_6-C_4	Naphthoquinones	 Juglone
C_6-C_1-C_6	Xanthones	 2-Hydroxyxanthone
C_6-C_2-C_6	Stilbenes, anthraquinones	 Resveratrol
C_6-C_3-C_6	Flavonoids	 Quercetin
$(C_6$-$C_3)_2$	Lignans	 Pinoresinol
$(C_6$-C_3-$C_6)_2$	Biflavonoids	 Amentoflavone
$(C_6)_n$	Catechol melanins	Polymeric structures
$(C_6$-$C_1)_n$:Glc	Hydrolizable tannins	
$(C_6$-$C_3)_n$	Lignins	
$(C_6$-C_3-$C_6)_n$	Condensed tannins	

Plant phenolics are synthesized from three distinct biogenetic routes: The shikimate/arogenate, the polyketide and the acetate/mevalonate pathways [Strack, 1997]. The shikimate/arogenate pathway (Figure 1.11) links carbohydrate metabolism to the biosynthesis of aromatic compounds [Herrmann *et al.*, 1999]. This pathway occurs in plants and bacteria and leads to the majority of plant phenolics (the phenylpropane derivatives - phenylpropanoids), by producing the aromatic amino acid L-phenylalanine (as well as the amino acids tyrosine and tryptophan). The shikimate/arogenate pathway is thought to occur in different locations, since it is involved in phenylpropanoid biosynthesis as well as in protein synthesis. Therefore, it has been postulated that one plastidial pathway is mainly responsible for protein synthesis while the other, occurring in the cytosol associated with other membrane-bound enzymes (multiprotein complex), is responsible for the phenylpropanoid biosynthesis [Strack, 1997; Hartmann, 2007].

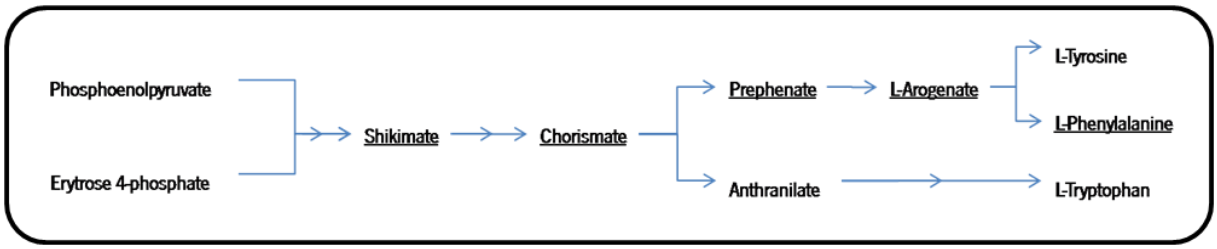


Figure 1.11: Outline of the shikimate/arogenate pathway [Strack, 1997].

Following phenylalanine production, the first step of the "general phenylpropanoid pathway" takes place (Figure 1.12). This first reaction is catalyzed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which converts phenylalanine to cinnamate via deamination. Moreover it is known that another amino acid from the shikimate pathway, tyrosine, can also be converted by PAL, although with lower efficiency, directly into *p*-coumaric acid. Because of its key role in shifting the flux of carbon from the primary to the secondary metabolism, PAL has been extensively studied. This tetrameric protein was the first enzyme of the phenylpropanoid pathway for which detailed structure–function information was made available [Ferrer *et al.*, 2008]. Cinnamate is then converted, by cinnamate 4-hydroxylase (C4H, EC 1.14.13.11), into *p*-coumaric acid, which can be the substrate for a series of enzymes that undergo hydroxylation and methylation reactions, leading to sequential formation of the other common hydroxycinnamates caffeic acid and ferulic acid (Table 1.10) as well as 5-hydroxyferulic acid and sinapic acid [Strack, 1997]. These intermediates are the substrates for 4-coumaroyl:CoA ligases (4CLs, EC

6.2.1.12). Isoforms from this enzyme activate hydroxycinnamate intermediates by the formation of a thioester bond between a CoA and their carboxyl groups [Ferrer *et al.*, 2008].

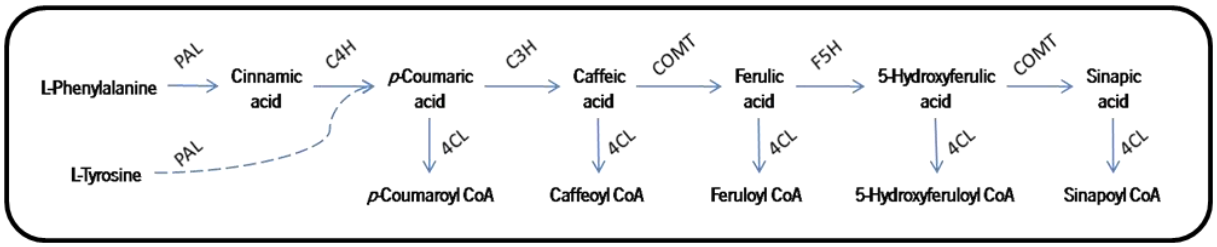


Figure 1.12: Outline of the general phenylpropanoid pathway and the hydroxycinnamate intermediates. PAL - Phenylalanine ammonia-lyase; C4H - Cinnamate 4-hydroxylase; 4CL - 4-Hydroxycinnamoyl CoA ligase; C3H - *p*-Coumarate 3-hydroxylase (EC 1.14.13.-); COMT - Caffeate/5-hydroxyferulate *O*-methyltransferase (EC 2.1.1.68); F5H - Ferulate 5-hydroxylase (EC 1.14.13) [Harakava, 2005 (adapted)].

From this step on, another common pathway for the synthesis of plant phenolics, the polyketide pathway, channels the distinct, activated hydroxycinnamic intermediates into specific branch pathways for the formation of several classes of phenylpropanoids like lignins, xanthones, stilbenes or flavonoids [Dixon *et al.*, 2002; Ferrer *et al.*, 2008]. Although some classes of phenylpropanoids are present in all plants, such as the hydroxycinnamic acids from the "general pathway", flavonoids or lignins, other classes, such as the xanthones or stilbenes, may be limited to particular plant families [Dixon *et al.*, 2002].

Flavonoids belong to a large, structurally diverse class, comprising nearly 9000 distinct phenolic compounds, found in all higher plants. Flavonoids played a key role in Mendel's classic discovery of the laws of heredity and are known to be of major importance in the biochemical ecology of plants [Ferrer *et al.*, 2008]. The biosynthetic pathway of flavonoids starts with the condensation, in a polyketide reaction, of the activated hydroxycinnamic acid *p*-coumaroyl CoA, with three molecules of malonyl CoA. This reaction is catalyzed by chalcone synthase (CHS; EC 2.3.1.74), yielding naringenin chalcone (2',4,4'6'-tetrahydroxychalcone) [Strack, 1997]. This chalcone is then isomerized by chalcone isomerase (CHI, EC 5.5.1.6), producing a flavanone. Flavanones are intermediates for the biosynthesis of all flavonoid subclasses such as flavones, flavonols and isoflavanoids, but also anthocyanidins and anthocyanins (Figure 1.13) [Farag *et al.*, 2008].

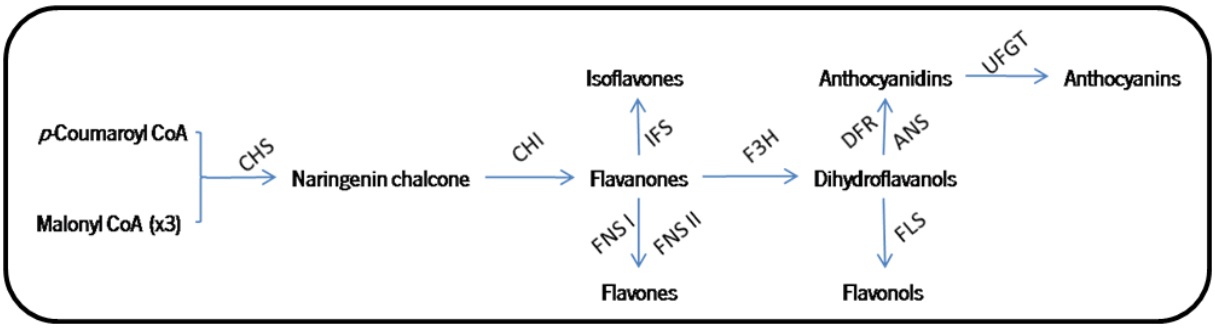


Figure 1.13: Biosynthetic pathway leading to major classes of flavonoid aglycones, anthocyanidins and anthocyanins. CHS - Chalcone synthase; CHI - Chalcone isomerase; FNS I and II – Flavone synthases (EC 1.14.11.22); IFS - Isoflavone reductase (EC 1.3.1.45); F3H - Flavanone 3-hydroxylase (EC 1.14.11.9); FLS - Flavonol synthase (EC 1.14.11.23); DFR - Dihydroflavonol 4-reductase (EC 1.1.1.219); ANS - Anthocyanidin synthase (EC 1.14.11.19); UFGT - Flavonoid 3-O-glucosyltransferase (EC 2.4.1.91) [Ferrer *et al.*, 2008 (adapted)].

Lignins are probably the most ubiquitous class of phenylpropanoids in plants. They represent a major carbon sink in vascular plants [Rest *et al.*, 2006], being the second most abundant plant polymer, after cellulose [Ferrer *et al.*, 2008]. Like for most phenylpropanoids, the synthesis of their monomers starts from activated hydroxycinnamic acids, produced in the general phenylpropanoid pathway. From a branch pathway that includes cinnamoyl-CoA reductase (CCR; EC 1.2.1.44) and cinnamyl-alcohol dehydrogenase (CAD, EC 1.1.1.195) (Fig. 1.14), each activated hydroxycinnamic compound is then converted to lignin monomers, the monolignols coniferyl, 5-hydroxyconiferyl, sinapyl and *p*-coumaryl alcohols, precursors of lignin. Another compound (caffeyl alcohol) is also produced although it is not a constituent of lignin in plants [Strack, 1997], being converted into some of the previous compounds (coniferyl, 5-hydroxyconiferyl or sinapyl alcohols). Finally, each of the four monolignol precursors is converted by lignin peroxidases (LiP, EC 1.11.1.7) into four basic lignin subunits (p-hydroxyphenyl, guaiacyl, 5-hydroxyguaiacyl and syringyl lignin) that will later be polymerized into the complex lignin structure. The ratio between these lignin subunits dictates the degree and nature of polymeric cross-linking, which is responsible for profound consequences in the interaction of plants and their environment [Ferrer *et al.*, 2008].

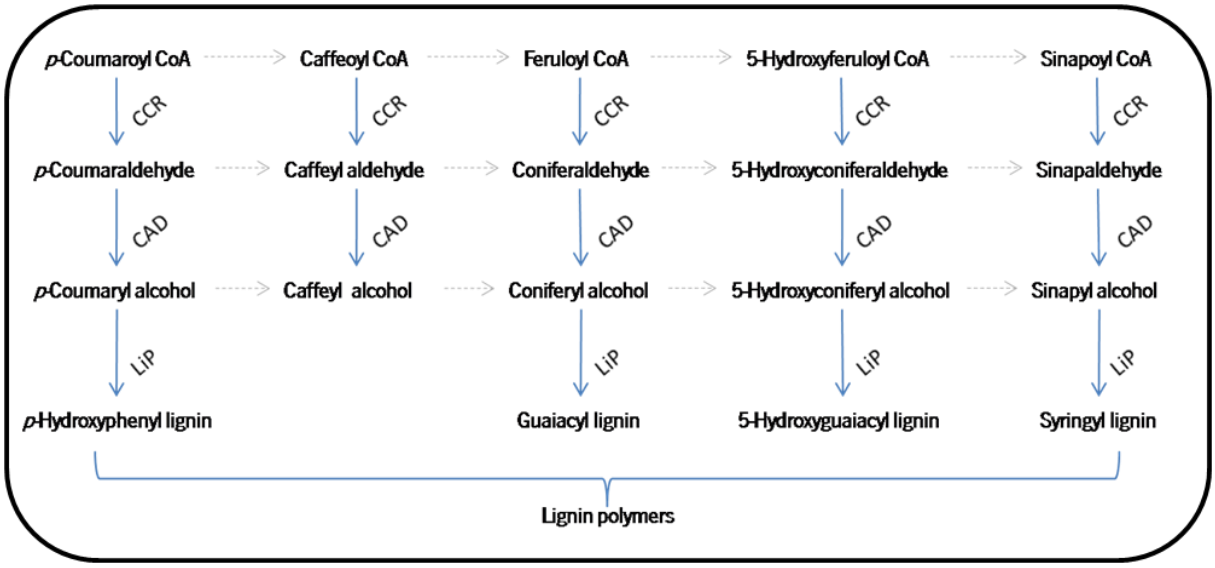


Figure 1.14: Outline of the lignin biosynthetic branch pathway. Dotted gray arrows show possible conversions between lignin biosynthesis intermediates [URL 15 (adapted)].

1.3.5. Chemical composition of *H. perforatum*: Characteristic and major secondary metabolites.

It is difficult to define a standard chemical composition for *H. perforatum* since both quantity and quality of the phenolic compounds accumulated varies greatly between breeding lines [Franke *et al.*, 1998] or even in the same cultivar [Büter *et al.*, 1998]. Nonetheless, a spectrum of six major natural product groups are usually found in *H. perforatum* dry alcoholic extracts, prepared with ethanol and the upper, aerial parts of the plant: naphtodianthrones, phloroglucinols, flavonol glycosides, biflavones, proanthocyanidins and phenylpropanes [Erdelmeier *et al.*, 2000]. The most relevant of these groups are described below, in detail.

Naphtodianthrones

The chemical study of this species had begun in 1830, when Buchner isolated the first naphtodianthrone, hypericin, from *H. perforatum* [Dias, 2000]. When compared to other major phenolic compounds, naphtodianthrones are present in relatively smaller amounts in the plant. Nonetheless hypericin, as well as the other minor naphtodianthrones (Fig. 1.15), also called *hypericins* [Dias, 2000], are the most noticeable compounds from *H. perforatum* due to their distinctive blood-red colour. In *H. perforatum*, hypericin is found in the dark glands as well as in some secretory canals [Maggi *et al.*, 2004]. Nonetheless, the plant cell tissues involved in the synthesis, as well as the biosynthetic pathway

of hypericin, is still not fully understood. The initial steps of the pathway may occur in the photosynthetic, green leaf tissues were acetate and/or malonate photosynthates (precursors of emodin) are produced. These compounds are then transported to the dark glands, were emodin is synthesized. The following steps of synthesis occur in these glands and hypericin is finally produced through the conversion of protohypericin in a simple biochemical process, in the presence of light [Zobayed *et al.*, 2006].

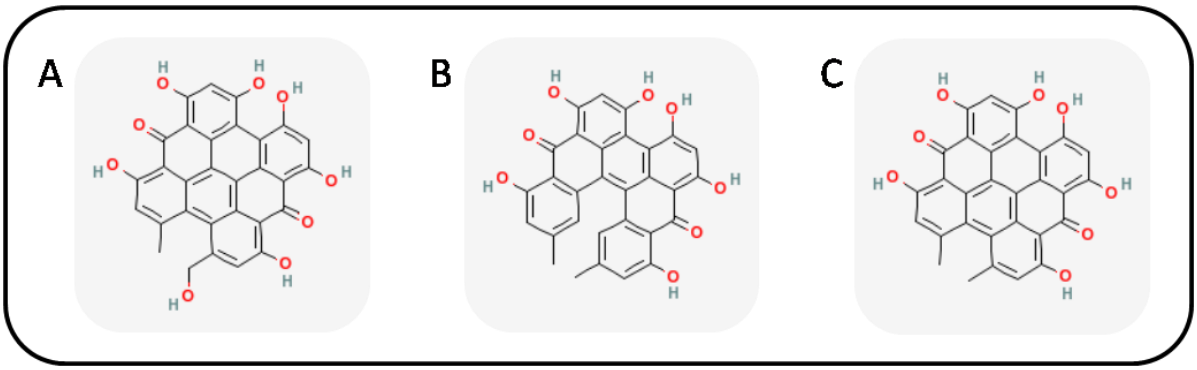


Figure 1.15: Some naphtodianthrones from *H. perforatum*: **(A)** pseudohypericin, **(B)** protohypericin, the non-toxic precursor of **(C)** hypericin [URL 14].

Although hypericin is usually associated with *H. perforatum*, this phenolic compound is also present in other species from the genus *Hypericum* and, interestingly, has also been found in a protozoon, the blue-green ciliate *Stentor coerulus*, associated in a photoreceptor complex. Moreover, hypericin has also been found in insects from the *Coccoidea* family [Erdelmeier *et al.*, 2000].

Hypericin is one of the compounds from *H. perforatum* most extensively screened for biological activities. Some *in vitro* results suggest a positive antiviral activity against several viruses, including HSV, Influenza A or HIV. Despite these promising results obtained *in vitro*, especially against HIV, clinical studies are still inconclusive about the effects of hypericin for the treatment of AIDS. Moreover, many studies found that hypericin was responsible for phototoxic side-effects, observed in numerous patients [Dias, 2000]. Despite this adverse effect observed in antiviral treatments, photoactivation of hypericin has shown to have a promising potential in quimioterapy treatment of several types of carcinoma [Miskovsky, 2002]. Clinical studies have found that hypericin was selectively effective against melanoma cells, after topical application and light activation [Davids *et al.*, 2008]. Other studies, focusing on the antidepressant activity of *H. perforatum*, have shown that hypericin plays no role in this therapeutic propriety [Dias, 2000].

Phloroglucinols

Phloroglucinols represent the largest group of compounds found in *H. perforatum*. The most important compound from this group is hyperforin (Fig.1.16), which comprises 2-5% of *H. perforatum* crude extract composition [Erdelmeier *et al.*, 2000; Beerhues, 2006]. Like hypericin, hyperforin accumulates only in secretory tissues but, while hypericin can be found only in the dark glands, hyperforin occurs only in minute amounts in these structures. Therefore, the preferred accumulation sites for hyperforin are known to be the translucent glands. In accordance to this fact, a recent theory proposes that the synthesis of this phloroglucinol takes place in the chloroplasts of cells delimiting the translucent glands, through the same biosynthetic machinery used for monoterpenes synthesis [Soelberg *et al.*, 2007].

Several phloroglucinols are known to display antibacterial, antifungal and antimalarial activities [Erdelmeier *et al.*, 2000]. The most prominent phloroglucinol in *H. perforatum*, hyperforin, is nowadays known to play a central role in the antidepressive activity of this plant, by interfering with the reuptake of many neurotransmitters [Müller, 2003]. Moreover, it has also been suggested that hyperforin displays anti-inflammatory [Schempp *et al.*, 2000] and antitumoral [Schempp *et al.*, 2002] properties.

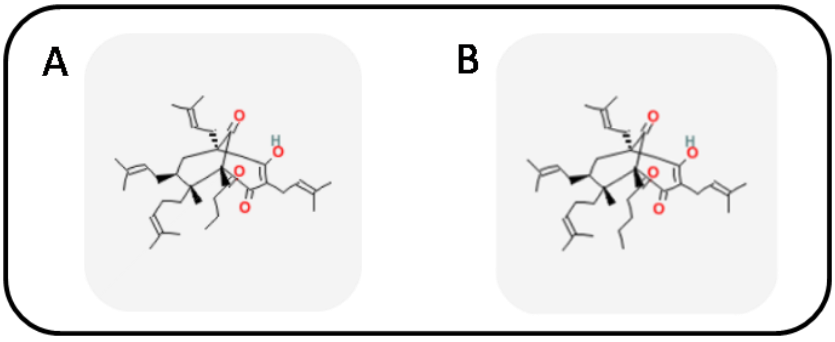


Figure 1.16: Two major phloroglucinols from *H. perforatum* L.: **(A)** hyperforin and **(B)** adhyperforin [URL 14].

Flavonoids

Flavonoids constitute one of the most representative classes of phenolic compounds from *H. perforatum* and may account for 1-7% of the dry biomass from this plant [Dias *et al.*, 1998]. The most common flavonoids belong to the quercetin-based flavonol glycosides, like hyperoside, rutin, quercitrin and isoquercitrin (Fig. 1.17). The most relevant aglycones found in *H. perforatum* are quercetin, kaempferol and luteolin. Moreover, some biflavones are usually referred in the literature as minor components [Erdelmeier *et al.*, 2000] present exclusively in the flowers of *H. perforatum* [Dias *et al.*,

2000]. Quercetin is known to display antiviral [Castrillo *et al.*, 1986], anti-inflammatory [Ueda *et al.*, 2004] and anticancer activities [Lu *et al.*, 2006]. Some other flavonoids, in particular the quercetin-based ones, are known to display *in vitro* inhibitory activity of monoamine oxidase A and B (MAO-A and -B) and catechol-O-methyl transferase (COMT), enzymes responsible for the catalysis of several neurotransmitters. Therefore, these flavonoids may be implicated in the antidepressant activity of *H. perforatum*. Although the amounts usually present in the extracts are not enough to display this antidepressant activity alone [Dias, 2000], a synergistic combination of flavonoids and other compounds, like hyperforin, may be responsible for the antidepressant activity of *H. perforatum* whole extracts [Simmen *et al.*, 2001].

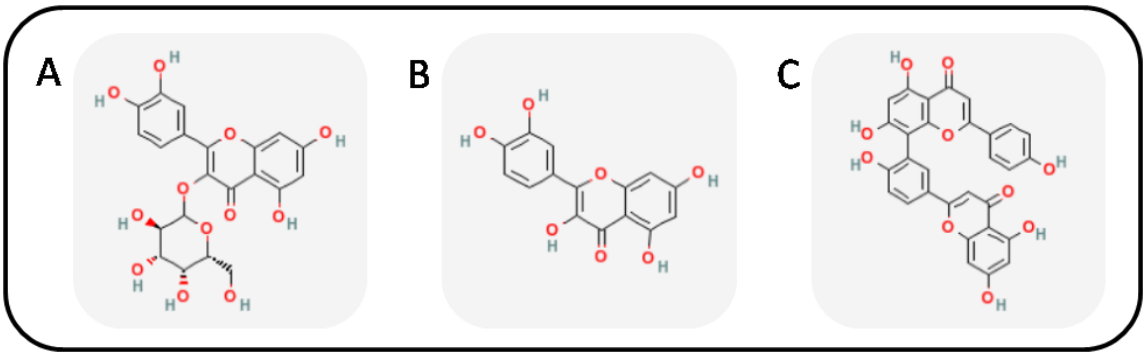


Figure 1.17: Some flavonoids from *H. perforatum*: The flavonols **(A)** hyperoside and **(B)** quercetin and the biflavone **(C)** amentoflavone [URL 14].

Proanthocyanidins

Other components usually present in the phenolic composition of *H. perforatum* include proanthocyanidins. These compounds are constituted of catechin and epicatechin (Fig. 1.18) units and account for nearly 8% of the crude ethanolic extract of *H. perforatum* [Erdelmeier *et al.*, 2000]. Some catechin derivatives, are known to display antimicrobial [Veluri *et al.*, 2004] and antiviral properties [Barnes *et al.*, 2001].

Xanthones

Xanthones (Fig. 1.18) are a very typical class of metabolites, present in plants from the *Guttiferae* family. Xanthones are usually found in the roots but trace amounts of this class of phenolic compounds can also be found in the aerial parts of the plants [Erdelmeier *et al.*, 2000].

Several pharmacological properties have been attributed to xanthones. Considering antidepressive activity, some xanthones have a remarkable and selective inhibitory activity upon MAO-A.

Nonetheless, the amounts present in the plant are not enough to develop this inhibition alone [Dias, 2000], as already observed for other classes of *H. perforatum* compounds. Other therapeutic properties of xanthones from *H. perforatum* include hepatoprotective, anti-inflammatory, anticancer and antimicrobial activities [Franklin *et al.*, 2007].

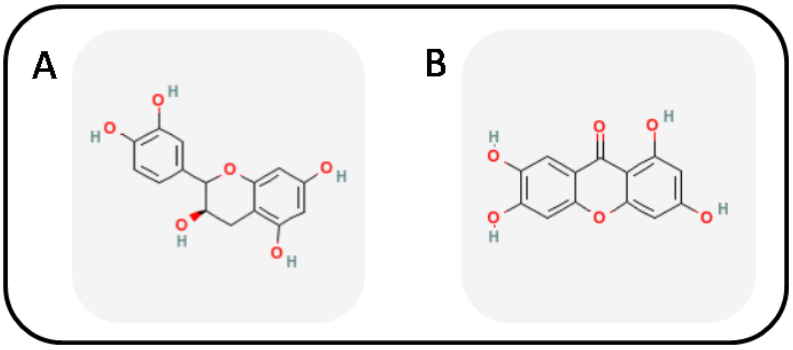


Figure 1.18: The proanthocyanidin **(A)** epicatechin and the xanthone **(B)** 1,3,6,7-tetrahydroxyxanthone *from H. perforatum* [URL 14].

Chapter 1.4

Plant defense mechanisms against biotic stress

1.4. Plant defense mechanisms against biotic stress

1.4.1. Introduction

Despite the sessile, inert-like nature of plants, these organisms are constantly fighting against environmental stresses, both biotic and abiotic. In fact, their lack of mobility has shown not to be a lethal situation, as plants thrive on virtually all ecosystems. Their success could only be achieved by a long-term co-evolution of defense mechanisms, particularly against their also rapidly evolving pathogens. In the short-term, the dynamic and elaborated defense mechanisms of plants can be found acting in three distinct ways. First, constitutive defenses are always present, regardless of the presence of a pathogen. The other two types of resistance are known to be activated upon pathogen attack, inducing several defense responses locally (at the site of infection) and/or systemically (across the whole plant). Due to the presence of these constitutive and/or induced defenses, which will be later pointed in this chapter, most plants are able to resist to most organisms, regardless their origins (fungi, bacteria, viruses, animals) and nutrition models [Dangl *et al.*, 2001].

1.4.2. Plant resistance models

Non-host resistance

Plants avoid diseases from most attacking organisms through a non-host resistance. The non-host resistance, common to an entire plant species against a particular organism, is more common and durable than host resistance, which can be particular to a cultivar from a given plant species [Cunha, *et al.*, 2006]. Despite the importance of this basal response to plant survival, the mechanisms underlying non-host resistance are still poorly understood [Mysore *et al.*, 2004]. One of the reasons may be the fact that, while host resistance can be determined by a single dominant gene locus [Yang *et al.* 1997], non-host resistance appears to be a multigenic trait [Holub *et al.*, 2004].

Non-host resistance can be classified in two types, according to the symptoms developed (or not) by the pathogen (Fig. 1.19). While non-host resistance type I does not produce visible symptoms (such as necrosis) in the plant, type II resistance is known to include an hypersensitive response (HR), developed by the plant against the pathogen, with rapid localized necrosis at the site of infection. Therefore, type II resistance resembles to the typical "gene-for-gene" host resistance, being more sophisticated than type I resistance. Although type I non-host resistance is characterized by the absence of visible symptoms, several molecular changes occur in the plant. After contact with the plant, the pathogen may be deterred by the preformed, constitutive barriers or by newly synthesized induced defenses, such as secondary metabolites, cell wall thickening or papilla formation. The type of non-host resistance varies according to the plant and the pathogen species. A given plant species can show a type I response to one pathogen and a type II response to another. In a similar way, a given pathogen can develop both type I or II non-host resistance on distinct plant species [Mysore *et al.*, 2004].

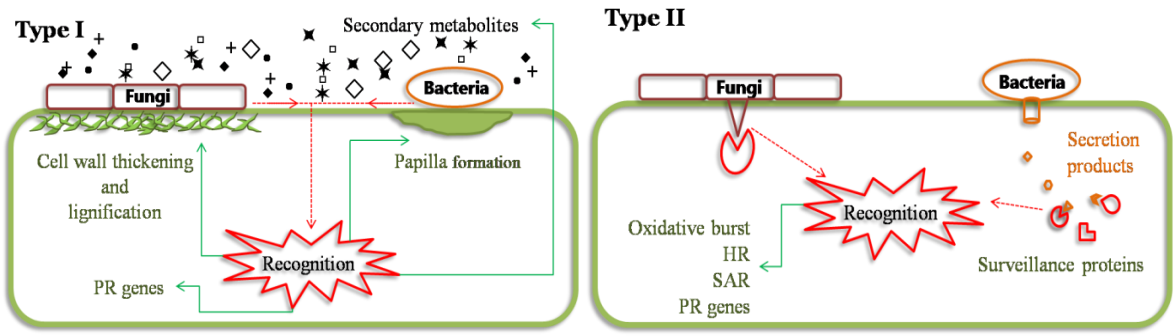


Figure 1.19: Models for non-host resistance types I and II [adapted from Mysore *et al.*, 2004 and Azevedo, 2005].

Host resistance

Even after breaching through the physical barriers and surviving to the constitutive defensive chemical compounds, the candidate pathogen still has to avoid recognition by the plant "surveillance" mechanisms, before effectively infect the host [Maor *et al.*, 2005]. Plants recognize several types of elicitors from the pathogen, as will be discussed later. According to the widely accepted "gene for gene" theory, proposed by H. H. Flor in the 1940's, a successful disease resistance, known as "incompatible interaction", is triggered because a resistance (*R*) gene product in the plant recognizes a specific "avirulence" (*Avr*) gene product. Moreover, the plant *R* proteins are thought to act either by detecting the *Avr* protein from the pathogen directly, in a "receptor-ligand" model, or by sensing changes on possible targets of the pathogen *Avr* proteins, the so-called "guard hypothesis" [Dangl *et al.*, 2006]. When either the plant or the pathogen fail to develop an *R* or *Avr* gene products, respectively, a virulent, compatible interaction occurs, causing the spread of the disease in the host plant. Due to the high complexity of both host and non-host resistance mechanisms, it is not clear why a pathogen fully virulent on one plant species is nonpathogenic on others [Mysore *et al.*, 2004].

Simple boundaries and divisions between host and non-host defense mechanisms may be too fragile to explain the complex web of plant-pathogen interactions [Heath, 2001]. Many of the plant defense responses are similar in both host and non-host resistance: Hypersensitive response, ROS production or lignification may occur in both resistance models, although timing and amounts may be slightly different [Mysore *et al.*, 2004].

1.4.3. Constitutive and induced plant resistance mechanisms

Plants dispense a large amount of their energy in the synthesis of defensive barriers against their pathogens. The costs of development and maintenance of toxic compounds in special storage structures are also thought to be quite high. Evidence of this energy cost comes from many plants overexpressing resistance mechanisms that are less fertile and show "stunted" or "dwarfed" phenotypes, when compared to normal plants [Heil *et al.*, 2002]. One way plants have to reduce these costs is the production of defensive compounds only after pathogen contact. Although this induced defense method seems more efficient at first, this strategy could prove to be risky. If the initial pathogen attack is too fast or severe the plant may have no time to deploy such induced defenses effectively [Wittstock *et al.*, 2002]. Therefore, plants must optimize and share their energies between three major

strategies: (i) Basal defense mechanisms, including constitutive physical barriers, accumulation of pre-formed toxic compounds (phytoanticipins) and antimicrobial proteins (ii) constitutive accumulation of inactive toxic compounds intermediates and (iii) induced production *de novo* of both physical barriers, chemical defensive compounds (phytoalexins) and defense-related proteins [Hartmann, 2007].

Constitutive physical and chemical barriers

The constitutive defense mechanisms are the first barriers that pathogens have to face before the successful establishment of a virulent interaction can occur. Although less understood, these constitutive barriers are responsible for the survival of plants against most of the existing pathogenic organisms. One of the first constitutive barriers that pathogens may have to face is the cuticle. As already explained in chapter 1.2, the cuticle is composed by waxes, polysaccharides and cutin polymers that may prevent a nonpathogenic organism from establishing an infection. Their wax composition also includes fatty acids combined with terpenoids or simple phenolics, acting also as a chemical defense [Chassot *et al.*, 2005]. Apart from the cuticle, other structures may increase the thickness of the plant surface. Cell walls (primary and secondary) and, in some cases, bark depositions, also help avoiding a possible infection. Moreover, actin microfilaments are also known to play a role in plant defense against several non-host fungal pathogens [Mysore *et al.*, 2004]. Physical barriers may also include the stomata, which morphology and position may prevent pathogen infection. Additionally, the waxy nature of the cuticle, as well as the vertical leaf orientation present in some plant species, may also prevent the formation of moisture films, therefore reducing the water available for germination or motility of some pathogens.

Plants constitutively produce a wide array of chemical compounds, to be used in defense against pathogens. These pre-formed compounds are known as phytoanticipins and include a long list of plant secondary metabolites [Dixon, 2001], as previously described in chapter 1.3. Among these compounds we can find terpenoids, such as the saponins that affect membrane integrity of pathogens, but also a plethora of phenols and phenolic glucosides, unsaturated lactones, sulphur compounds, alkaloids, cyanogenic glycosides or glucosinolates [Osbourn, 1996]. After their synthesis, phytoanticipins may be stored in active or inactive forms, according to their specific function and/or toxicity [Wittstock *et al.*, 2002]. Moreover, these compounds may be readily released to the exterior upon production or may accumulate inside dead or living cells or even in specialized external structures, as already described for hypericin, accumulated in *H. perforatum* glands, scattered across the aerial parts of the plant. Upon pathogen attack and plant cell collapse, these stored compounds are released to the environment and

activated (if necessary), usually killing both the invading pathogen and the surrounding plant cells. Some examples of constitutive defensive compounds activated upon plant cell compartmentalization collapse include the formation of HCN from cyanogenic glycosides or the formation of mustard oils from glucosinolates [Hartmann, 2007].

Induced physical and chemical barriers

After facing the first, constitutive barriers, the pathogen may have to face inducible defense responses. Upon recognition of specific or general (non specific) elicitors, plants respond with the synthesis *de novo* of defensive compounds and proteins, implicated in both physical and chemical resistance. A great variety of elicitors, from biotic or abiotic origin, are recognized by plants [Zhao *et al.*, 2005]. Some of these elicitors may be the result of host degradation by the pathogen (such as pectin fragments or cutin monomers) [Chassot *et al.*, 2005]. Other elicitors are synthesized by the pathogen during infection or simply make part of their structural composition, like the bacterial protein flagellin, lipopolysaccharides (LPSs), lipooligosaccharides, peptides, as well as other pathogen surface molecules, also referred to as pathogen-associated molecular patterns (PAMPs) [Mysore *et al.*, 2004; Zhao *et al.*, 2005; Cunha, *et al.*, 2006]. Although generally connected to non-host defense mechanisms, PAMPs can also contribute to host resistance. In a similar way, hypersensitive responses may be related to both host and non-host resistance. Rather than strictly divided, plant "surveillance systems" seem to recognize host and/or non-host elicitors using at least a few similar mechanisms. Therefore, the final defense phenotype likely depends on which and how many defense pathways are triggered as well as how strongly those pathways are activated [Cunha, *et al.*, 2006].

The plant elicitors can be perceived by a variety of receptors and, despite the difficulty in their distinction, plant receptors are generally "divided" into *R* proteins (for host resistance) or PAMP receptors (for non-host resistance). The location of these plant receptors is variable as well as their structure. Nonetheless a "broad division" into three families is usually accepted. One group includes cytoplasmic proteins with a nucleotide binding site (NB) and leucine rich repeats (LRRs). Another group of receptors include proteins with extracellular LRRs and an intracellular kinase domain (receptor-like kinases, RLK). Finally, a third group includes membrane-spanning proteins with extracellular LRRs (RLP). Curiously, some similarity exist between animal and plant immune receptors, leading room for speculation that these defense systems diverge from a common, ancient evolutionary surveillance system [Cunha, *et al.*, 2006].

The responses induced by elicitor recognition are numerous and include both physical and chemical strategies. One of the most common is hypersensitive response, discussed in more detail later. Cell wall thickening and lignification can reinforce cell walls in order to stall or prevent pathogen penetration. Moreover, papilla formation and callose deposition may occur at the site of infection, encapsulating the pathogen haustorial complexes, also preventing their penetration [Maor *et al.*, 2005]. Increased production of secondary compounds previously referred as constitutive defenses, such as phenolics and saponins, may also occur. Plant response to infection also includes the synthesis of several new compounds, known as phytoalexins [Dixon, 2001]. The ability of plants to produce diffusible antimicrobial compounds *de novo* was first discovered in 1911, when the French botanist Noel Bernard found that two orchid species became resistant to further fungal attack, after they had been infected by the fungus *Rhizoctonia repens*. Despite this early finding, the term "phytoalexin" was only created decades later by Müller (1940), after the identification of some diffusible compounds from potato tubers, infected by *Phytophthora infestans* [Grayer *et al.*, 2001]. Since then, a multitude of plant defensive compounds were identified. A short list of phytoalexins, known to have antimicrobial or feeding deterrent activities, is shown in table 1.11. As observed for phytoanticipins, the diversity of phytoalexins produced is enormous and, although some of them are common to various plants species, other phytoalexins are quite specific. Moreover, the distinction between phytoalexin and phytoanticipin is not always obvious since some compounds may be phytoalexins in one plant species and phytoanticipins in others [Dixon, 2001].

Table 1.11: Some examples of defensive compounds produced by plants against biotic stress.

Metabolite	Origin	Target organism	References
Brussalexin A	*Brassica oleracea*	*Leptosphaeria maculans* *Alternaria brassicicola* *Sclerotinia sclerotiorum*	Pedras *et al.*, 2007
Luteolinidin Apigeninidin	*Sorghum bicolor*	*Colletotrichum graminicola*	Hipskind *et al.*, 1990 Snyder *et al.*, 1991
Luteolin	*Medicago sativa*	*Verticillium albo-atrum*	Picman *et al.*, 1995
Cassiaflavan	*Narcissus pseudonarcissus*	*Botritis cinerea*	Iwashina, 2003
Naringenin	*Prunus cerasus*	*Cystospora persoonii*	Geibel, 1995
Desoxyhemigossypol	*Gossypium hirsutum*	*Fusarium oxysporum* (*sp. vasinfectum*)	Zhang *et al.*, 1993
Daidzein	*Glycine max*	*Rhizobium spp.*	Bassam *et al.*, 1998
Scoparone	*Citrus spp*	*Phytophthora citrophthora* *Penicillium digitatum*	Kuniga *et al.*, 2006
Licoisoflavone B Luteone Wighteone	*Lupinus angustifolius*	*Colletotrichum gloeosporioides* *Cladosporium cladosporioides* *Heteronychus arator*	Iwashina, 2003
Isoneorautenol	*Erythrina mildbraedii*	*Staphylococcus aureus*	Mitscher *et al.*, 1988
Vestitol	*Lotus pedunculatus*	*Costelytra zealandica*	Lane *et al.*, 1987
Methylhildgardtol A	*Tephrosia hildebrandtii*	*Spodoptera exempta*	Simmonds *et al.*, 1990

Apart from the synthesis of secondary compounds implicated in physical and chemical barriers, plant responses to pathogen presence also include the production of several antimicrobial proteins [Mysore *et al.*, 2004], proteases (such as the pectinase-inhibiting proteins - PGIPs) [Chassot *et al.*, 2005], as well as syntaxins. These last proteins belong to the SNARE family of receptors, involved in membrane fusion events [Collins *et al.*, 2003; Maor *et al.*, 2005]. While some PR proteins are known to display antimicrobial activities, such as chitinase or glucanase activities, the functions of many of them in plant defense are still unknown [Ferreira *et al.*, 2007]. Moreover, despite their connection to defense responses, their presence may actually be unnecessary for successful resistance, as occurs with PR-1 and ß-glucanase 2 [Greenberg *et al.*, 2000]. Other changes induced by pathogen recognition do not include production or activation of compounds with direct effect on pathogen spread and survival. A transient increase in cytosolic Ca^{2+} levels, for example, is known to be responsible for triggering some defense signaling pathways, like oxidative burst and hypersensitive response, as discussed below.

1.4.4. Oxidative burst and Hypersensitive response (HR)

The oxidative burst is generally defined as a rapid production of high levels of ROS in response to external stimuli [Wojtaszek, 1997]. When attacked by incompatible pathogens, plants respond by activating a variety of defense responses, including the ROS-generating enzyme complex. The increase of cellular concentration of ROS is a key event in plant and animal programmed cell death (PCD) and occurs as a result of many biotic and/or abiotic stresses. Independently of the stress source, an oxidative burst is known to be an essential prerequisite for induction of plant hypersensitive cell death [Yakimova *et al.*, 2005].

Under normal conditions, most cells possess the ability to produce and detoxify ROS which appear in cells as inevitable by-products formed as a result of successive one-electron reductions of molecular oxygen (O_2) (Fig. 1.20) [Wojtaszek, 1997], being mitochondria, chloroplasts and peroxisomes the main sites of ROS production [Noctor *et al.*, 1998]. Clearing ROS from the cells is carried by both enzymatic and non-enzymatic means. While several enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) or glutathione peroxidase (GPX) are known to metabolize ROS [Rouhier *et al.*, 2002], secondary metabolites, like some flavonoids, alkaloids, or carotenoids, also

display ROS-scavenging abilities, therefore contributing for the maintenance of ROS homeostasis inside the cell [Wojtaszek, 1997; Apel *et al.*, 2004].

In some cases, however, especially under stress conditions, these protective mechanisms are overridden by the rapid, transient, production of huge amounts of ROS, namely the oxidative burst [Wojtaszek, 1997]. Increased ROS accumulation enhances the lipid catabolism, resulting in peroxidation of polyunsaturated fatty acids in the cell membranes, leading to structural decomposition and change in permeability. Moreover, ROS also induces extensive damage by alterations of essential proteins as well as DNA [Yakimova *et al.*, 2005].

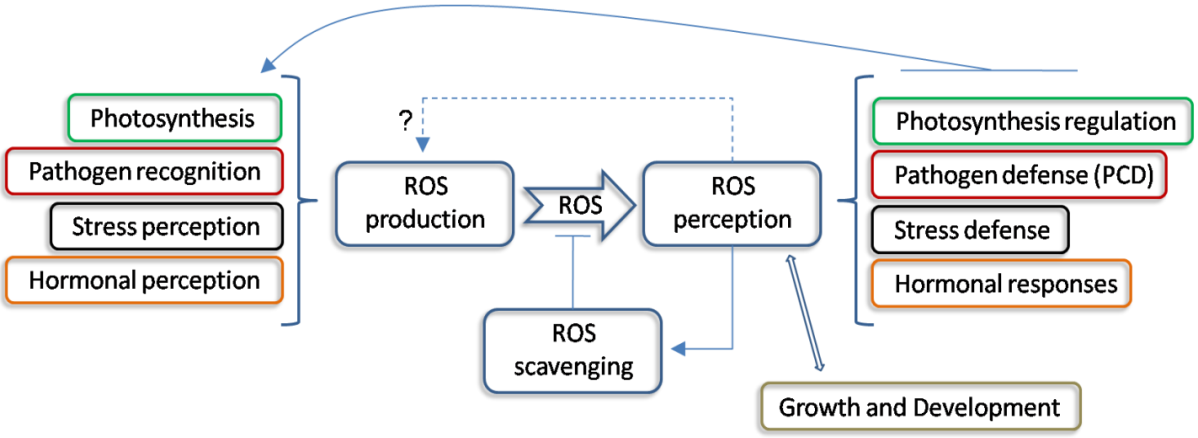


Figure 1.20: The modulation of ROS signaling by the ROS gene network of plants. ROS are a natural consequence of aerobic metabolism, but can be induced during environmental conditions that disrupt cellular homeostasis, resulting in increased ROS levels. During plant-microbe interactions, this increase is promoted by ROS-generating mechanisms following pathogen perception (oxidative bursts). ROS sensing is integrated into stress sensing pathways, which dictate the activation of ROS scavenging mechanisms or the induction of positive loops of ROS production [Mittler *et al.*, 2004].

Considering the case of biotic stress, while a virulent race fails to stimulate O_2^- production, incompatible interactions have shown to be responsible for ROS production, usually observed as a biphasic process. Phase I is very similar in its timing to the reaction of plant cells to fungal elicitors, and is considered as a non-specific response. In incompatible interactions, however, the weak, transient Phase I is accompanied by a second, massive and prolonged oxidative burst (Phase II), occurring 1.5 - 6 h after elicitation, depending on the plant species as well as the pathogen [Wojtaszek, 1997; Allan *et al.*, 2001]. The incompatible Phase II is dependent on *avr* expression in the race-host cultivar interaction or the expression of the *hypersensitive response and pathogenicity* (*Hrp*) gene cluster in the non-host interaction [Lamb *et al.*, 1997].

The ROS-generating systems in plants and animals have a great deal of similarity. However, the basic differences involving these two kingdoms (cell mobility and the presence of a structural cell wall in

plants) play an important role in the modes of their defense responses. In mammals, the accumulation of ROS is observed in a specific compartment, the phagocytotic vacuole where the invading organisms are killed, while the phagocyte remains alive. In plants, however, ROS generation often leads to HR and plant cell death in the vicinities of the infection, thus trapping the pathogens among dead cells and limiting their spread trough non-infected tissues [Wojtaszek, 1997; Heil *et al.*, 2002].

The production of ROS is thought to occur mainly by two models. According to one model, the elicitor is recognized by appropriate receptors located in the plasma membrane which in turn activates several signaling components, involving GTP-binding proteins, protein kinases and protein phosphatases, ion channels, phospholipases A and C and possibly cyclic AMP, finally leading to the activation of NADPH oxidase. In fact, the plasma-membrane NADPH oxidase is a multi-component complex composed of membrane-bound and cytosolic proteins and was further identified as a major contributor to the bactericidal capacity of phagocytes. In adition to NADPH oxidase, a second model for ROS synthesis considers the possibility that a receptor triggers the activation of ion channels (Ca^{2+}, K^{+}, H^{+}, Cl), upon elicitation. The movement of the ions results in a transient alkalinization of the apoplastic space, activating pH-dependent peroxidases, ionically or covalently bound to cell wall polymers [Wojtaszek, 1997; Apel *et al.*, 2004]. These peroxidases can act in two different catalytic modes. If H_2O_2 is present, the peroxidatic cycle is activated, engaging the cross-linking of the cell wall phenolic polymers. However, if the phenolic substrates are replaced by NADPH or related reduced compounds, H_2O_2-producing NADH oxidase activity of peroxidases is activated instead [Apel *et al.*, 2004]. Most probably, both NADPH oxidase and cell wall peroxidases are responsible for the synthesis of superoxide, which is later dismuted, leading to the formation of other ROS, especially H_2O_2. Moreover, other mechanisms responsible for ROS generation have been proposed including a lipoxygenase acting on polyunsaturated fatty acids, copper amine oxidase, flavin polyamine oxidases and oxalate oxidase [Gara *et al.*, 2003].

Hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) are thought to be the most important species associated with the oxidative burst but singlet oxygen ($^1O_2$) and the hydroxyl radical ($HO^{\cdot}$) may also be present [Mittler *et al.*, 2004]. However, the inherent interrelationship between H_2O_2 and $O_2^{\cdot-}$ makes it sometimes difficult to identify clearly the ROS behind the oxidative burst. Most important or not, H_2O_2 can easily diffuse in the cell (unlike $O_2^{\cdot-}$) [Cardenas *et al.*, 2001] and has been found to display direct antimicrobial activity (due to its toxicity) as well as indirect effects on pathogen resistance. Some of these indirect functions include the previously described (i) activation of peroxidases responsible for

the oxidative cross-linking of cell wall polymers (therefore reducing their susceptibility to enzymatic degradation), (ii) induction of systemic acquired resistance (as described later), (ii) diffusion as a signal molecule [Yakimova *et al.*, 2005] and (iv) coordination of the hypersensitive cell-death response [Wojtaszek, 1997].

The term hypersensitive response (HR) was first used in the 1900's by Stakman, when describing a rapid host cell death in pathogen infected plants. Since then, much effort was put in understanding the roles of HR, not only in plants, but also in the animal kingdom. In both cases HR is considered an active process of cell suicide (programmed cell death – PCD), leading to controlled elimination of cells that are harmful, unwanted or misplaced in specific structures and organs. Despite this role in elimination of "unexpected mistakes", PCD (and therefore HR) plays an essential role during "normal" development and morphogenesis. This cellular suicide is implicated, for example, in xylogenesis, plant reproduction, aerenchyma formation, senescence or endosperm cell death during germination [Yakimova *et al.*, 2005]. Finally, and concerning this work, PCD plays also a role in plant resistance to pathogen attack. The HR is activated by ROS, nitric oxide, calcium and proton pumps, mitogen-activated protein kinases (MAPKs) or salicylic acid (SA). Upon activation, HR is characterized by a rapid, localized death of tissues at the site of infection, limiting further pathogen multiplication and spread. During the HR, dying plant cells strengthen their cell walls, synthesize defense related compounds, such as phytoalexins and accumulate antimicrobial proteins as well as other pathogenesis related proteins. Moreover, during HR, cells also produce phytohormones (SA, JA and ethylene) responsible for the signaling of both local and systemic responses against pathogen attack [Yakimova *et al.*, 2005].

1.4.5. Plant systemic defense signaling pathways

Upon contact between elicitors and their corresponding plant receptors, several metabolic changes associated with the activation of signaling cascades occur. Ion channels, GTP binding proteins and protein kinases are some of the first components from plant cells to activate and amplify the signals for pathogen presence. Most of their actions include changes on ion fluxes through the plasma membrane, phosphorylation/dephosphorylation of plasma membrane proteins, production of reactive oxygen species (ROS) and other signaling molecules such as jasmonates (JA), nitric oxide (NO), ethylene or salicylic acid (SA) [Zhao *et al.*, 2005]. The variety of sequential signaling reactions taking

place during signal transduction is not only great but also quite intricate. Although SA, JA and ethylene are all involved in basal resistance, it has been suggested that SA-dependent responses are closely related to biotrophic pathogens while necrotrophs and herbivores are more sensitive to JA or ethylene responses [Walters *et al.*, 2007]. Most of these pathways interact with each others at some stage of signal transduction, leading to distinct target responses, specifically adapted to the stress situation perceived by the plant [Zhao *et al.*, 2005].

Systemic acquired resistance (SAR)

As defined by van Loon, induced resistance represents a physiological "state of enhanced defensive capacity" where plant basic defenses are potentiated against further biotic challenges, sometimes for the lifetime of the plant [van Loon *et al.*, 1998; Vallad *et al.*, 2004]. One of these induced resistance mechanisms in known as "Systemic Acquired Resistance" (SAR). Plants can develop SAR both locally at the site of infection and systemically, throughout the plant's tissues, in response to a broad spectrum of virulent pathogens, from viruses to herbivores. Some of the biological processes related to SAR include the synthesis of SA, changes in redox status, as well as the coordinated induction of gene expression, including some related to PR proteins. While the recognition of the pathogen is responsible for the local triggering of SAR, SA is known to be the signal required for the systemic response [Durrant *et al.*, 2004]. Apart from defense mechanisms, this phytohormone is known to be connected with other physiological processes including flowering, thermogenesis, stomatal closure and response to abiotic stress. Nonetheless, it is SA role as a signaling molecule in defense responses against biotic stress that has been most intensely studied. During the 1990's, the first evidences of SA central role in SAR arose, when increased amounts of this molecule were found in both local and systemic tissues of tobacco infected with TMV. Moreover, concentration levels of this molecule were also considerably high in the phloem sap, leading researchers to believe that SA might be a systemic signal for SAR [Durrant *et al.*, 2004]. Nowadays a few studies suggest that some derivatives of SA, such as the volatile methyl salicylate, may be responsible for an "extreme" signaling method, inducing resistance not only in the infected plants but also in the neighboring ones [Durrant *et al.*, 2004].

As previously referred, plant response mechanisms are known to interact with each other and SAR is no exception. It was recently found that "microbursts" (connected to H_2O_2 accumulation) occur in small groups of cells from uninoculated tissues of *Arabidopsis*, after pathogen attack. It was further

demonstrated that these ROS microbursts, triggered by the oxidative burst at the site of infection, may activate defense responses at a low level throughout the plant, contributing to the SAR-induced state. Moreover, some studies also pointed that low concentrations of SA may also potentiate the production of ROS and HR cell death. In fact, SA may inhibit antioxidant enzymes, causing an increase in ROS concentration [Durner *et al.*, 1995]. This has led to the hypothesis that the accumulation of low levels of SA, together with the development of microbursts of ROS, could amplify responses to secondary, systemic infections, thus contributing to SAR [Durrant *et al.*, 2004]. A point of convergence between SAR and ISR, another systemic resistance mechanism, has already been found and well characterized. Despite being considered as independent pathways, both SAR and ISR require the function of the regulatory protein NPR1. Although the expression of the corresponding gene *NPR1* (nonexpressor of PR genes1) is known to increase two to threefold after pathogen infection or SA treatment [Walters *et al.*, 2007], NPR1 is constitutively expressed in an inactive multimeric state. During oxidative burst the monomers are released, becoming active due to changes in redox homeostasis. Monomeric NPR1 accumulates in the nucleus and activates gene expression by association with TGA transcription factors (Fig. 1.21) [Mou *et al.*, 2003]. The convergence between these two key signaling pathways and the central role of NPR1 can be observed using *npr1* mutant plants. When SA and JA are applied together to leaves, the presence of SA inhibits JA synthesis and signaling. This inhibition is alleviated in the *npr1* mutant, indicating that NPR1 is part of the crosstalk control between signaling pathways [Dong, 2004].

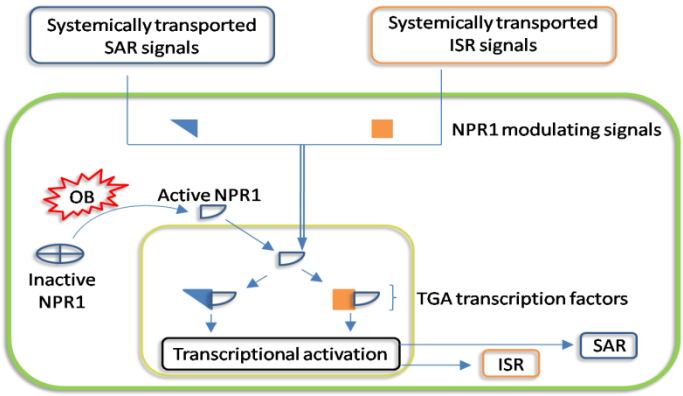


Figure 1.21: A proposed model for the signal transduction network controlling SAR and ISR [adapted from Walters *et al.*, 2007].

Induced systemic resistance (ISR)

Another systemic resistance pathway found in plants is known as "Induced Systemic Resistance" (ISR). This pathway plays an important role in plant defense, especially against herbivore pathogen

attack and wounding. These challenges are usually responsible for the release of oligosaccharides and oligogalacturonides from the damaged cell walls, thus eliciting the plant to develop a wound resistance response. Another elicitor of ISR is the 18-amino acid polypeptide systemin, released from a 200-amino acid precursor (prosystemin) upon wounding. Systemin is responsible for the release and further conversion of linolenic acid into jasmonates by activating the octadecanoid signalling cascade [Heil *et al.*, 2002]. The jasmonate-family molecules originate from oxidation of linolenic acid, present in high amounts in the chloroplastidial membranes. After a few enzymatic reactions, the resulting 12-oxophytodienoic acid (OPDA) produced in the chloroplast is transferred to peroxisomes, where jasmonic acid (JA) is finally produced. Although JA is usually assumed as "the" signal molecule for ISR, some of its precursors (eg. OPDA), as well as some derivatives (eg. methyl-jasmonate), are also known to display signaling functions in defense resistance [Staswick, 2008], being collectively called *jasmonates*. Both systemin and JA are known to be transported through the phloem, therefore both have the ability to act as systemic signals. This ISR systemic signaling triggers the activation of genes encoding proteinase inhibitors (PIs), proteins responsible for the synthesis of phenolic compounds and other secondary metabolites, as well as other defense-related proteins [Heil *et al.*, 2002].

Like for SAR, the cross-talk between ISR and other signaling pathways depends on the plant species and the particular pathogen. As an example, JA biosynthesis in tomato is related to the induction of ROS production and Ca^{2+} influx, as well as the production of PIs. On the other hand, in parsley cultures JA synthesis is Ca^{2+} dependent but ROS-independent. ISR is also known to interact with SAR defense mechanisms as well and strong evidence suggests a potent inhibition of JA synthesis by SA in some species [Zhao *et al.*, 2005], as previously described. Per instance, acetylsalicylic acid strongly reduces PI accumulation in tomato in response to wounding or to the action of systemin. By contrast, the synthesis of several SA-induced PR proteins from tobacco are inhibited by JA [Heil *et al.*, 2002]. Apart from ROS production and SAR, ISR is known to interact also with ethylene signaling pathway. Once again, both enhanced and antagonistic effects result from ethylene and JA actions. While synergistic stimulation of volatiles can be observed in corn, ethylene suppresses JA induction of nicotine gene expression in tobacco [Zhao *et al.*, 2005].

Interactions like those observed between SAR, ISR, ethylene and the oxidative burst give scientists a glimpse on how complex the cross-talk among signaling pathways may be (Figure 1.22) and how a resistance response can be specific, according to the precise challenge facing the plant.

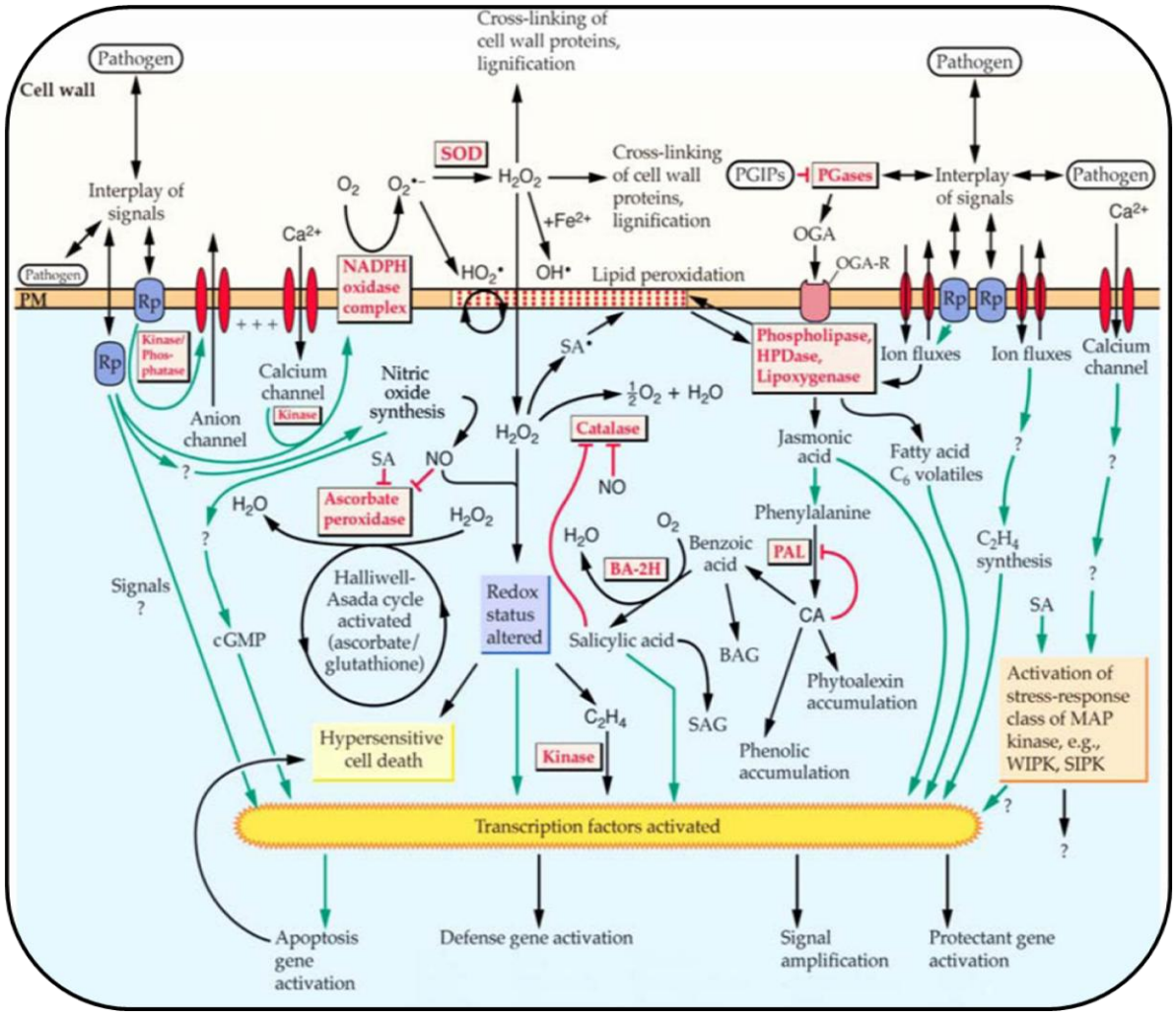


Figure 1.22: Overview of signal transduction pathways involved in plant defense responses. The intermediate downstream signaling events are not known but involve kinases, phosphatases and ion fluxes. Several distinct and rapidly activated outcomes are recognized, including ROS and direct induction of defense gene transcription. Amplification of the initial defense response occurs through the generation of additional signal molecules (e.g. other ROS, lipid peroxides, salicylic and jasmonic acids). These, in turn, induce other defense–related genes. Concomitant alterations of cellular redox status or cellular damage will activate ROS-scavenging mechanisms (e.g., ascorbate-glutathione cycle). Cross-talk between the various induced pathways appears to coordinate the responses. ACC, 1-aminocyclopropane-1-carboxylic acid; BAG, benzoic acid glucoside; BA-2H, benzoid acid 2-hydroxylase; CA, cinnamic acid; cGMP, cyclic guanosine 5'-monophosphate; CHS, chalcone synthase; EFE, ethylene-forming enzyme; GP, glutathione peroxidase; GST, glutathione S-transferase; HMGR, 3'-hydroxy-3-methyl-glutaryl-CoA reductase; $HO_2\bullet$, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; MAP, mitogen-activating protein; NO; nitric oxide; $OH\bullet$, hydroxyl radical; OGA and OGA-R, oligogalacturonide fragments and receptor; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PM, plasma membrane; $SA\bullet$, salicylic acid radical; SAG, salicylic acid glucoside; SIPK, salicylic acid-induced protein kinase; WIPK, wound induced protein kinase [Kosack *et al.*, 2000].

Chapter 1.5

Aims and Outline

1.5. Aims and outline of the thesis

Hypericum perforatum is a medicinal herb used across the world for centuries. Currently, the main application of *H. perforatum* extracts concerns the treatment of mild to moderately severe depressions. The efficacy of this plant has been supported by pharmacological and clinical studies [Erdelmeier *et al.*, 2000; Izzo *et al.*, 2003; Butterweck, 2003], attracting the pharmaceutical industry's interest. In fact, *H. perforatum* is nowadays one of the leading medicinal plants sold in the USA and EU [Erdelmeier *et al.*, 2000].

The medicinal value and economic relevance of *H. perforatum* extracts increased the pressure on raw-material suppliers. Due to the growing demand, collection of plants from the wild is no longer an option, not only for reasons of ecological sustainability but also because higher variability in phytochemical composition occurs, leading to products of variable or even unfit commercial quality. At present, field cultivation is the main source of *H. perforatum* biomass, covering several hundred hectares in Europe [Gaudin *et al.*, 2003]. Cultivation was responsible for an enhancement on both quantity and phytochemical consistency of biomass produced, leading to *H. perforatum* extracts of increased quality. Nowadays, most *H. perforatum* cultures are grown organically, following a worldwide

agricultural tendency. Without the support of pest-control chemicals, such as fungicides or herbicides, cultures are increasingly exposed to biotic attack. As for *H. perforatum*, one of the main problems concerning long-term cultivation is the fungal contamination by *Colletotrichum gloeosporioides* [Pank, 1998; Gaudin *et al.*, 2003]. This fungus is responsible for the development of anthracnose disease in several plant species, many of them being of major economic relevance. These include plants as diverse as corn, strawberry or mango, as well as other fundamental crops distributed worldwide. Regarding *H. perforatum* plantations, *C. gloeosporioides* is responsible for great losses by lowering yields and eventually modifying the chemical composition of the extracts obtained.

A considerable effort has been done in order to find or develop *H. perforatum* plants resistant to anthracnose disease. Nonetheless, little is known about the defense responses of *H. perforatum* against pathogen attack. One *H. perforatum* accession, *Helos*, has shown to display some resistance to *C. gloeosporioides* infection. Despite this promising result, the chemical composition of *Helos* plants is not as valuable as that found in other *H. perforatum* accessions [Pank, 2000].

The main aim of this work was to study some of the defense mechanisms developed by *H. perforatum* upon *C. gloeosporioides* elicitation. Namely, both phenylpropanoid metabolism and hypersensitive response were evaluated, in cell suspension cultures obtained from two *H. perforatum* accessions, distinct in their susceptibility to *C. gloeosporioides* infection *in vivo*. Furthermore, we also studied the possible influence of the phytohormones methyl-jasmonate (MeJ) and salicylic acid (SA), related to two distinct plant systemic defense signaling pathways (SAR and ISR), in *H. perforatum* - *C. gloeosporioides* interaction.

The outline of this thesis is composed of 8 main chapters and, besides the general introduction (Chapter 1), the following chapters are described below.

Chapter 2 lists the materials and methodologies applied in the work described in this thesis.

Chapter 3 describes the establishment and characterization of *H. perforatum* cell suspension cultures, the plant model used along the work. Growth and survival parameters, as well as major nutrients consumption, were studied in cell cultures from both *H. perforatum* accessions available.

Chapter 4 describes the effects of pathogen elicitation, with or without prior treatment with the phytohormones SA or MeJ, on the parameters previously described in chapter 3. The effects of MeJ or SA alone were also monitored, on both *H. perforatum* suspension cell cultures.

Chapter 5 presents the results associated to reactive oxygen species accumulation, oxidative burst development and hypersensitive response, observed under the treatments previously described in chapter 4. Furthermore, the antioxidant potential of *H. perforatum* extracts, as well as the enzymatic ROS-scavenging capacity of *H. perforatum* cells were also monitored.

Chapter 6 presents the differential accumulation and identification of major soluble phenolic compounds, found on *H. perforatum* cultures from the more anthracnose-susceptible accession (HPS), when faced with each of the treatments described in chapter 4.

Chapter 7 compares the accumulation of lignin and soluble phenolic compounds on both *H. perforatum* accessions available, upon the several treatments previously described. Furthermore, PAL enzymatic activity and expression of some key phenylpropanoid pathway genes, related to *H. perforatum* defense mechanisms against *C. gloeosporioides*, are also described in this chapter.

Chapter 8 comprises the main, general conclusions that can be drawn from the work presented in this thesis.

Chapter 2

Materials and Methods

Chapter 2.1

Biological materials, reagents and culture media

2.1.1. Plant material

Hypericum perforatum L. (var. *HPS*) plants were collected from the National Park of Peneda-Gerês (Portugal) and used for the development of suspension cell cultures, established by Dias (2001). *H. perforatum* suspension cell cultures from *Helos* accession were obtained from commercially available seeds (Richters® seeds, ON, Canada) and sterilized as described below.

Helos seeds were water embedded overnight, at 4°C. Seed surface was disinfected by immersion in ethanol 70% (v/v) for 2 min, followed by bleach treatment, for 8 min. Seeds were then thoroughly rinsed using sterile H_2O. Germination was carried out in culture flasks containing 20 mL of MS medium [Murashige *et al.*, 1962] without any hormonal supplementation, as described below in table 2.2. Seedlings were grown in a culture chamber at 26°C with a 16h/8h photoperiod (cool white fluorescent light of 450-500 $\mu W/cm^2$) for approximately one month. Seedlings obtained *in vitro* were used for establishing *Helos calli* and suspension cell cultures, as described in chapter 2.1.6.

2.1.2. Fungal strain

A *Colletotrichum gloeosporioides* strain (CG1159), isolated from *H. perforatum* plants *in vivo*, was already available in the lab and maintained as described in chapter 2.1.7.

2.1.3. Bacterial strains

Table 2.1 presents the *Escherichia coli* strains that were used in several molecular biology procedures, including cDNA library screening and bacterial transformation, described in this thesis.

Table 2.1. *Escherichia coli* strains used in this work.

Organism	Strain	Genotipe	Reference
E. coli	XL1 Blue MRF'	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'proAB lacIqZΔM15 Tn10 (tetr)]	Jerpseth *et al.*, 1992
	XLOLR	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)] Su- (nonsuppressing) λr (lambda resistant)	Short *et al.*, 1992
	DH5α	F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Hanahan, 1983
	DH5α (DB3.1)	F- gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(rB–, mB–) supE44 ara-14 galK2 lacY1 proA2 rpsL20(SmR) xyl-5 λ– leu mtl1	Hanahan, 1983

2.1.4. Vectors and bacteriophages

H. perforatum L. cDNA library was previously prepared using the *ZAP Express* vector, provided with the *ZAP Express cDNA Synthesis Kit* (Stratagene). This vector possesses increased cloning capacity and the ability of both eukaryotic and prokaryotic expression. The main characteristic of lambda ZAP vectors is the ability to excise the cDNA insert in a phagemid (pBK-CMV), without the requirement for subcloning [Short *et al.*, 1992].

The *ZAP Express* vector was packaged into the *Gigapack III Gold* packaging extract (Stratagene) in order to create bacteriophage particles.

The *ExAssist* helper bacteriophage (Stratagene) was used to auxiliate the *in vivo* excision of the phagemid pBK-CMV from the Zap Express vector (Stratagene). This bacteriophage was provided with the *ZAP Express cDNA Synthesis Kit* (Stratagene).

DNA fragments obtained from PCR amplifications were cloned onto *pGEM-T Easy* vector (Promega), *pJET1.2/blunt* vector (Fermentas) or *pCR2.1 TOPO*® cloning vector (Invitrogen), which are designed to conveniently clone PCR products. Another vector used was the *pDONR*®*207* (Invitrogen), required in middle steps of Gateway® (Invitrogen) cloning techniques, as described in the manufacturer instructions manual [URL 16].

2.1.5. Culture media

2.1.5.1. H. perforatum L. culture media

A cell culture medium was developed in order to induce *H. perforatum calli* from *Helos* accession and subsequently produce and maintain a suspension cell culture. The definite culture medium chosen for the maintenance of both *HPS* and *Helos* cell suspension cultures ("MS-NAA") was based on the Murashige and Skoog (MS) medium [Murashige *et al.*, 1962]. Table 2.2 shows the detailed composition of MS-based medium, together with the hormonal supplementations tested for the development of *calli* and suspension cell cultures. Moreover, all culture media were sterilized by autoclaving recipients for 15 min at 121°C and 1 atm.

Table 2.2. Composition of MS-based media used in the maintenance of *H. perforatum* seeds, *calli* and suspension cell cultures.

	Composition	Murashige and Skoog, 1962 (MS)
Macronutrients (mg.L^{-1})	$MgSO_4.7H_2O$	370.00
	$CaCl_2.2H_2O$	440.00
	KNO_3	1900.00
	NH_4NO3	1650.00
	KH_2PO_4	170.00
Micronutrients (mg.L^{-1})	Na_2EDTA	37.300
	$FeSO_4.7H_2O$	27.800
	$MnSO_4.4H_2O$	22.300
	KI	0.800
	$CoCl_2.6H_2O$	0.025
	$ZnSO_4.7H_2O$	8.600
	$CuSO4.5H_2O$	0.025
	H_3BO_3	6.200
	$Na_2MoO_4.2\ H_2O$	0.250
Carbon source (g.L^{-1})	Sucrose	30.0
Vitamins (mg.L^{-1})	Nicotinic acid	0.50
	Tiamine-HCl	0.10
	Piridoxine-HCl	0.50
	Glicine	2.00
	Myo-inositol	100.00
Other components (g.L^{-1})	Agar	8.00
pH 5.8		

	Hormonal supplementation					
Hormones (mg.L^{-1})	**"NK"**	**"NK2"**	**"2,4-D"**	**"NAA"**	**"IBA"**	**"IK"**
Kinetin	0.5	0.1	0.02	-	-	0.5
NAA	1.0	0.1	-	0.5	-	-
2,4-D	-		0.2	-	-	-
IAA	-		-	-	0.5	0.8
BA	-		-	-	0.5	-

2.1.5.2. Microbiology culture media

Composition of the culture media used in growing, maintaining or operating fungal mycelium and bacterial cultures is depicted in table 2.3. All agarized media were obtained by adding 1.5% (w/v) agar to the medium's broth composition. Recombinant selection using the *lacZ* gene was performed by supplementing the appropriate agar plaques with 40 µg.mL^{-1} of IPTG (0.5 M in water) and 40 µg.mL^{-1} of X-gal (50 mg.mL^{-1} in DMF). The culture media were sterilized by autoclaving for 20 min at 121°C and 1 atm.

Table 2.3. Composition of culture media used for growing fungi and bacteria strains.

Culture medium	Composition	Aim	Strain
LB	1% (w/v) NaCl 1% (w/v) bacto-tryptone 0.5% (w/v) yeast extract pH 7.0	Growth and maintenance	*E. coli*

LB$^{-,-}$	LB 10 mM $MgSO_4.7H_2O$ 0.2% (w/v) maltose	Phage infection	*E. coli* XL1 Blue MRF' *E. coli* XLOLR
LB-Tet	LB 12.5 µg.ml^{-1} Tetracyclin	Growth and maintenance of transformants carrying Tet resistance mark	*E. coli* XL1 Blue MRF' *E. coli* XLOLR
LB-Kan	LB 50 µg.ml^{-1} Kanamycin	Growth and maintenance of transformants carrying Kan resistance mark	*E. coli* XLOLR *E. coli* DH5α
LB-Amp	LB 100 µg.ml^{-1} Ampicillin	Growth and maintenance of transformants carrying Amp resistance mark	*E. coli* DH5α
LB-Gen	LB 25 µg.ml^{-1} Gentamicin	Growth and maintenance of transformants carrying Gen resistance mark	*E. coli* DH5α
NZY	0.5% (w/v) NaCl 0.2% (w/v) $MgSO_4.7H_2O$ 0.5% (w/v) yeast extract 1% (w/v) NZ amine (casein hydrolysate), pH 7.5	Phage infection	*E. coli* XL1 Blue MRF' *E. coli* XLOLR
NZY Top agarose	NZY 0.7% (w/v) agarose	Phage infection	*E. coli* XL1 Blue MRF'
PDA	0.4% (w/v) potato infusion 2% (w/v) dextrose 1.5% (w/v) agar	Growth and maintenance	*C. gloeosporioides*
Modified Liquid Mathur	0.2% (w/v) yeast extract 0.2% (w/v) bacto-peptone 0.5% (w/v) $MgSO_4.7H_2O$ 0.54% (w/v) KH_2PO_4 2% (w/v) Sucrose	Growth for elicitor preparation	*C. gloeosporioides*

2.1.6. Establishment and maintenance of *H. perforatum* L. (*Helos*) suspension cell cultures

H. perforatum (*Helos*) suspension cell cultures were established after induction of *calli* tissues in agarized MS medium. For this, 10 mm length sections of roots, stems and leaves were obtained under aseptic conditions. Three to five explants were transferred to a culture flask containing 20 mL of agarized MS medium with the hormonal supplementations described in table 2.2. *Calli* were allowed to develop in a culture chamber at 26°C with a 16h/8h photoperiod (cool white fluorescent light of 450-500 µW/cm^2) and were subcultured to fresh medium monthly.

Suspension cultures were initiated from 3-week old *calli* by transferring 2-5 g of biomass to 250 mL Erlenmeyer flasks containing 25 mL of MS liquid medium, with the corresponding hormonal supplementation. *Calli* fragments were incubated at 26°C with a 16/8h photoperiod (cool white fluorescent light of 450-500 µW/cm^2) on an orbital shaker, at 110 rpm. Additional 25 mL were added when biomass started to increase. Initial subcultures were performed according to the morphological characteristics (browning and biomass accumulation) observed. After stabilization, the subsequent subculture cycles were performed at late exponential phase (approximately 12 days), by transferring 10

mL of the culture into 70 mL of fresh MS medium. Moreover, both *Helos* and HPS suspension cell cultures were maintained in the same medium supplementation (NAA) and subcultured every 14 days.

2.1.7. *C. gloeosporioides* growth, maintenance and elicitor preparation

C. gloeosporioides cultures were maintained in potato dextrose agar (PDA) plates at room temperature, and subcultured by mycelium transfer to fresh medium every month. For long term storage, glycerol stocks were created (chapter 2.1.11.).

The elicitor suspension was prepared by transferring 4-5 PDA agar plugs containing *C. gloeosporioides* mycelium to a modified liquid Mathur's medium [Freeman *et al.*, 2000b], as described in chapter 2.1.5.2. The fungus was allowed to grow for 14 days at 25°C on an orbital shaker at 250 rpm. The biomass from cultures at late exponential growth phase was recovered by vacuum-filtration and lyophilized for 48 h in a *Christ Alpha RVC Lyophilizer* (B-Braun). Dried *C. gloeosporioides* biomass was crushed with a mortar and pestle and the powder stored, at room temperature, in falcon tubes. Elicitor suspension was prepared fresh, before every experiment, by autoclaving the powder in distilled water to a final concentration of 20 $mg.mL^{-1}$. Autoclaving conditions were the same as described for microbiology culture media, in chapter 2.1.5.2. To confirm its sterility, 200 µL of elicitor preparation were inoculated in PDA plates and kept at the same conditions as described above for the maintenance of *C. gloeosporioides* cultures.

2.1.8 Methyl-Jasmonate and Salicylic Acid *priming* solutions

Commercially available phytohormones methyl-jasmonate (MeJ) and salicylic acid (SA) (Sigma-Aldrich) were dissolved in ethanol 100% (v/v) to final concentrations of 35 $mg.mL^{-1}$ and 20 $mg.mL^{-1}$, respectively, and stored in screw-cap glass vials, for up to two months, at 4°C.

2.1.9. Treatment of *H. perforatum* suspended cells with *C. gloeosporioides* elicitor and/or phytohormones (MeJ or SA) priming solutions

H. perforatum suspension cultures from both HPS and *Helos* accessions were grown until early exponential phase and divided into 6 groups, as described below (Table 2.4). One group of flasks was

kept as control. Two sets were treated, on the 5th day of growth, with MeJ or SA priming solutions, to a final concentration of 100 µM and 25 µM, respectively. The remaining three groups were elicited on the 6th day with the fungal suspension. Two of those sets were primed with MeJ (100 µM) or SA (25 µM), 24 h before fungal elicitor treatment. The final concentration of the fungal biomass in the suspension cultures was 0.25 $g.L^{-1}$. The priming and elicitation procedures were done in cells during their exponential phase, the growth period at which they were most responsive to treatments. Cell suspension culture samples were collected at desired times, according to the subjacent methods used.

Table 2.4: Main elicitation assay scheme, used in most of the work reported in this thesis.

Experimental Set	Days of growth (HPS and *Helos*) 5	6	Notes
"Ct"	-	-	Control cultures
"MeJ"	+ MeJ 100 µM	-	Cultures treated with Methyl-jasmonate only
"SA"	+ SA 25 µM	-	Cultures treated with Salicylic acid only
"SA+Cg"		+ Cg	Cultures treated with SA, prior to Cg elicitation
"MeJ+Cg"	+ MeJ 100 µM		Cultures treated with MeJ, prior to Cg elicitation
"Cg"	-		Cultures treated with *C. gloeosporioides* biomass only

2.1.10. Growing of bacterial strains

E. coli strains were grown in the appropriate medium as indicated in table 2.3. For isolating single colonies, the strains were stroke onto an appropriate agarized medium and incubated overnight at 37°C. Liquid cultures were obtained by inoculating a single colony into the medium and incubating at 37°C with agitation (150 - 250 rpm).

2.1.11. Glycerol stock preparation

Long term viable stocks of *E. coli* strains were prepared by inoculating single colonies in the appropriate liquid medium (Table 2.3.), followed by growth in the appropriate conditions (chapter 2.1.10.) until reaching late exponential growth phase. Culture aliquots were then added to sterile glycerol-containing criotubes to a final 20% (v/v) glycerol concentration.

C. gloeosporioides glycerol stocks were prepared by growing mycelium cultures in PDA medium or modified Mathur's medium for 30 or 15 days, respectively, when a reasonable amount of biomass

had accumulated. Mycelium aliquots were then transferred to sterile glycerol-containing criotubes at a final 30% (v/v) glycerol concentration.

All vials were immediately stored at -80°C. To maintain cell viability, stocks were recovered by scraping off splinters of solid ice with a sterile wire loop.

2.1.12. Reagents

All chemicals used for molecular biology methods and nucleic acid extractions were *Molecular Biology* grade. Solvents and chemical compounds used in HPLC studies were all HPLC grade. The remaining chemicals were *p.a.* grade.

2.1.13. Material treatment

RNA manipulation was carried out under special conditions to prevent RNase contamination. The specifications are described in table 2.5. DEPC was destroyed by autoclaving, for 20 min, at 121°C and 1 atm.

Table 2.5. List of basic laboratory practices used in order to promote an RNase free environment.

Reagents and Material	Treatment
Water	u.p. treated overnight with 0.1% (v/v) DEPC and autoclaved
Solutions	Prepared using u.p. water. Treated overnight with 0.1% (v/v) DEPC and autoclaved
Glass and ceramics	Treated at 180°C for 6h
Disposable materials	Autoclaved at 1 atm for 1h
Electrophoresis material	Treated overnight with 0.1% (v/v) DEPC or for 2h with 0.1 M NaOH

Chapter 2.2

Biochemical methods

2.2.1. Characterization of *H. perforatum* suspension cell cultures

2.2.1.1. Determination of dry weight

Three independent *H. perforatum* suspension cell cultures, in MS-NAA medium, were started using the same inoculum, grown under the conditions described in chapter 2.1.6. Aliquots of 5 mL of suspended cells were harvested under sterile conditions, over a total period of 20 days (for experiments shown in chapter 3), or over 12 and 14 days for *Helos* and HPS, respectively (for the experiments shown in the following chapters, unless stated otherwise). Samples were centrifuged at 4000 *g* for 4 min using half strength deceleration to avoid cell resuspension. The supernatant was separated and cells were filtered using pre-weighted *GF/C glass microfiber filter* (Whatman). Dry weight was determined after lyophilization of the cells for 48 h in a *Christ Alpha RVC Lyophilizer* (B-Braun). The pH value of each supernatant was immediately measured and stored at -20°C for further studies.

2.2.1.2. Determination of cell viability

Viability of *H. perforatum* suspended cells was determined using two distinct methods. In the *trypan blue* exclusion method, aliquots of the cell culture were mixed with an identical volume of 0.4% (w/v) *trypan blue*, and incubated in the dark, for 10 min. Cells were observed under a light microscope. Non-viable cells were stained in blue.

H. perforatum cell viability was also checked by fluorescein diacetate (FDA) and propidium iodide (PI) double staining. Briefly, after thorough mixing using cut pipette tips, 1 mL of cell suspension was transferred to an Eppendorf tube. To each sample, 10 µL of FDA (500 µg.µL^{-1}, Sigma) and 1 µL of PI, (500 µg.µL^{-1}, Sigma) were added, thoroughly mixed and incubated in dark, at room temperature (25°C). After 10 min of incubation, 100 µL of cell suspension were spread on a glass slide and observed under a *Leica DM 5000B Microscope* (Leica Microsystems, Wetzlar, Germany) equipped with an *AF6000 fluorescent lamp* (Leica Microsystems). Microscope was programmed for excitation at 490 nm and emission at 510 nm (for FDA) and excitation at 543 nm and emission at 570 nm (for PI). Light microscopic and fluorescent images were acquired using a *DFC350 Camera* (Leica Microsystems) attached to the microscope. Viable cells were stained in green while non-viable ones stained red.

2.2.1.3. HPLC quantification of sugar content

Sucrose, fructose and glucose levels were determined by HPLC in a *Gilson* system composed of a *piston pump model 307* and a *refractive index detector model 132*, coupled with a *block heater model 7970* (Jones Chromatography). The HPLC system was connected through the *Gilson 506C System Interface Module* to a CPU containing the *System Controller Gilson 712* software. The mobile phase consisted in a solution of 0.125 $g.L^{-1}$ calcium nitrate in u.p. water, filtrated through a 0.2 µm nylon membrane and vacuum degasified. Supernatant samples obtained (chapter 2.2.1.1) were filtrated for cell debris removal, and an equal volume of 10 $g.L^{-1}$ arabinose was added, serving as the internal standard. For sample running, 20 µL were injected, through a 0.5 $mL.min^{-1}$ flow, into a *HyperRez H+ Carbohydrate LG* column (Hypersyl) at 37°C.

2.2.1.4. Phosphate quantification

The orto-phosphate ion was quantified spectrophotometrically using the ascorbic acid method, as described by Adams (1991). The working solution was composed of 50 mL of *Armstrong reagent*, added to 10 mL of freshly made 3% (w/v) ascorbic acid solution. Supernatant samples obtained in chapter 2.2.1.1 were unfrozen and diluted 2 - 20 fold. A one-mL aliquot was added to 120 µL of working reagent, vortexed and allowed to react for 20 min. The presence of phosphate in the sample was detected by the appearance of blue coloration and absorbance was determined at 880 nm, in a UV-VIS double beam spectrophotometer *Cary 1E UV-Vis Spectrophotometer* (Varian). A calibration curve was produced using KH_2PO_4 solutions with linearity being observed between 30 - 4.500 $\mu g.L^{-1}$.

Armstrong reagent	11.8% (v/v) H_2SO_4; 0.03% (w/v) $K(SbO)C_4H_4O_6.1/2H_2O$; 1.05% (w/v) ammonium molibdate

2.2.1.5. Ammonium and nitrate quantification

Ammonium was quantified using a *Spectroquant Ammonium Test* kit (Merck). For this purpose, 5mL of *H. perforatum* suspension cell cultures were harvested and weighted, as described in chapter 2.2.1.1. According to the manufacturer instructions, 200 µL of the supernatant obtained were added to

a falcon tube containing 5 mL of NH-1 reagent and mixed at room temperature. Reagent NH-2 was then added (30 mg or *1 level blue microspoon*, as described in the product's manual) and the mixture was shaken vigorously, until the reagent was completely dissolved. After 15 min incubation at room temperature, ammonium was quantified spectrophotometrically (A_{690}). A calibration curve was made using NH_4Cl, showing linearity between 10 and 300 $mg.L^{-1}$.

As for nitrate quantification, an adaptation of the sulphamic/perchloric acid method [Carvalho *et al.*, 1998] was performed. Briefly, 400 µL of the supernatant were added to 100 µL of sulphamic acid (20% v/v, Merck) at room temperature, vigorously shaken and allowed to rest for 2 min. Samples were vortexed once more and 500 µL of perchloric acid (10% w/v, Pronalab) were added. After vigorous vortex shaking, absorbance was measured spectrophotometrically (A_{210}). A calibration curve was made using KNO_3, showing linearity between 0.1 and 1.0 $mg.L^{-1}$.

2.2.2. Determination of lignin content

The lignin content in cell walls of *H. perforatum* suspension cultures was determined through the acetyl bromide method, adapted from Fukushima (2001). *H. perforatum* suspension cells were harvested, washed twice in d.d. water by centrifugation at 5000 *g* for 5 min, lyophilized for 48 h in a *Christ Alpha RVC Lyophilizer* (B-Braun) and grinded to a fine powder. Between 0.1 - 0.2 g of cell material were transferred onto a 15 mL Falcon tube and precision weighted. Cells were added 5 mL of 90% (v/v) methanol, vortexed thoroughly and allowed to extract for 24 h in the dark, at room temperature. The methanol extract was removed by centrifugation (5000 *g* for 5 min). Cell material was consecutively extracted with water, acetone and hexane as previously described, after which it was dried overnight at 60°C. Ten mg of cell wall were added to 500 µL of acetic acid and 500 µL of 25% (v/v) acetyl bromide in acetic acid and incubated at 50°C, for 2 hours with agitation (150 rpm). Samples were centrifuged and 100 µL of the supernatant were mixed with 200 µL of acetic acid, 150 µL of 3 M NaOH and 50 µL of 0.5 M hydroxylamine hydrochloride. Finally, 500 µL of acetic acid were added, and the absorbance was determined at 280 nm in a spectrophotometer. A standard calibration curve was generated with lignin (Aldrich), showing linearity within the range of 0.005 - 1.0 $mg.mL^{-1}$.

2.2.3. Determination and identification of the soluble phenolics content by HPLC-DAD and HPLC-MS-MS

Dried *H. perforatum* biomass from both cell suspension cultures was lyophilized for 48 h in a *Christ Alpha RVC Lyophilizer* (B-Braun) and extracted with an aqueous methanolic solution (90%) (at approximately 50 mg dw.mL^{-1}), with sonication for 20 min, in the dark, at room temperature. The liquid phase was filtered and submitted to HPLC–DAD analysis, as described elsewhere [Dias *et al.*, 1999].

Quantification of phenolic compounds was performed by the external standard method. Xanthones were quantified as mangiferin equivalents at 260 nm. Flavonols and flavones were quantified at 350 nm as quercetin and luteolin-7-glucoside equivalents, respectively.

Phenolic identification was performed by HPLC–MS–MS. Chromatographic separation was carried out on an *RP C18 column* (25×0.4 cm, particle size 5 µm, Merck, Germany), using water/formic acid (99:1) and methanol as the mobile phases. Elution was performed as described elsewhere [Dias *et al.*, 1999]. The HPLC system was an *Agilent HPLC 1100* instrument series equipped with an *Agilent DAD detector G1315B* (Agilent Technologies, Germany), and mass detector in series, controlled by software from Agilent Technologies (Germany). The mass detector used was an ion-trap mass spectrometer *G2445A* (Agilent Technologies, Germany), equipped with an electrospray ionization (ESI) system. The heated capillary and voltage were maintained at 350°C and 4 kV, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. Mass scan (MS) and daughter (MS–MS) spectra were measured from 100au to *m/z* 1.500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired, both in the negative and positive modes.

2.2.4. Quantification of reactive oxygen species

2.2.4.1. Detection of $O_2^{\cdot-}$

The intracellular production of the superoxide radical ($O_2^{\cdot-}$) was quantified by the reduction of the tetrazolium dye sodium,3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT; Molecular Probes) to a soluble formazan [Able *et al.*, 1998].

Immediately before suspended cell elicitation with *C. gloeosporioides*, *H. perforatum* cells were added 0.5 mM XTT and incubated in the dark, at room temperature with agitation. Aliquots were removed periodically, and the reduced XTT form was readily quantified by reading the absorbance of the supernatant at A_{470}. Similar assays were performed using MeJ, SA or a combination of one of these phytohormones with *C. gloeosporioides*, as previously described in section 2.1.9.

2.2.4.2. Detection of total ROS

The overall oxidative stress of the cell was quantified using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes) as previously described [Parsons *et al.*, 1999; Allan *et al.*, 2001]. H_2DCFDA is converted by nonspecific cellular esterases to H_2DCF, which oxidizes in the presence of H_2O_2 or reactive oxygen intermediates. The end product 2',7'-dichlorofluorescein is highly fluorescent [Cathcart *et al.*, 1983] and able to diffuse out of the cell. This property was used to quantify the intracellular production of 2',7'-dichlorofluorescein, by performing a spectrofluorimetric analysis of the supernatant.

During the time course of *H. perforatum* suspended cells elicitation with *C. gloeosporioides* biomass, 1 mL aliquots were removed and mixed with 10 µL of 20 µM H_2DCFDA. Cells were incubated in the dark, at room temperature, for 30 min with agitation. Samples were centrifuged at 8000 *g* for 5 min and the supernatant recovered. Relative fluorescence was quantified using a *LS 50 Luminescence Spectrometer* (Perkin Elmer) at an excitation wavelength of A_{488} and an emission wavelength of A_{525}.

Similar assays were performed using MeJ, SA or a combination of one of these phytohormones with *C. gloeosporioides*, as described in section 2.1.9.

2.2.4.3. Detection of H_2O_2

The quantification of H_2O_2 concentration in cell suspension medium was performed spectrophotometrically by the xylenol orange method [Bellincampi *et al.*, 2000]. The reaction is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the formation of a complex between Fe^{3+} and xylenol orange (*o*-cresosulfonephthalein 3',3''-bis[methyl-imino] diacetic acid, sodium salt).

Aliquots of *H. perforatum* suspended cells were harvested during the time course of the elicitation with *C. gloeosporioides* biomass and/or the phytohormones MeJ or SA. The supernatant was

recovered after centrifugation at 8000 *g* for 5 min. A 500 µL aliquot of cell medium was added to 500 µL of assay solution. The reaction mixture was incubated for 45 min at room temperature and analyzed in a spectrophotometer (A_{560}). A calibration curve was constructed using H_2O_2 dilutions in MS-NAA medium.

Assay solution	500 µM ammonium ferrous sulfate; 50 mM H_2SO_4; 200 µM xylenol orange; 200 mM sorbitol

2.2.5. Detection of lipid peroxidation

H. perforatum suspension cells were grinded to a fine powder in liquid nitrogen with a mortar and stored at -80°C. Samples (approximately 250 mg of fresh weight) were thawed in 2 mL of protein extraction buffer, vortexed and incubated on ice, for 5 min. After centrifugation for at 13.000 g for 15 min at 4°C, the supernatant was recovered and immediately used for enzyme assays or stored at -80°C. Protein was quantified using the *Coomassie Blue* method [Sedmak *et al.*, 1977]. The reaction mixture consisted of 1 mL of *Coomassie Blue reagent* and 100 µL of a suitable dilution of protein extract. Samples were incubated for 10 min at room temperature in the dark, after which absorbance was read at 595 nm. Bovine serum albumine (0.5 - 10 µg) was used as standard.

Lipid peroxidation was quantified spectrophotometrically by the MDA-TBA method [Loreto *et al.*, 2001], which quantifies the end product of lipid peroxidation malondialdehyde (MDA) by a reaction at low pH and high temperature with 2-thiobarbituric acid (TBA). The reaction was initiated by adding 250 µL of protein extract from *H. perforatum* suspension cells, to 750 µL of chilled reaction solution. The mixture was incubated at 95°C for 30 min and placed immediately on ice. Samples were centrifuged at 10.000 g for 5 min at 4°C, and the supernatant was recovered. Quantification of the MDA-TBA complex was performed by determining the absorbance of the supernatant at 532 nm and deducting non-specific absorbance at 600 nm. The molar extinction coefficient of MDA-TBA complex, at 532 nm, is 155 $mM^{-1}.cm^{-1}$.

Extraction buffer	50 mM Tris-HCl, pH 8.5; 1 mM EDTA
Reaction solution	0.5% (w/v) TBA 20% (w/v) TCA

2.2.6. Enzymatic activity measurements

2.2.6.1. Phenylalanine ammonia-lyase (PAL) enzymatic activity assay

Alternatively to the method described in chapter 2.2.5, PAL activity assays were performed using a distinct protein extraction method, adapted from Mizukami *et al.* (1991). Briefly, cells were collected by centrifugation and immediately frozen in liquid nitrogen. Biomass was then ground to a fine powder in a cold mortar and stored at -80°C until preparation of the enzyme extract. All the following operations were carried out at 4°C. The frozen cells were mixed with *Polyclar AT* and 5 mL of the extraction buffer and thawed with stirring for 15 min. The mixture was centrifuged at 10.000 *g* for 15 min, at 4°C, and the resulting supernatant was used as crude enzyme extract. Protein concentration of the crude enzyme was determined by the Bradford method (1976), using bovine serum albumin as a standard protein.

PAL activity measurements were done according to Saunders *et al.* (1974). Briefly, a 400 µL aliquot of the protein extract was added to 600 µL of reaction mixture and incubated at 40°C. Samples were collected and absorbance was measured at 290 nm to determine cinnamic acid formation. Blank assay was performed by using 400 µL of d.d. water instead of protein extract. A control was prepared by boiling the protein extract for 10 min, prior to reaction mixture addition.

Extraction buffer	50 mM Tris-HCl, pH 8.5; 1 mM EDTA
Reaction mixture	100 mmol.L^{-1} Tris-HCl, pH 8.5; 50 mmol.L^{-1} L-phenylalanine

2.2.6.2. Superoxide dismutase (SOD) and catalase (CAT) enzymatic activity assays

Total protein crude extract was prepared and quantified as described by Jebara (2005), with modifications. The biomass was grinded with liquid nitrogen in a mortar. The fine powder (5 g) was then mixed with 5 mL of the extraction buffer.

Catalase activity was measured by following the decline in A_{240} as H_2O_2 (E = 36 M^{-1} cm^{-1}) was catabolised, according to the method of Aebi (1984) in a reaction mixture containing 200 µL of enzyme extract in 50 mM potassium phosphate buffer, at with pH 7.5. The reaction was started by addition of H_2O_2 at the final concentration of 15 mM, and its consumption was measured for 10 min, at 240 nm.

SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) as described by Giannopolitis (1977). A 2 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 75 mM NBT, 2 mM riboflavin, 10 mM methionine, 0.1 mM EDTA was added to 0.1 mL of enzyme extract. The reaction mixture was illuminated for 15 min at light intensity of 75 μmol m^{-2} S^{-1} and NBT reduction was monitored at A_{560}.

Extraction buffer	10 mM DTT 50 mg PVP 0.1 mM EDTA 50 mM KH_2PO_4 buffer, ph 7.5

2.2.7. Antioxidant potential of *H. perforatum* extracts

The antioxidant potential of methanolic extracts from *H. perforatum* suspension cells was determined by the 1,1–diphenyl–2–picrylhydrazyl (DPPH) assay, as described previously [Silva *et al.*, 2005]. The methanolic extract was obtained as described in section 2.2.3. The antiradical activity of each sample was evaluated using a series of dilutions, in order to obtain a large spectrum of sample concentrations. The reaction mixture consisted of 100 μL of diluted sample and 1.4 mL of 80 μM DPPH (dissolved in 100% ethanol). The absorbance was monitored continuously at 515 nm with a *UV/VIS Spectrometer Lambda2* (Perkin–Elmer), assuring that the reaction was complete (plateau state). The percentage of reduced DPPH at steady state (DPPH–R) was calculated and these values were plotted against the concentrations methanolic extracts. A decrease by 50% of the initial DPPH concentration was defined as the half maximal effective concentration (EC_{50}).

2.2.8. TUNEL assay

TUNEL assay was performed according to the procedure described by Gavrieli *et al.* (1992). The reaction is based on the presence of single and double-stranded breaks in genomic DNA during apoptosis. Terminal deoxynucleotidyl transferase (TdT) polymerizes fluorescein labelled nucleotides to free 3'-OH termini, in a template independent manner. The TUNEL reaction was performed using the *In Situ Cell Death Detection Kit - Fluorescein* (Roche Applied Science), according to the manufacturer's instructions, and as described by Sgonc *et al.* (1994). *H. perforatum* suspended cells were washed

once with PBS and subsequently fixated for 1 h at 20°C in newly prepared fixation solution. Cells were washed in PBS and incubated for 10 min at 20°C in blocking solution. After washing in PBS, permeabilisation solution was added, followed by 2 min of incubation on ice. The TUNEL reaction solution was prepared by mixing *Label and Enzyme solution* (Roche Applied Science). Suspended cells were washed twice in PBS and carefully dried, after which 50 µL of TUNEL solution were added to approximately 50 µL of cells. The mixture was incubated for 60 min at 37°C in the dark. Finally, cells were washed three times with PBS and transferred to a glass slide. Fluorescein has a detection range of 515-565 nm (green). Cells were analysed for TUNEL reaction under a UV light with an excitation wavelength of 450-490 nm and an emission filter of 510 nm in a fluorescence microscope.

PBS solution	0.2 $g.L^{-1}$ KCl 8 $g.L^{-1}$ NaCl 1.44 $g.L^{-1}$ Na_2HPO_4 0.24 $g.L^{-1}$ KH_2PO_4
Fixation solution	4% Paraformaldehyde in PBS, pH 7.4
Blocking solution	3% H_2O_2 in methanol
Permeabilization solution	0.1% (v/v) Triton X-100 0.1% (w/v) sodium citrate

Chapter 2.3

Molecular biology methods

2.3.1. RNA purification

2.3.1.1. Total RNA purification (High quantity)

This protocol was developed for the purification of high amounts of RNA from *H. perforatum* suspension cells, suitable for northern blotting experiments. Cells were ground to a fine powder in liquid nitrogen with a mortar and pestle. In a 15-mL Falcon tube, 1 g of frozen powder was mixed with 3 mL of cold extraction buffer and vortexed vigorously. 1 vol of phenol-chloroform-isoamyl alcohol (25:24:1 [v/v/v]) was added and the mixture was vortexed, prior to centrifugation at 12.000 rpm for 5 min, at 4ºC to separate phases. The top aqueous phase was transferred to another tube and the phenol extraction procedure was repeated once more. The supernatant was collected in a new falcon tube and extracted by adding 1 vol of chloroform-isoamyl alcohol (24:1[v/v]), followed by thorough vortexing and centrifugation at 12.000 rpm for 15 min at 4°C to separate phases. The top phase was recovered and 0.5 vol of cold ethanol 96% (v/v) was added. Samples were incubated on ice for 30 min to precipitate polysaccharides and then centrifuged at 10.000 rpm, for 10 min at 4ºC. The supernatant was collected to a new tube and 1 vol of 4 M LiCl was added. Samples were incubated on ice overnight, for RNA precipitation, and then centrifuged at 12.000 rpm, for 30 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 2 vol of cold ethanol 96% (v/v). After 20 min precipitation at -20ºC, the samples were centrifuged at 12.000 rpm, for 30 min, at 4°C. The pellet was dissolved in 50 - 200 µL of DEPC-treated water and the RNA's concentration and purity were determined spectrophotometrically. RNA samples were immediately frozen in liquid nitrogen and stored at -80°C.

RNA extraction buffer	25 mM Tris-HCl, pH 8.0 25 mM $EDTANa_2$, pH 8.0 75 mM NaCl 1% (w/v) SDS 2% (v/v) ß-mercaptoethanol 4% (w/v) Polyvinylpolypyrrolidinone (PVPP, Sigma P-6755)

2.3.1.2. Total RNA purification (High quality)

This protocol was used for the purification of high-quality RNA in lower amounts, suitable for cDNA synthesis. *H. perforatum* suspension cells were filtered using pre-weighted *GF/C glass microfiber filter* (Whatman) and immediately ground to a fine powder in liquid nitrogen with a mortar and pestle. In an Eppendorf tube, 100 mg of frozen biomass and 450 µL of buffer RLT (including 4.5 µL of ß-

mercaptoethanol) were added and thoroughly vortexed. The lysate was transferred to a *QIAshredder spin column* (Qiagen) and centrifuged at 8000 *g*, for 2 min, at 4°C. The supernatant was transferred to a new microtube and 0.5 vol of absolute ethanol were added, before mixing. The sample was then transferred to an *RNeasy spin column* (Qiagen) and centrifuged for 15 s at 8000 *g* and 4°C. The column containing the sample was washed once with 700 µL Buffer RW1 and twice with 500 µL buffer RPE. Centrifugations between washes were done for 30 s, at 8000 *g* and 4°C. The column was then transferred to a new microtube and centrifuged as before, but for 2 min, in order to remove any traces of the washing buffers used. The dry column was finally transferred to a new microtube and 50 µL of pre-warmed DEPC-treated water were added. After 1 min incubation, the column was centrifuged for 1 min at 8000 *g*, at room temperature. The total RNA's concentration and purity were determined spectrophotometrically. The RNA was immediately used or frozen in liquid nitrogen and stored at -80°C.

2.3.2. DNA purification

2.3.2.1. Plasmid isolation - quick boiling miniprep

For purifying small amounts of DNA, the boiling method [Holmes *et al.*, 1981] was used. Bacteria were grown in appropriate culture medium, until end exponential phase was reached. An aliquot of 1.5 mL of the culture was removed and centrifuged at 8000 *g* for 5 min to collect the cells. The pellet was resuspended in 400 µL of STET supplemented with 25 µL of freshly prepared lysozyme solution. Lysis was promoted by incubation at room temperature for 10 min, followed by incubation at 95°C for 1 min. Denaturated proteins and chromosomal DNA was removed by centrifugation at 14.000 *g* for 15 min. The supernatant was recovered and mixed with 300 µL of isopropanol to precipitate plasmid DNA. After centrifugation under the same conditions, the supernatant was discarded. The plasmids were then resuspended in 20 - 100 µL of TE and stored at – 20°C.

STET	10 mM Tris-HCl (pH 8.0); 100 mM NaCl; 1 mM EDTA; 5% (v/v) Triton X-100
Lysozyme solution	10 mM Tris-HCl (pH 8.0); 10 mg.mL^{-1} lysozime
TE	10 mM Tris-HCl (pH 8.0); 1 mM EDTA

2.3.2.2. Plasmid purification - miniprep kit

Small scale isolation of high purity plasmid DNA was performed using the *QIAGEN Plasmid Mini Kit* (Qiagen), according to the supplier's instructions. Bacteria were grown in appropriate culture medium, until end exponential phase was reached.

A 3 mL sample was centrifuged at 8000 *g* for 5 min to collect the cells. The bacterial pellet was resuspended in 300 µL of resuspension buffer and an equal volume of lysis buffer was added, followed by gentle mixing and incubation at room temperature for 5 min. The lysate was neutralized by the addition of 300 µL of neutralization buffer, followed by gentle mixing and incubation on ice for 5 min. Cell debris, denatured proteins and chromosomal DNA were removed by centrifugation at 14.000 *g* for 10 min.

The supernatant was loaded onto a *Qiagen-tip 20* column (Qiagen), previously equilibrated with 1 mL of equilibration buffer. The column was washed with 4 x 1 mL of wash buffer and plasmid DNA was eluted by loading 800 µL of elution buffer. To precipitate plasmid DNA, 0.7 vol. of isopropanol were added. After centrifugation at 8000 *g* for 15 min, the supernatant was discarded and the plasmid pellet briefly washed in 70% (v/v) ethanol. Plasmid DNA was resuspended in 20 - 50 µL of u.p. sterile water and stored at -20°C.

Resuspension buffer	50 mM Tris-HCl (pH 8.0); 10 mM EDTA; 100 µg.mL^{-1} of RNase A
Lysis buffer	200 mM NaOH, 1% (w/v) SDS
Neutralization buffer	3.0 M KOAc (pH 5.5)
Equilibration buffer	50 mM MOPS (pH 7.0); 750 mM NaCl; 15% (v/v) isopropanol; 0.15% (v/v) Triton X-100
Wash buffer	50 mM MOPS (pH 7.0); 1 M NaCl; 15% (v/v) isopropanol
Elution buffer	50 mM Tris-HCl (pH 8.5); 1.25 M NaCl; 15% (v/v) isopropanol

2.3.2.3. Plasmid purification – midiprep kit

Medium scale isolation of high purity plasmid DNA was performed using the *Wizard™ Plus Midipreps DNA Purification System* (Promega), according to the supplier's instructions. Bacteria were grown in appropriate culture media until end exponential phase was reached.

The bacterial culture (100 mL) was centrifuged at 8000 *g* for 5 min. The cells were resuspended in 3 mL of resuspension buffer and an equal volume of lysis buffer was added, followed by gentle mixing and incubation until the cell suspension cleared. The lysate was neutralized by the addition of 3 mL of neutralization buffer, followed by gentle mixing. Cell debris were removed by centrifugation at 14.000 *g* for 15 min at 4°C.

The supernatant was combined with 10 mL of the *Wizard® Midiprep DNA Purification Resin* (Promega) and loaded onto the midicolumn. Vacuum was applied to the column to promote packaging of the resin and 2x15 mL of washing buffer were subsequently added. The midicolumn was placed inside a 1.5 mL microtube and centrifuged at 8000 *g* for 2 min to remove any residual wash buffer. The midicolumn was transferred to a new microtube and plasmid DNA was eluted by loading 300 µL of u.p. sterile water at 70°C. After a 1 min incubation, the column was centrifuged at 8000 *g* for 20 sec to collect the eluted plasmid. The column was discarded and the plasmid DNA solution was centrifuged at 8000 *g* for 5 min to precipitate column debris. The supernatant was stored at -20°C.

Resuspension buffer	50 mM Tris-HCl (pH 7.5); 10 mM EDTA; 100 µg.mL^{-1} of RNase A
Lysis buffer	200 mM NaOH, 1% (w/v) SDS
Neutralization buffer	1.32 M KOAc (pH 4.8)
Wash buffer	8.3 mM Tris-HCl (pH 7.5); 80 mM KOAc; 40 µM EDTA; 55% (v/v) ethanol

2.3.2.4. cDNA purification from phage library

The DNA from *H. perforatum* cDNA library was isolated by adding 1 vol of phenol-chloroform-isoamyl alcohol (25:24:1 [v/v/v]) to 1 mL phage suspension. The mixture was vortexed, centrifuged at 7500 *g*, for 5 min and the aqueous phase recovered. The DNA was precipitated by adding 2 vol. of

ethanol, 1/10 vol of 3 M NaAc (pH 5.2) and incubating the mixture at -20°C for 4h. The sample was centrifuged at 7500 *g*, for 10 min at 4°C and the pellet was resuspended in u.p. water and stored at -20°C.

2.3.3. cDNA library screening

2.3.3.1. Plating and titering the cDNA library

Most *E. coli* strains contain the McrA and McrB (modified cytosine restriction systems), which are responsible for digesting hemimethylated DNA [Raleigh *et al.*, 1998]. Therefore, an *E. coli* strain (XL1-Blue MRF') lacking this system was necessary to plate the *ZAP Express* cDNA library. Once the library is amplified using XL1-Blue MRF' cells, the DNA is no longer hemimethylated and can be grown on strains like XLOLR, that contain the McrA and McrB restriction systems.

XL1-Blue MRF' cells were grown in LB^{++} (Table 2.3) and harvested when A_{600} had reached 0.5 - 1.0. The cells were centrifuged at 5.000 *g* for 10 min, and resuspended in 10 mM $MgSO_4$ to a final A_{600} of 0.5.

For platting the cDNA library, serial dilutions (10^{-2}-10^{-5}) of the packaged reaction mixture were performed. Aliquots of 100 µL of each dilution were added to 200 µL of XL1-Blue MRF' cells. After phage infection, the tubes were incubated at 37°C for 15 min, to allow the adsorption of the phage particles to bacterial cell wall. Then, 3 mL of melted Top Agarose NZY medium supplemented with 15 µL of 0.5 M IPTG and 50 µL of X-gal (250 $mg.mL^{-1}$ in dimethylformamide) were added to each sample, and immediately poured onto NZY agar plates. After incubation at 37°C for 12 h, phage plates were counted to determine the library's titer. Phagic plates containing non-recombinant clones were stained in blue.

To titer the amplified secondary library, 25 µL aliquots of dilutions 10^{-6}-10^{-11} were incubated with XL1-Blue MRF' cells under the conditions described for primary library, with the exception that Top Agarose NZY was not supplemented with IPTG and X-gal.

2.3.3.2. cDNA library screening

Amplified *H. perforatum* cDNA library was plated at a density of approximately $5x10^4$ plaques in 150 mm NZY agar plates. The phage suspension was combined with 600 µL of XL1-Blue MRF' cells.

For this purpose, XL1-Blue MRF' cells were prepared as previously described (chapter 2.3.3.1.). The tubes were incubated at 37°C for 15 min, and 6.5 mL of melted Top Agarose NZY medium were added. The mixture was quickly distributed onto the NZY agar plates, incubated at 37°C for 12 h and then transferred to 4°C for 2 h.

Phage particles were transferred from plaques to duplicate nylon filter discs (*Hybond-N+*; Amersham). The first disc was placed onto the agarized plaque for 2 minutes to allow the transfer of phage particles to the membrane. The replica disc was placed for 4 min to compensate for the smaller number of phage particles. Release of the phage DNA from the phage particle was promoted by incubating the membrane in denaturation solution for 2 min. Plaque lifts were then transferred to the neutralization solution for 5 min, rinsed in washing solution for 30 seconds and air dried on Whatman® 3MM paper. DNA was crosslinked to the membranes by UV light in a *Stratalinker® UV crosslinker* (Model 1800, Stratagene) using the autocrosslink setting (1200 mJ). Hybrization with a radiolabeled probe was identical to what is described further, in section 2.3.8. Positive signals matching in both replicas were removed from the NZY agar plaque and resuspended in 500 µL of SM buffer containing 20 µL of chloroform in order to kill *E. coli*, precipitate cell debris and prevent further library contamination. Tubes were vortexed and Lambda phages were re-plated at low density in 100 mm agar plaques, using for the purpose 200 µL of XL1-Blue MRF' cells and 3 mL of melted Top Agarose NZY in the conditions previously described. For second screening, *Hybond-N* (Amersham) nylon filter discs were used, but no duplicates were performed. Positive individualized clones were cored from the agar plates and resuspended in 500 µL of SM buffer containing 20 µL of chloroform, vortexed and stored at 4°C until further use.

Denaturation solution	1.5 M NaCl; 0.5 M NaOH.
Neutralization solution	0.5 M Tris-HCl (pH 8.0); 1.5 M NaCl
Washing solution	0.2 M Tris-HCl (7.5); 2x SSC
SM buffer	50 mM Tris-HCl (pH 7.5); 100 mM NaCl; 10 mM $MgSO_4.7H_2O$; 0.01% gelatine

2.3.3.3. In vivo *excision of recombinant pBK-CMV phagemids*

The excision *in vivo* of the pBK-CMV phagemid from the *ZAP Express* vector was initiated by combining 250 µL of the previously isolated single-clone lambda phage solution, with 1 µL of *ExAssist* helper phage ($1x10^6$ pfu/µL) and 200 µL of XL1-Blue MRF' cells. These cells were prepared as previously described (chapter 2.3.3.1.) but were resuspended to a final A_{600} of 1.0. The mixture was incubated at 37°C for 15 min to permit phage attachment to the bacterial cell wall, and 3 mL of NZY medium were added. After growing overnight at 37°C with agitation, the cultures were heated at 70°C for 20 min to inactivate the parent lambda phage and kill XL1-Blue MRF' cells. The samples were centrifuged at 1000 *g* for 15 min. The supernatant contained the recombinant pBK-CMV phagemid packaged in f1 phage particles, as well as the f1 helper phage.

To eliminate the helper phage, 25 µL of the supernatant were combined with 200 µL of XLOLR cells. These had been grown in NZY and resuspended in $MgSO_4$, as previously described for XL1-Blue MRF' cells. The mixture was incubated at 37°C for 15 min. After adding 300 µL of NZY broth, the samples were incubated at 37°C for 45 min with agitation. LB-kan plates were used to plate 25 µL of the cell suspension. Colonies containing the pBK-CMV phagemid were obtained after incubation of the cultures at 37°C for 12 h.

2.3.3.4. Selection of cDNA clones of interest

To discard the false-positive clones among the putative positive cDNA clones obtained by cDNA library screening, the corresponding phagemids were isolated by the quick boiling miniprep method and digested with *EcoR* I and *Xho* I restriction endonucleases. Digestion products were separated through a 1.2% (w/v) agarose gel electrophoresis and transferred to nylon membranes (*Hybond-N+*, Amersham) and hybridized with the same labeled probe used for library screening. The cDNA inserts of positive clones of interest were then sequenced.

2.3.4. Spectrophotometric quantification of nucleic acids

Quantification of nucleic acids was performed spectrophotometrically by measuring the A_{260} of sample solution on a *Cary 1E UV-Vis Spectrophotometer* (Varian) using quartz cuvettes. For estimation of the nucleic concentration, it was considered that 1 A_{260}= 50 ng/µL DNA; 1 A_{260}= 40 ng/µL RNA. To

determine the purity of the samples the values of A_{230} and A_{280} were also determined [Sambrook *et al.*, 1989; Krieg, 1996].

2.3.5. Digestion with endonucleases

The digestion of DNA with restriction endonucleases was performed according to standard procedures [Sambrook *et al.*, 1989; Ausubel *et al.*, 1996] and to the supplier's instructions. Acetylated BSA (Stratagene) was used to stabilize endonuclease activity. Enzyme buffers were chosen according to standard buffer efficiency tables supplied by the manufacturers. Reactions were performed at 37°C for 1.5h to overnight, depending on experimental needs.

20 µL reaction	2 µL enzyme buffer (10x); 2 µL BSA (5 $mg.mL^{-1}$); *x* µL DNA; 0.5 µL restriction enzyme; complete with H_2O

2.3.6. Nucleic acid electrophoretic separation

2.3.6.1. Agarose gel electrophoresis

DNA fragments were resolved by electrophoretic separation using horizontal slab gel apparatus. Agarose concentration (0.5% - 1.2%) was chosen depending of the fragment length range. Gels were made by melting agarose in 0.5x TBE, also used as running buffer. DNA samples, including molecular weight markers (*1 kb DNA Ladder*, Invitrogen) were mixed with 0.25 vol. of loading buffer and 1 µL of EtBr (1 $mg.mL^{-1}$). Electrophoresis was carried out at 50 - 100 V, until the bromophenol blue dye had migrated two thirds the length of the gel.

10x TBE buffer	0.89 M Tris; 0.89 M boric acid; 20 mM EDTA
Loading buffer	30% (w/v) Glycerol; 0.1 M EDTA; 0.25% (w/v) bromophenol blue

2.3.6.2. Denaturing formaldehyde agarose gel electrophoresis

The electrophoretic separation of RNA was performed on a denaturing formaldehyde agarose gel to prevent the formation of secondary structures [Krieg, 1996]. Samples were added an equal volume of loading buffer and, when required for visualization, 1 µL of 1 $mg.mL^{-1}$ of EtBr was also added. Denaturation was promoted by incubating samples at 65°C for 10 min, followed by cooling on ice. After loading samples into the gel, electrophoresis was carried out at 60 V until the dye front had migrated two-thirds the length of the gel. EtBr-stained DNA agarose gels were rinsed several times in DEPC-treated water to remove formaldehyde, visualized under long wave UV light (*Transilluminator 2020E*, Stratagene) and analyzed using the *Eagle Eye® II Still Video System* (Stratagene) through corresponding software (*EagleSight™ 3.2*, Stratagene).

Agarose gel	0.8% or 1.2% (w/v) agarose; 1× MOPS (pH 8.0); 6.7% (v/v) formaldehyde
Loading buffer	66% (v/v) formamide; 3% (v/v) formaldehyde; 0.1% (p/v) bromofenol blue; 1× MOPS (pH 7.0)
Running buffer	1× MOPS (pH 7.0)
10x MOPS	2 M MOPS; 0.5 M Sodium acetate; 100 mM EDTA

2.3.7. Northern blotting

For Northern blot analysis, 20 µg of RNA were separated by denaturing formaldehyde agarose gel electrophoresis. The agarose gel was rinsed in DEPC-treated water (2x20 min) to remove formaldehyde, and equilibrated in 20x SSC for 30 min. RNA was transferred to *Hybond-N+* nylon membranes (Amersham) by capillary transfer using a 20x SSC solution. RNA was crosslinked to the filters by UV light, according to what was described in chapter 2.3.3.2.

20x SSC	0.3 M Na citrate (pH 7.0); 3 M NaCl

2.3.8. Hybridization with ^{32}P-labelled DNA probes

2.3.8.1. Labeling of DNA probes

In the present work, heterologous and homologous DNA probes were used for cDNA library screening and for hybridization after Northern blotting. Plasmids containing DNA inserts to be used as probes were digested using restriction enzymes belonging to the corresponding polylinker sites or used as templates for PCR amplification of the desired insert.

Digestion or PCR products were separated through gel electrophoresis and the fragments of interest were recovered from the agarose gel using DEAE membranes or appropriate gel extraction kits (Section 2.3.9), quantified spectrophotometrically and stored at -20°C.

DNA fragments were ^{32}P-labeled by random oligonucleotide priming, using the *Rediprime II DNA labeling system* (Amersham) and [α-^{32}P] dCTP (*Redivue*, Amersham). Each DNA probe (100 - 200 ng) was diluted to a final volume of 45 µL in TE buffer, denaturated at 95°C for 5 min and cooled on ice for 5 min. The solution was then used to reconstitute the *Rediprime II* labeling mix, after which 5 µL of [α-^{32}P] dCTP (50 µCi) were added. Radioactive nucleotide incorporation was promoted by incubating the reaction mixture at 37°C for 1h.

TE buffer	10 mM Tris-HCl (pH 7.6); 1 mM EDTA

2.3.8.2. Purification of ^{32}P-labeled DNA probes

Radiolabeled probes were purified from unincorporated nucleotides, by gel filtration through a *Sephadex G-50* (Pharmacia) mini column [Sambrook *et al.*, 1989]. A sterile Pasteur pipette was partially blocked with a glass bead and filled with the resin equilibrated in TE buffer. The column was washed with 3 mL of TE and the radiolabeled probe loaded. Size separation by gravity flow was promoted by loading into the column one 450 µL TEN fraction followed by twelve 150 µL TEN fractions. All fractions were successively collected in microcentrifuge tubes and evaluated for radioactivity with a mini-monitor (*Series 900*, Morgan). The first 4-5 fractions to present radioactivity were pooled for further use.

TE buffer	10 mM Tris-HCl (pH 7.6); 1 mM EDTA
TEN buffer	10 mM Tris-HCl (pH 7.6); 1 mM EDTA; 100 mM NaCl

2.3.8.3. Hybridization and washings

Membrane filters were pre-hybridized in hybridization buffer at 42°C, for 3h, in a hybridization oven (Amersham). The probe was heat denatured for 5 min, cooled on ice and added to the hybridization buffer. Hybridization was allowed to proceed overnight at 42°C, with shaking.

After hybridization, filters were successively washed for 20 min in the following solutions: 2x SSC, 0.1% SDS, at 45°C; 2x SSC, 0.1% SDS, at 50°C; 1x SSC, 0.1% SDS, at 50°C. For heterologous probes, the final washing step was 1x SSC, 0.1% SDS, at 55°C. For homologous probes, the final washing step was 0.5x SSC, 0.1% SDS, at 65°C.

Hybridization buffer	50 mM sodium phosphate (pH 7.0); 0.9 M NaCl; 5 mM EDTA; 10x Denhardt reagent; 0.1% SDS; 250 µg.mL^{-1} denatured salmon sperm DNA; 30% (v/v) formamide.(heterologous probe) or 50% (v/v) formamide (homologous probe)
50x Denhardt's Reagent	5% (w/v) Ficoll 400; 5% (w/v) polyvinylpyrrolydone 360; 5% (w/v) BSA (fraction V)
20x SSC	0.3 M Na citrate (pH 7.0); 3 M NaCl

2.3.8.4. Autoradiography

Radioactive membrane filters were enveloped in plastic film and placed in cassetes (*Hypercassete*, Amersham) in direct contact with an autoradiographic film (*BioMax MS*, Kodak; *BioMax MR*, Kodak; *Cronex ortho-S*, Sterling), within two intensifying screens (*Hyperscreen*, Amersham). Exposure was performed at -80°C for a suitable time (overnight to one week). The autoradiographic film was developed in a dark room by submerging in *X-ray Developer D-19* (Kodak) for up to 5 min. Development was stopped by rinsing in 3% (v/v) acetic acid stop solution and fixation was performed by

submerging the film in *Rapid Fixer* (Ilford) for 5 min. After rinsing with running water for 5 min, the film was dried at room temperature.

Alternatively, the autoradiographic film was substituted by an *ImageScreen – K* (BioRad) and placed in an *Exposure Cassette – K* (BioRad). Exposure was performed at -80°C (1h to 5h). Scanning was done in a *Personal Molecular Imager FX* (BioRad), using the *Quantity One* software package (v. 4.5.2; BioRad).

2.3.9. DNA fragment recovery from agarose gels

2.3.9.1. DEAE membrane-based method

Recovery and purification of DNA fragments was performed using DEAE membranes [Dretzen *et al.*, 1981]. After electrophoresis, incisions were made on the agarose gel, above and below the DNA fragment band of interest. Strips of DEAE membrane (*NA-45*, Schleicher & Schuell) were placed in the incisions. Membranes had been previously activated by soaking in 10 mM EDTA (pH 7.6) for 10 min, followed by soaking in 0.5 M NaOH for 5 min and extensive washing in sterile u.p. water. The electrophoresis was then resumed until the DNA fragment reached the lower DEAE membrane. This membrane strip was removed from the gel and briefly washed in LSB to remove any agarose debris. The DNA fragment was recovered by incubating the membrane strip in 400 µL of HSB at 60°C for 1h. The isolated fragment was further purified by performing a phenol/chloroform/IAA [25:24:1 (v/v/v)] extraction followed by an ethanol precipitation (2 vol. ethanol, 1/10 vol. 3M NaAc pH 5.2).

LSB	20 mM Tris-HCl (pH 8.0); 0.15 M NaCl; 0.1 mM EDTA
HSB	20 mM Tris-HCl (pH 8.0); 1.0 M NaCl; 0.1 mM EDTA

2.3.9.2. DNA purification from agarose gel

Recovery of DNA from agarose gels was also performed using the *GFX PCR DNA* or the *QIAquick Gel Extraction Kit* (Qiagen). Both kits follow a similar protocol; therefore only GFX methods will be outlined. Briefly, a maximum of 300 mg of agarose gel, containing the DNA band of interest, were sliced in small pieces and placed in a microcentrifuge tube. An equal volume (1 mg = 1 µL) of capture

buffer was added. After vortexing vigorously, the mixture was incubated at 60°C until the agarose gel was completely dissolved (aprox. 10 min). During the incubation, a GFX column was placed in a collection tube.

The sample was loaded onto the GFX column and incubated at room temperature for 1 min, followed by centrifugation at 8000 *g* for 30 sec. The flow-through was discarded by emptying the collection tube. The GFX column was placed back into the collection tube and 500 µL of washing buffer were loaded. After centrifugation at 8000 *g* for 30 sec the collection tube containing the washing buffer was discarded and the GFX column placed in a new microcentrifuge tube. DNA recovery was promoted by applying 50 µL of autoclaved u.p. water directly to the top of the glass fiber matrix in the GFX column. The sample was incubated at room temperature for 1 min and eluted by centrifugation at 8000 *g* for 1 min.

2.3.10. Reverse transcription and cDNA synthesis

Synthesis of cDNA from *H. perforatum* mRNA was performed using the *SuperScript® II First-Strand Synthesis System* (Invitrogen). According to the manufacturer instructions, 5 µg of total RNA (chapter 2.3.1.2), 100 µM dNTP mix, 2.5 µM oligo(dT)$_{20}$ and DEPC-treated water (up to 10 µL) were incubated at 65°C for 5 min and transferred to 4°C for 1 min. 10 µL of cDNA Synthesis Mix was added to each sample and incubated at 50°C for 1 h. cDNA synthesis reactions were terminated after incubation, for 5 min, at 85°C. Total RNA was degraded after addition of 2 units of RNase H to each sample, followed by incubation at 37°C, for 20 min. Synthesized cDNA was stored at -20°C.

cDNA Synthesis Mix	2x RT buffer; 100 mM $MgCl_2$; 0.2 M DTT; 40 U RNaseOUT™; 200 U SuperScript™ II RT

2.3.11. Amplification of cDNA fragments by PCR

H. perforatum L. cDNA was obtained from the phage library (chapter 2.3.2.4) or from mRNA reverse transcription (chapter 2.3.10) and used as template in a polymerase chain reaction (PCR) using degenerate primers. Degenerate primers were designed based on conserved gene regions, as

determined by the multiple sequence alignment of homologous genes. Primer design obeyed several parameters as suggested by Griffin *et al.* (1994) and was performed with the assistance of specific software (*PrimerSelect*, *Lasergene*, DNASTAR). Primers were 18-25 bases long and 3' ends contained G/C nucleotides to ensure correct annealing (G.C clamp). The G/C content was maintained between 45-55%, and melting temperatures (T_m) between primer pairs were as similar as possible. Annealing sites between primer pairs were distanced between 300-2.200 bp. The extent of primer self-homology was minimized with the aid of software analysis. Redundancy in degenerate primers (combination of nucleotide sequences synthesized) was kept as low as possible. In order to minimize sequencing errors, primers were designed approximately 100 bp prior to the end of the template sequence. Primers were synthesized by *Invitrogen* or *StabVida*. Primers used for Gateway cloning included *att*B1 and *att*B2 flanking sequences, as described in chapter 2.3.13.2.

Each 20 µL PCR reaction mixture contained 40 ng of cDNA, 1 µM of each primer, 200 µM of dNTP mix (Boehringer Mannheim), 2 mM $MgSO_4$ (Invitrogen) and 1x PCR buffer (Invitrogen). The enzymatic reaction was started by adding 2 units of *Taq* polymerase or *Pfu* polymerase (Invitrogen). PCR amplification was carried out on a *Mastercycler Gradient* (Eppendorf) with a gradient of 5 annealing temperatures. PCR steps were as follows (unless stated otherwise): **(1)** denaturation for 5 min at 94°C; **(2)** 35 cycles of denaturation for 1 min, at 94°C, annealing for 1 min at 42.2°C, 46.3°C, 49.6°C, 53.8°C or 59.8°C, polymerization for 1 min of at 72°C; **(3)** extension for 10 min at 72°C.

2.3.12 DNA sequencing

Plasmid inserts were sequenced by *BigDye* terminator chemistry (ABI Prism), using universal primers that flank the plasmid's polylinker. cDNA inserts in the pBK-CMV plasmid were sequenced in both directions using T3 and T7 primers. When required for obtaining full sequence, new specific primers were designed.

2.3.13 Cloning of PCR fragments

2.3.13.1 PCR cloning kits

PCR fragments were separated by agarose gel electrophoresis and the fragments of interest recovered using the *GFX PCR DNA* purification kit or the equivalent *QIAquick Gel Extraction Kit* (Qiagen),

as previously described. DNA fragments were cloned onto the *pGEM-T Easy vector* (Promega), *TOPO pCR2.1* cloning vector (Invitrogen) or *pJET1.2/blunt* cloning vector (Fermentas). All these vectors are suited to efficiently clone PCR products. *pGEM-T* and *TOPO* vectors contain 3'-dT overhangs at the insertion site that prevent recircularization of the vector and provide a compatible ligation site to PCR products obtained from A-tailing thermostable DNA polymerases such as *Taq* polymerase. On the other hand, *pJET1.2/blunt* was used for cloning blunt-end PCR products generated by proofreading DNA polymerases, such as *Pfu* polymerase. Due to the similarity between the methods associated to these cloning vectors, only *pGEM-T Easy vector* protocol (Promega) will be outlined here.

In 0.5 mL tubes, a 10 µL ligation reaction was set up using 5 µL of 2x *Rapid Ligation Buffer* (Promega), 1 µL of *pGEM-T Easy vector* (50 ng), 1-3 µL of purified PCR product and 1 µL of T4 DNA Ligase. The reaction was incubated overnight at 4°C to maximize the number of transformants.

An aliquot of the ligation reaction (5 µL) was used to transform *E. coli DH5α* cells. Cells were transformed using the method described below (section 2.3.14) and plated onto selective LB plates, containing X-gal and IPTG (when necessary) for recombinant selection.

2.3.13.2 PCR Cloning using GATEWAY® Technology

The *Gateway® Technology* is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda, providing a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression [Hartley *et al.*, 2000; URL 16]. In order to use this technology, the PCR primers were designed as described before (chapter 2.3.11) but included specific extra flanking sequences (*attB*1 and *attB*2), as described in the product's manual.

The *attB*-PCR product was then used in a *BP reaction* for cloning into a *pDONR®207* vector. Briefly, the *BP reaction* was assembled, vortexed and incubated, at room temperature, for 1 h. Proteinase K (4 µg) was added and the mixture was incubated, at 37°C, for 10 min. 5 µL of the mixture was then used in *E. coli DH5α* transformation, as described in chapter 2.3.14. Liquid cultures were made for vector propagation as described in chapter 2.1.10. Glycerol stocks were made as previously described (chapter 2.1.11).

For cloning in expression vectors, the propagated *pDONR®207* vector was used in a *LR reaction*, according to the manufacturer instructions. Briefly, the *LR reaction* was assembled, vortexed

and incubated, at room temperature, for 1 h. Proteinase K (4 µg) was added and the mixture was incubated, at 37°C, for 10 min. 5 µL of the mixture was then used in *E. coli DH5α* transformation, as described in chapter 2.3.14.1. Liquid cultures were made for vector propagation as described in chapter 2.1.10. Glycerol stocks were made as previously described (chapter 2.1.11).

BP reaction	5 µL *att*B-PCR product 300 ng *pDONR*™ vector 1X *BP Clonase*™ reaction buffer TE Buffer, pH 8.0 (up to 16 µL) 4 µL *BP Clonase*™ enzyme mix
LR reaction	100-300ng Entry clone 300 ng Destination vector 1X *LR Clonase*™ reaction buffer TE Buffer, pH 8.0 (up to 16 µL) 4 µL *LR Clonase*™ enzyme mix

2.3.14 Transformation of *E. coli*

Chemically competent *E. coli DH5α* cells were obtained by inoculating 250 mL of SOC medium with a single colony of *E. coli DH5α*. Cells were grown at 18°C with vigorous shaking (200-250 rpm) until A_{600} = 0.6 was observed. The culture was placed on ice for 10 min and cells were collected by centrifugation at 2500 *g* for 10 min at 4°C. The pellet was resuspended in 80 mL of ice-cold TB medium, and left on ice for 10 min. Cells were centrifuged for at 2500 *g* for 10 min at 4°C, and gently resuspended in 20 mL of ice-cold TB medium. DMSO to a final concentration of 7% (v/v) was carefully added. The preparation was left on ice for 10 minutes and distributed in 200 µL aliquots. Competent cells were immediately place in liquid nitrogen and stored at -80°C. Another strain of chemically competent *E. coli DH5α* (*DB3.1*) was purchased from Invitrogen.

Transformation of both *E. coli* strains followed the same protocol. The *DB3.1* strain was used for propagation of vectors containing the lethal *ccdB* gene (such as *pDONR*). This *E. coli* strain contains a gyrase mutation (*gyr*A462) that renders it resistant to the CcdB effects [URL 16].

Transformation was initiated by thawing competent cells on ice. The DNA sample (up to 1 µg) was added to 200 µL of chemically competent cells by gentle mixing, and the mixture was incubated at 4°C for 30 minutes. Cells were heat-shocked by incubation at 42°C for 30 s with minor agitation, followed by 10 min on ice. After addition of 0.8 mL of SOC medium and incubation for 1 hour at 37°C with vigorous shaking (200-250 rpm), cells were spinned down for a few seconds at 10.000 *g* and the

pellet resuspended in 50 µL of the supernatant. Finally, cells were transferred to appropriate selective plates and grown overnight, at 37°C.

In order to confirm the transformation, colony PCR screening was done, using primers specific for the cloning vectors included in each cloning kit used (chapter 2.3.13.1). PCR conditions vary according to the vector used and the DNA insert size.

TB	10 mM PIPES; 15 mM $CaCl_2$; 250 mM KCl; 55 mM $MnCl_2$. Mix all components except $MnCl_2$ and adjust pH to 6.7 with KOH. Dissolve $MnCl_2$ and sterilize solution through a 0.45 µm filter
SOC	2% (w/v) Tryptone; 0.5% Yeast extract; 2.5 mM KCl; 10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 20 mM glucose

2.3.15 Bioinformatics

2.3.15.1. Sequence analysis

Nucleotide and amino acid sequence editing and analysis were performed using the *Lasergene* suit of sequence analysis software from DNASTAR. Within the software package, *EditSeq* was used to edit, translate and back-translate sequences, locate ORFs, as well as create base-files for the remaining softwares. *MegAlign* produced multiple sequence alignments and phylogenetic trees.

2.3.15.2. NCBI Tools and GenBank Database

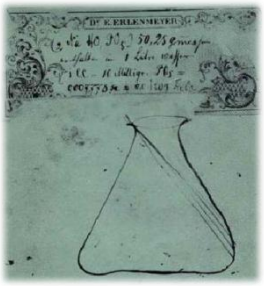
Nucleotide sequences isolated from *H. perforatum* cDNA library, as well as the corresponding amino acid sequences, were submitted to the GenBank Database at NCBI [URL 17]. Database search for specific nucleotide and protean sequences was performed using Entrez search engine [URL 18]. The database search for highly similar sequences was carried out using the BLAST algorithm [URL 19; Altschul *et al.*, 1997].

Chapters 3 - 7

Results and Discussion

Chapter 3

Establishment and characterization of *H. perforatum* L. suspension cell cultures



3. Establishment and characterization of *Hypericum perforatum* L. suspension cell cultures.

3.1. Introduction

Cell cultures have been used for a long time in the study of several biochemical and molecular aspects associated with plant metabolism, including in *Hypericum* species (Table 3.1). The advantages of these *in vitro* cultures are many, including the possibility of maintaining a controlled environment, independent from climatic and soil variable conditions, as well as controlling possible biological influences, such as pathogen attack [Mulabagal *et al.*, 2004]. Climate, nutrient availability, biotic interactions or abiotic stresses can therefore be minimized and/or manipulated, in order to reduce variables affecting the proposed study. As for cell suspension cultures, other advantages, such as a fast growth, reduce the time of an experiment that, in plant biology, could take several months or years before useful biological material could be obtained. Moreover, due to their unicellular/undifferentiated nature, cell suspension cultures can provide a simpler tool for the study of basic plant metabolism, from which biosynthetic pathway intervenients can be more easily regulated/accessed [Bourgaud *et al.*, 2001]. Due to the large surface area in contact with the external medium, responses are usually

concerted, with minor variability observed [Stafford, 1991; Eshita *et al.*, 2000]. Nonetheless, their simplicity should be regarded with caution before extrapolating results to a multicellular level. Depending on the aim of the study, the information obtained from these undifferentiated cell cultures should be seen as "clues" rather than definite knowledge.

Table 3.1 – Some studies using *in vitro* plant cell cultures of *Hypericum* species.

***Hypericum* species**	**Tissue culture type**	**Study**	**Reference**
H. patulum	Suspended cells	Xanthone identification	Ishiguro *et al.*, 1993, 1995
H. erectum	*Calli* and *shoot* cultures	Procyanidin production	Yasaki *et al.*, 1990
H. canariensis	*Shoot* cultures	Hypericin accumulation	Mederos *et al.*, 1996
H. brasiliense	*Calli* and *shoot* cultures	Micropropagation	Cardoso *et al.*, 1996
H. androsaemum	Suspended cells	Xanthone biosynthesis	Schmidt *et al.*, 1997
H. perforatum	Suspended cells	Phenylpropanoid metabolism	Gadzovska *et al.*, 2007
		Elicitation – Phenolic metabolism	Conceição *et al.*, 2006
		Elicitation/Hypericin accumulation	Walker *et al.*, 2002
			Xu *et al.*, 2005
			Kirakosyan *et al.*, 2000; 2001
	Shoot cultures	Hyperforin and secohyperforin accumulation	Charchoglyan *et al.*, 2007

3.2. Establishment of suspension cell cultures of *H. perforatum* L. (var. *Helos*)

To study the relevance of *H. perforatum* defense responses upon pathogen attack, cell suspension cultures from two *H. perforatum* accessions were used. One accession (HPS) was already available in the lab, obtained from *H. perforatum* plants known to be sensitive to *C. gloeosporioides* infection *in vivo*. A second cell suspension culture was later established, from commercial seeds (Richters®, Canada) of *H. perforatum* (var. *Helos*), which is considered to be the less susceptible accession to *C. gloeosporioides* infection *in vivo*. The seeds from *Helos* were sterilized (as described in chapter 2.1.1) and grown in "MS" medium [Murashige *et al.*, 1962] without hormonal supplementation. The roots, leaves and stems from the seedlings obtained were segmented and transferred to MS media, supplemented with diverse hormonal combinations (Chapter 2.1.5.1), in order to develop *calli* cultures. Results for the most relevant hormonal combinations are described below (Table 3.2 and figure 3.1).

Table 3.2: Differences in morphology of *calli* obtained from the combination of three distinct hormonal supplementations and three types of explants from *H. perforatum* (var. *Helos*). Other supplementations led to organogenesis or *shoot* development.

Hormonal supplementation (mg/L)				Explants					
				Roots		Leaves		Stems	
"2,4-D"	0.2 (2.4D)	0.02 (Kin)	*Calli*	Non-friable	Browning	Non-friable	Browning	Non-friable	Browning
"NK"	1.0 (NAA)	0.5 (Kin)	*Calli*	Friable	Low Browning	Non-friable	Browning	Non-friable	Browning
"NAA"	0.5 (NAA)	-	*Calli*	Friable	Low Browning	Friable	Low Browning	Non-friable	Browning
"IK"	0.8 (IAA)	0.5 (Kin)	*Shoots / organogenesis*						
"IBA"	0.5 (IAA)	0.5 (BA)	*Shoots / organogenesis*						
"NK2"	0.1 (NAA)	0.1 (Kin)	*Shoots / organogenesis*						

As described in table 3.2, the *"2,4-D"* hormonal supplementation failed to develop friable *calli* cultures for all explants used and was also responsible for their browning. Although small adjustments on the auxin/cytokinin ratio were performed (data not shown), no significant improvements were observed. The browning observed during *H. perforatum calli* development is a common feature, found in many other plant tissue cultures. According to some authors, the browning is usually associated to increased accumulation of phenolic compounds, but also to an increased cell death [Heath, 2000; Kobayashi *et al.*, 1990]. Similar results were observed when leaves and stems were grown on *"NK"* hormonal supplementation. Despite the results observed for these explants, roots under *"NK"* supplementation have shown to develop *calli* with moderate friability, as well as lower browning. Finally, hormonal supplementation *"NAA"* was found to be responsible for both the lowest accumulation of phenolics (in roots and leaves) and the induction of the most friable *calli* cultures. The *calli* cultures obtained with these two hormonal supplementations (*NAA* and *NK*) were subcultured to fresh MS medium monthly. During the following four months, no significant change on these parameters was observed, although a general, slight decrease in browning occurred, in all experimental conditions studied. The most representative results found for *calli* cultures (obtained from *Helos* roots), under the hormonal supplementations tested, are shown below (Fig. 3.1 – B). Other hormonal supplementations were tested (Table 3.2), being responsible for organogenesis and shoot development in *H. perforatum Helos* cultures. These results are out of the scope of this chapter and won't be discussed further.

Considering the results achieved, friable *calli* obtained from leaves and roots of *Helos* were transferred to liquid MS media, with *"NK"* or *"NAA"* hormonal supplementations. Suspension cell

cultures were initiated in a stepwise process, keeping a minimal cell concentration to allow the cultures to surpass the lag phase. According to the explants and medium composition, the first 5 - 10 subculturings of the initiated cell cultures showed unstable growth as well as increased browning. Nonetheless, six months after the first transfer to liquid MS medium, suspension cell cultures obtained from root explants, grown with *"NAA"* hormonal supplementation, achieved a stable and constant growth. Additionally, browning was observed only after exponential growth phase, as referred later. On the other hand, cell suspension cultures obtained from leaves grown in *"NAA"* medium, as well as those grown on *"NK"* supplementation (root and leaf explants), failed to achieve stable growth and decrease browning, during those six months, and were no longer subcultured.

Helos suspended cells obtained from roots under *"NAA"* supplementation were therefore selected for all the following work, together with the already available HPS cell cultures. While HPS suspension cultures showed formation of cell clumps, *Helos* cultures showed a low tendency for aggregation, as observed in figure 3.1 (D and F). Cell clump formation is a common (and usually "unwanted") feature, observed in many plant cell suspension cultures [Kato *et al.*, 1994; Edahiro *et al.*, 2006]. Due to this result, a change on HPS media composition was tried, in order to achieve lower aggregation levels. HPS suspended cells were transferred from their original *"NK"* supplementation to *"NAA"* medium but no decrease on clump formation was observed. Moreover, no changes on other parameters, such as growth, viability or phenolic compounds accumulation in HPS cells were found, after moving cells to *"NAA"* hormonal supplementation.

In order to minimize the variables to be considered in further experiments, both HPS and *Helos* were maintained in *"NAA"* medium. New modifications in the media composition could have been made to diminish cell aggregation, such as inclusion of colchicine [Umetsu *et al.*, 1975] or AOPP [Edahiro *et al.*, 2006]. Nonetheless, since aggregation is known to be associated to several phenolic compounds, such as feruloyl polysaccharides [Kato *et al.*, 1994], most treatments against clump formation include the inhibition of plant secondary metabolism. Therefore, and considering the scope of this work, no further modifications were made in order to control cell aggregation. Moreover, when we consider that HPS cell cultures were also obtained from root explants, we can expect that any differences in response to treatments should be mainly related to the inherent differences existing between those two *H. perforatum* accessions.

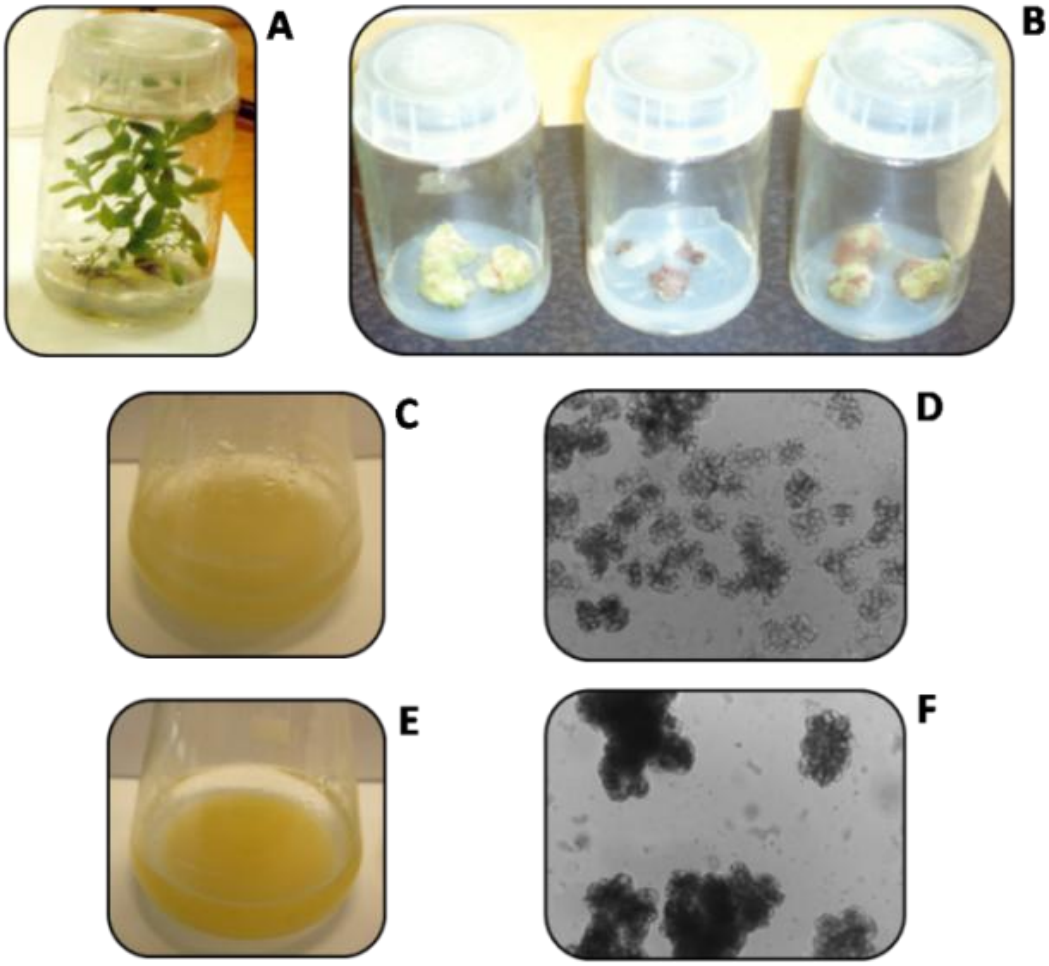


Figure 3.1: Establishment of **(A)** *Shoots*, **(B)** *calli* and **(C, D)** suspended cells from *H. perforatum* (var. *Helos*). **(E, F)** Suspension cultures from *H. perforatum* (var. HPS). *Calli* obtained from roots of *Helos* shown in **(B)** are representative of *"NAA"*, *"2,4-D"* and *"NK"* hormonal supplementations (left to right, respectively).

3.3. Characterization of *H. perforatum* L. suspension cell cultures.

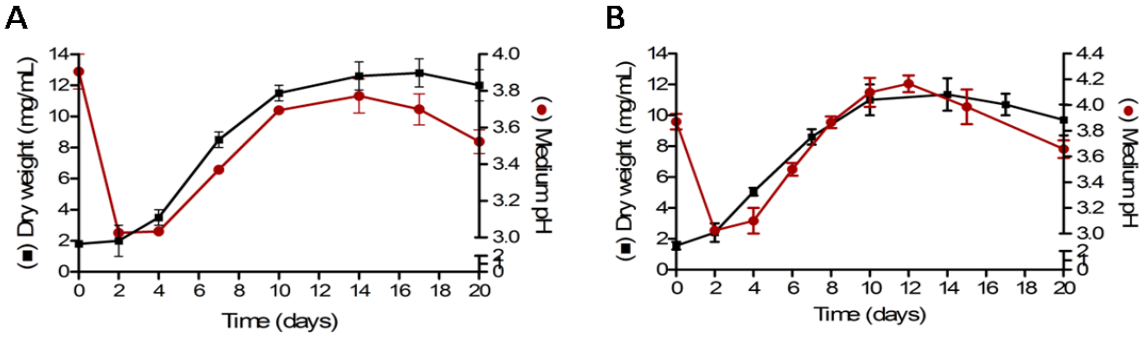
In order to characterize growth and survival in both *H. perforatum* cell suspension cultures some standard parameters were evaluated, namely, biomass accumulation, cell viability and medium pH. Moreover, cell suspension nutrition requirements were also analyzed, including the changes in the consumption of three major groups of nutrients: carbon, nitrogen and phosphate sources.

3.3.1. Characterization of growth and survival parameters

When we analyzed biomass accumulation, a similar pattern was observed in both HPS and *Helos* suspension cell cultures (Fig. 3.2 - A and B). As in most batch cultures, biomass accumulation

showed a typical sigmoid curve [King *et al.*, 1973; Botha *et al.*, 1998; Su-Hwan *et al.*, 2002], composed by a lag phase during the first days after subculturing, followed by a period of exponential (and then linear) growth and, finally, a stationary phase at the end of cell culture time. While the lag phase is usually considered as an adaptation period of the inoculum to fresh medium [Silveira *et al.*, 2002], the final steady-state is known to occur due to limitations in one or more nutrients in the liquid medium or an increased accumulation of toxic compounds. The absence of biomass accumulation, observed during the lag phase, does not mean a standstill in suspended cells' biochemical processes, but quite the opposite, with strong activity being associated with the initial acclimatization [Wilson, 1971]. Between the two lag phases, common to most plant cell suspensions, optimal growth condition occurs and is responsible for the exponential growth phase, where the highest growth rate (μ) is found. In the case of HPS, exponential growth was observed approximately between days 4 and 10, with a maximum growth rate of 0.13 d^{-1} achieved at the seventh day after subculturing (Fig. 3.2 – A and C; table 3.3). In *Helos* suspension cultures, exponential growth occurred between days 2 and 10 and the maximum growth rate was estimated to occur approximately at the fourth day after subculturing, with values close to 0.16 d^{-1} (Fig. 3.2 – B and C; table 3.3). Moreover, and considering these growth rate values, the shortest doubling time (T_d) can be estimated to occur in 56 h (2.3 days) for HPS while *Helos* cell division occurred in 45 h (approximately 1.9 days) (Table 3.3). These doubling times are within the normal range of values, between 20 to 100 h, observed for most plant cell suspension cultures [Facchini *et al.*, 1990]. More extreme values have already been described, for example, in some fast-growing cultures of *Nicotiana tabacum* (0.45 days) [Su, 2007], while the slow-growing suspension cells of *Cenchrus ciliaris* L. displayed doubling times as high as 30 days [Rogers *et al.*, 1993].

As observed in figure 3.2 (A and B), both HPS and *Helos* suspension cultures accumulated similar amounts of biomass, reaching maximum growth yield values (Ymax) of approximately 13 mg/mL and 11 mg/mL, at days 17 and 14 for HPS and *Helos*, respectively.



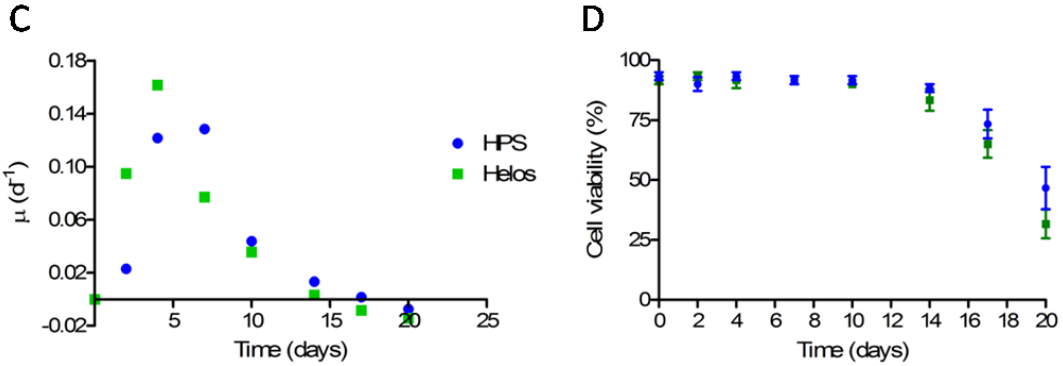


Figure 3.2: Growth parameters of both *H. perforatum* suspension cell cultures. Dry weight (■) and medium pH (●) observed for **(A)** HPS and **(B)** *Helos* cultures. **(C)** Estimated growth rates and **(D)** cell viability for HPS (●) and *Helos* (■) suspension cells.

Table 3.3: Growth parameters evaluated for both *H. perforatum* suspension cell cultures studied.

***H. perforatum* variety**	**Maximum growth rate**	**Shortest doubling time**	**Maximum yield**
HPS	0.13 d^{-1} (day 7)	2.3 days	13 mg/mL
Helos	0.16 d^{-1} (day 4)	1.9 days	11 mg/mL

After reaching their maximum yield values, none of the *H. perforatum* cultures displayed a clear stationary stage. Nonetheless, in the case of *Helos*, we can predict that to occur roughly between days 11 and 15, while for HPS a later stationary stage should occur, between days 14 and 18 (Fig. 3.2 – A and B). Until the beginning of this predicted stationary stage no significant changes on cell viability were observed, although a small transient decrease was detected between days 2 and 4 (Fig. 3.2 – D). The stationary stage was followed by a small decrease in biomass accumulation, tissue browning and a significant, sharp decrease in cell viability (Fig. 3.2 – D), for both suspension cell cultures, observed from days 14 and 17 (for *Helos* and HPS, respectively) until the end of the experiment, at day 20. As previously referred, these results are probably due to a decrease in availability of one or more nutrients in the medium. This possibility will be discussed in detail in chapter 3.3.2.

Another parameter analyzed during *H. perforatum* suspension cell culture growth was the medium pH. Both HPS and *Helos* cultures showed similar medium pH variations, as described in figure 3.2 (A and B). As observed for suspension cultures of many other plants [MacDonald *et al.*, 1989; Srinivasan *et al.*, 1995; Fleisher *et al.*, 1998], a fast decrease in the pH values was observed, just a few days after subculturing. In both HPS and *Helos* cultures, pH dropped from initial values of nearly 4.0 to values close to 3.0, during the 2 - 4 days after inoculation. Although plant cells are known to grow efficiently within a wide range of medium pH values, the absence of growth, observed in the first days

after subculturing, is sometimes associated with an increased influx of H^+ to the cytoplasm of cells, which may affect optimal growth conditions. Even though this could be a valid explanation for cell suspension cultures of many other plant species, the narrow range of pH values observed for our *H. perforatum* suspension cell cultures, during those initial days, does not seem to be enough to explain the initial lag phase. Moreover, some authors suggest that, during the initial adaptation of cells to the fresh medium, the drop in pH values could be a direct consequence of cell death and subsequent release of cell content into the medium [Srinivasan *et al.*, 1995; Mühlbach, 1998; Dias, 2000]. This can be a reasonable explanation for the decrease in pH and the stall in biomass accumulation. Nonetheless, since we observed only a minute decrease in cell viability during the initial days after subculturing, other mechanisms associated with acclimatization, such as nutrient uptake, may also contribute for these results, as discussed later. The sharp decrease in medium pH was followed, during exponential growth phase, by a gradual recovery to the original values and, approximately 12 days after inoculation, medium pH values were nearly restored (Fig. 3.2 – A and B). The medium pH observed at the stationary stage is usually considered as the "optimal pH" for cell growth [Hahlbrock *et al.*, 1972] which, for HPS cultures, is nearly 3.8 while for *Helos* is 4.2, approximately. After passing the stationary stage, another decrease on medium pH values was observed, until the end of the study, at day 20. Unlike the sharp pH drop observed in the initial lag phase, this new decrease may be a direct consequence of cell death alone, since a decrease in both biomass accumulation (Fig. 3.2 – A and B) and cell viability (Fig. 3.2 – D) was also observed during that time.

3.3.2 Time course changes on major nutrients uptake

Along with biomass accumulation, medium pH and cell viability, we also studied, for both *H. perforatum* suspension cells, the possible variations in the uptake of major nutrients present in the MS culture medium, namely, nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{2-}) and sugars (sucrose, glucose and fructose) and how these can influence the growth of HPS and *Helos* cell suspension cultures.

3.3.2.1. Sugar

Although photoautotrophic cultures can be established [Barz *et al.*, 1982; Yamada *et al.*, 1982] the most commonly used cell suspension cultures are heterotrophic. Therefore, these cultured plant

cells require carbohydrates to provide both a carbon skeleton and an energy source, able to sustain growth. Beside their metabolic roles, it is also attributed to carbohydrates an osmoregulatory function, as essential as the carbon-energy roles, since these compounds are normally the major constituents of any tissue culture medium, in terms of mass and molarity [Thompson *et al.*, 1987].

In *H. perforatum* cell cultures, as for suspension cells of many other plant species, sucrose was added to the medium (3%), to be used by the cells as their main source of carbon and energy. Sucrose is the most common and preferred carbohydrate since higher biomass accumulation is usually achieved with it, when compared to other energy sources like the disaccharide maltose or the monosaccharides mannose or galactose [Thompson *et al.*, 1987]. Moreover, while reducing the amount of sucrose added to the medium usually leads to lower biomass production, increasing to values higher than 4-5% would probably have no positive impact on *H. perforatum* biomass accumulation, as previously described for many other plant suspension cell cultures [Grey *et al.*, 1987].

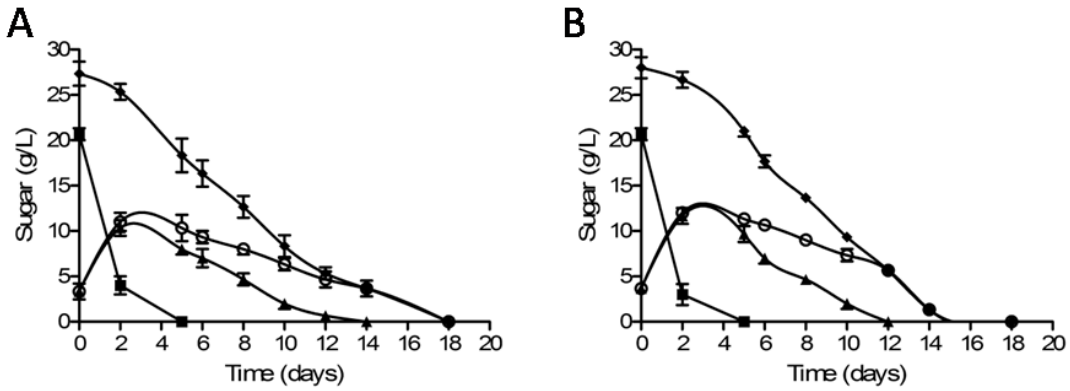


Figure 3.3: Sugar available in the medium of *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures. Total sugar (◆), sucrose (■), glucose (▲) and fructose (○) content in the medium.

As figure 3.3 shows, residual amounts of fructose and glucose were detected in the fresh medium, prior to subculturing. These are likely the result of hydrolysis of sucrose, during autoclaving process. After inoculation of the medium, sucrose concentration in both *H. perforatum* suspension cell cultures decreased sharply and in a similar pattern. While small amounts (4 g/L, approximately) could still be found at day 2, sucrose was no longer detected at day 5. This rapid conversion of sucrose into glucose and fructose, also described for other species [Zhang *et al.*, 1998; Srinivasan *et al.*, 1995], is known to be the action of extracellular or cell wall-bound invertases [Kanabus *et al.*, 1986]. The fast response to sucrose in the medium is not surprising if we consider that sucrose is the most common

transport and storage (and therefore metabolizable) saccharide in plants [Thompson *et al.*, 1987]. From figure 3.3 we can estimate that the highest glucose and fructose concentrations occurred between days 2 and 5, after the complete hydrolysis of sucrose. Moreover, although both fructose and glucose concentrations in the medium were progressively decreasing during cell suspension growth, glucose was found to be preferably consumed by both *H. perforatum* suspension cells. Similar results were found in plant suspension cultures from other species such as *Lavandula vera* [Ilieva *et al.*, 1997], *Olea europaea* [Oliveira *et al.*, 2002] or *Pinus pinaster* [Azevedo *et al.*, 2008]. As described by some authors, different affinities of hexose phosphorylating enzymes for fructose and glucose are known to be involved in the preferable consumption of glucose [Azevedo *et al.*, 2008; Krook *et al.*, 2000]. Interestingly, suspension cultures from a closely related species, *H. androsaemum*, are known to consume fructose preferably [Dias, 2000] while *Cupressus lusitanica* showed no preference for any of these monosaccharides [Yamada *et al.*, 2003]. In *H. perforatum*, glucose uptake was found to occur during the exponential growth phase, which ended when glucose was depleted from the medium, at days 12 and 14 for *Helos* and HPS, respectively. During the following stationary stage, only fructose was available and was rapidly consumed, within a few days. The depletion of all sugar sources from the medium also coincides with the decrease in cell viability and biomass accumulation, starting at days 14 and 17, for *Helos* and HPS cultures (respectively), as previously described. Therefore, the sugar content in *"NAA"* medium seems to play a decisive role as a growth limiting nutrient in both *H. perforatum* suspension cells studied. Increased relevance may be pointed to glucose, which seems to be directly responsible for the growth arrest. The decisive role of sugars on growth was already described for other plant suspension cell cultures, such as *Catharanthus roseus* [Rho *et al.*, 1991; Gulik *et al.*, 1993] or *H. androsaemum* [Dias, 2000]. On the other hand, growth of *Pinus pinaster* suspension cultures in MS medium was found to be arrested by phosphate medium depletion instead of carbohydrate exhaustion [Azevedo *et al.*, 2008].

3.3.2.2. Phosphate

Another major nutrient analyzed was the phosphate ion (PO_4^{2-}), which presence in the medium is critical for suspension cell growth. Phosphate consumption in both cell suspension cultures of *H. perforatum* show a similar pattern. In both cases, a descending sigmoidal curve was observed (Fig. 3.4), accompanying the upward growth curve previously referred (Fig. 3.2 – A and B). The straight connection between these two parameters occurs because phosphate is known to be a critical nutrient,

necessary for cell division [Sano *et al.*, 1999]. The lowest phosphate concentration in the medium was achieved at day 14, for both HPS (18 mg/L) and *Helos* (15 mg/L) suspension cultures. These values were achieved just after the exponential phase, when growth arrest was observed. Since phosphate was still available in the MS medium during the stationary stage it seems that, although relevant, phosphate is not a limiting nutrient, as previously referred for sugar, in both *H. perforatum* suspension cultures studied. After passing the stationary stage, when sugar depletion was observed, phosphate concentration in the medium increased sharply for both *H. perforatum* cultures, from day 14 until the end of the study, at day 20. The increase in phosphate concentration is most likely due to the release of cellular contents from the increasing number of dead cells [Archambault *et al.*, 1996], as predicted from the previously referred sharp decrease in cell viability (Fig. 3.2 – D). In fact, sugar starvation is known to be a trigger for programmed cell death and consecutive mobilization of at least part of the available nutrients, such as phosphate [Lino-Neto, 2001].

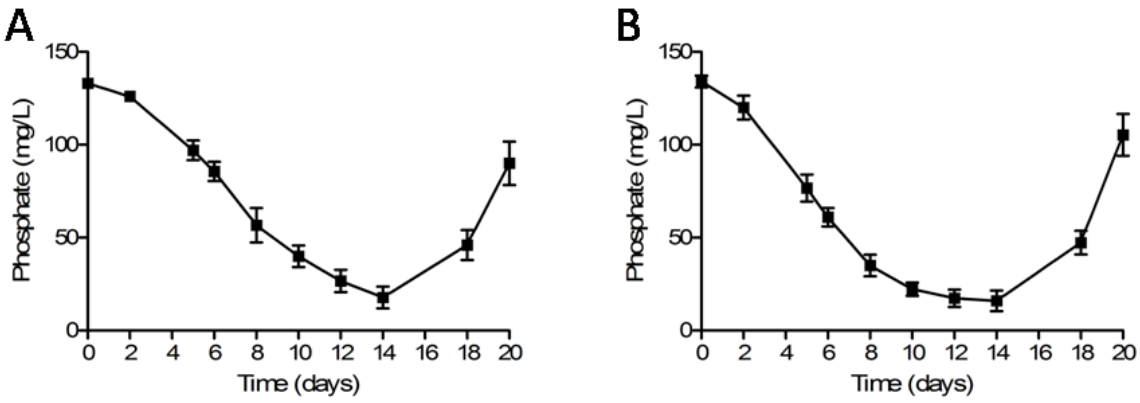


Figure 3.4: Phosphate ion (PO_4^{2-}) available in the medium of *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures.

3.3.2.3. Nitrate and Ammonium

Nitrogen is found in a myriad of plant components such as proteins, nucleic acids or phytohormones. Therefore, nitrogen sources, as well as carbohydrates and phosphate, are of singular importance for growth. Both the amount and the form of the nitrogen source in the medium are known to have significant effects on the growth rate, cell morphology and totipotency [Kirby *et al.*, 1987]. Usually, plant culture media includes both nitrate and ammonium salts as inorganic nitrogen sources [Gamborg *et al.*, 1981; Kirby *et al.*, 1987]. Due to the relevance of these two nitrogen sources in the

growth of tissue cultures, their uptake was also analyzed, for both *H. perforatum* suspension cells available.

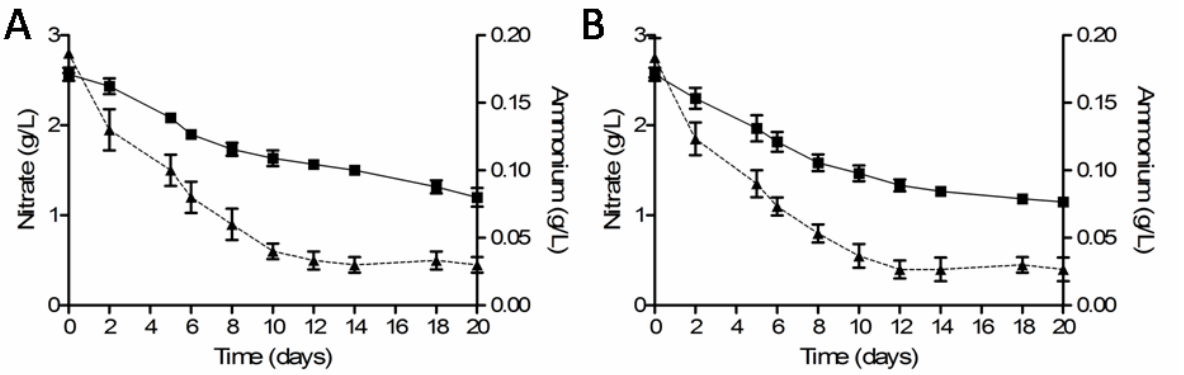


Figure 3.5: Consumption of nitrogen sources available in MS medium, namely, (▲) ammonium and (■) nitrate, observed in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures.

As previously described for sugar and phosphate, consumption of nitrogen sources was similar for both *H. perforatum* suspension cultures (Fig. 3.5). Both HPS and *Helos* showed an increased consumption of ammonium during the exponential growth period. This response was followed by a stall in consumption, starting at day 10 and observed until the end of the study, at day 20. Nitrate consumption, on the other hand, occurred during most of the period studied, although a tendency for increased consumption during the exponential growth period was also observed (Fig. 3.5). These results come in accordance to what was previously observed for *H. androsaemum* cell suspension cultures [Dias, 2000] and other plant models [Srinivasan *et al.*, 1995]. Although present in much lower amounts in the medium, ammonium was readily uptaken by *H. perforatum* suspension cells and only 20% (approximately) of the initial amounts were available at day 12. As for nitrate, 50% of this nitrogen source was still present at the end of the study, at day 20. The increased *preference* for ammonium could be explained by the fact that this nitrogen source is generally considered toxic to plant cells, being readily metabolized [Kirby *et al.*, 1987]. In fact, some plant models, such as *Vitis vinifera*, utilize nitrate only when ammonium is fully depleted from the medium [Pépin *et al.*, 1995]. While ammonium cannot be efficiently used in high amounts as a sole source of nitrogen, the utilization of nitrate alone presents other difficulties since nitrate reduction is an energetically expensive process. Although possible, growth rates in nitrate-only cell culture medium are usually lower than on medium containing both nitrate and ammonium [Kirby *et al.*, 1987]. Taken together, the results obtained suggest that neither one of the nitrogen sources studied are growth limiting nutrients, as previously observed for phosphate

consumption (Chapter 3.3.2.2), although culture growth can be directly associated to their consumption.

3.4. Discussion

The development of *calli* and cell suspension cultures from *H. perforatum Helos* accession was associated with an initial tissue browning, decreasing gradually upon subsequent subculturings. As previously referred, increased cell death and accumulation of phenolic compounds are known to be responsible for this feature in other plant tissue cultures. Both these responses are associated to plant stress, most likely due to the intense tissue manipulation and wounding, inevitable during micropropagation [Campos-Vargas *et al.*, 2005; Arencibia *et al.*, 2008]. Furthermore, adaptation to new growth conditions (such as hormonal supplementation) or nutrient availability may also be responsible for the stress, suffered by the cells.

Following the development of *Helos* suspension cultures, and from the analysis of all growth and nutrient consumption parameters, we can conclude that both HPS and *Helos* responded in a overall similar way, despite their morphological differences (clump formation) or any other inherent divergences existing between these two *H. perforatum* accessions. Nonetheless, and although statistically similar, *Helos* growth was faster but lower maximum yields of biomass were observed. While sugar consumption showed a similar pattern on both *H. perforatum* cell suspension cultures, phosphate ion (a key nutrient necessary for cell division) consumption in *Helos* was faster in the initial 8 - 10 days. Moreover, consumption of other minor medium components, also necessary for cell growth, were not monitored in this study. Therefore, the faster intake of phosphate and other nutrients (possibly due to an increased cell surface area in contact to the medium or due to higher metabolic rates), could be responsible for the faster growth, observed in *Helos*. On the other hand, the lower biomass yield observed in *Helos* cultures could be related to differences in metabolic channeling of some nutrients, between primary and secondary metabolism. In fact, as shown in chapter 7, *Helos* cell suspension cultures accumulate 10x more flavonols than HPS. The relevance of this channeling will be further referred in the next chapter.

The analysis of major nutrient consumption allow us to conclude that sugar was the limiting nutrient, responsible for the growth arrest of both suspension cells, namely after glucose depletion from the MS medium. Sucrose was the first nutrient to be uptaken, followed by glucose, fructose and

ammonium, while phosphate and nitrate were still available in the medium, in relatively high amounts, at the end of the study. Furthermore, the late increase in the phosphate available in the medium seems to be related to the increased autolysis of suspended cells, after passing their stationary stage. These results are in accordance to what is usually found in batch cell suspension cultures from other plant species [Cheng *et al.*, 2005].

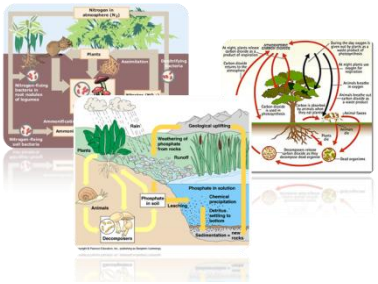
The medium pH shifts observed during the course of the study may have occurred, not only because of the increase in cell death, but also due to the complex interaction between *H. perforatum* suspension cells, the medium constituents and their differential consumption profiles, especially in the case of glucose and nitrogen sources. The fast, initial ammonium uptake could be responsible for the decrease in medium pH values observed a few days after inoculation, since the intake of this ion is known to be associated with a release of H^+ from the cell into the medium [Smith *et al.*, 1976; Minocha, 1987]. When present in the medium, this toxic nutrient is readily metabolized for amino acid biosynthesis [Salisbury *et al.*, 1992; Vance, 1997]. The transient medium acidification was followed by a slow, constant increase in medium pH, in a pattern similar of that found for glucose and nitrate consumption. In fact, the intake of glucose involves the co-transport of H^+ by the action of a monosaccharide H^+ symport system [Minocha, 1987; Azevedo *et al.*, 2006] while a similar mechanism exists for nitrate intake, therefore increasing the medium pH [Kirby *et al.*, 1987].

By comparing the consumption parameters for all the nutrients studied, we can predict that the lag phase, associated with the adaptation to the fresh medium, may be influenced not only by the change in nutrient availability but also by their effect in the osmolarity of the medium, especially in the case of sucrose, as previously described.

Considering the analysis of all these parameters, most of the following experiments shown in this thesis were performed only until the end of exponential growth phase, at days 12 and 14 (for *Helos* and HPS, respectively), in order to avoid other sources of plant stress than the ones we intend to study.

Chapter 4

Nutrient consumption and growth parameters on elicited *H. perforatum* L. suspended cells



4. Nutrient consumption and growth parameters on elicited *H. perforatum* L. suspended cells.

4.1. Introduction

When plants are faced with a possible pathogen, several biochemical and molecular changes occur, not only in the site of attempted infection and close vicinities, but also on other "distant" plant tissues. Our aim, during the course of the following experiments, was to evaluate possible changes on growth, survival and nutrition parameters when *H. perforatum* cell suspension cultures were challenged with the pathogen *C. gloeosporioides* and/or treated with two phytohormones, Salicylic acid (SA) and Methyl-jasmonate (MeJ), known to be related to plant defense mechanisms against biotic stress.

In one set of experiments, cell suspension cultures from both *H. perforatum* accessions were challenged with autoclaved biomass from the pathogenic fungus *C. gloeosporioides*. Two other sets of experiments were carried out by adding MeJ or SA to the suspension cells. These compounds play a significant role in induced plant defense responses, namely, in both systemic resistance pathways against pathogen infection known, SAR and ISR, as previously described in chapter 1.4. Finally, in two other sets of experiments cell cultures were treated with MeJ or SA, prior to elicitation with *C.*

gloeosporioides biomass. The elicitor and the phytohormones were prepared as described in chapters 2.1.7 and 2.1.8 (respectively) and administered to both HPS and *Helos* cell suspension cultures, at an early stage of their exponential growth phase (as described in chapter 2.1.9).

With these experimental models we intended to reproduce the most common situations occurring during plant-pathogen biotic interactions. In detail, suspension cultures treated only with the pathogen-derived elicitor may respond as the first plant tissues facing the incoming pathogen. On the other hand, MeJ and SA-treated cultures may represent plant tissues that were not in direct contact with the pathogen but were *primed* (or conditioned) for defense by one of the known systemic signaling pathways. The last two sets include those tissues that were primed for defense by systemic signaling and were, afterwards, faced directly with the spreading pathogen. These 5 experimental models, plus the control group, were used for all subsequent studies on *H. perforatum* defense mechanisms against *C. gloeosporioides* infection, described in this chapter and the following ones. Furthermore, all the following experiments were carried out on both suspension cultures from *H. perforatum* (HPS and *Helos*), unless stated otherwise. Our aim was to study possible divergences in their defense responses that could explain the differences in susceptibility to *C. gloeosporioides* infection, found between these *H. perforatum* accessions *in vivo*.

4.2. Changes on growth and survival parameters of elicited suspension cells

Cell suspension cultures of *H. perforatum* responded differently according to the elicitor treatment, systemic signaling treatment or the combination of both, when compared to control cultures. Both HPS and *Helos* accessions showed similar patterns of response to all the treatments they were subjected. Namely, a decrease in cell viability and culture growth was observed in *C. gloeosporioides*-elicited cultures while phytohormonal treatment had no effect in these parameters when applied alone, as described below. However, it is worthwhile to mention that, despite the pattern similarity, the negative impact on biomass accumulation and cell viability was predominantly higher in *Helos* suspension cultures than on HPS, the more anthracnose-susceptible *H. perforatum* accession (Fig. 4.1).

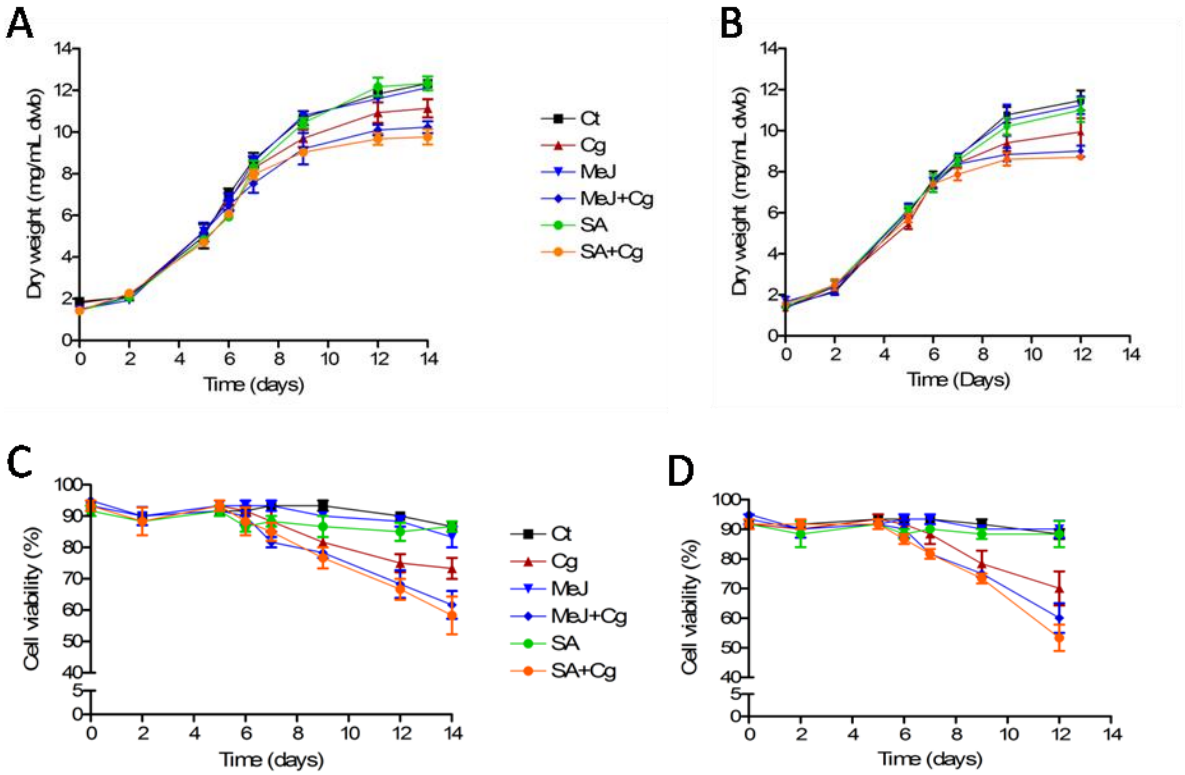
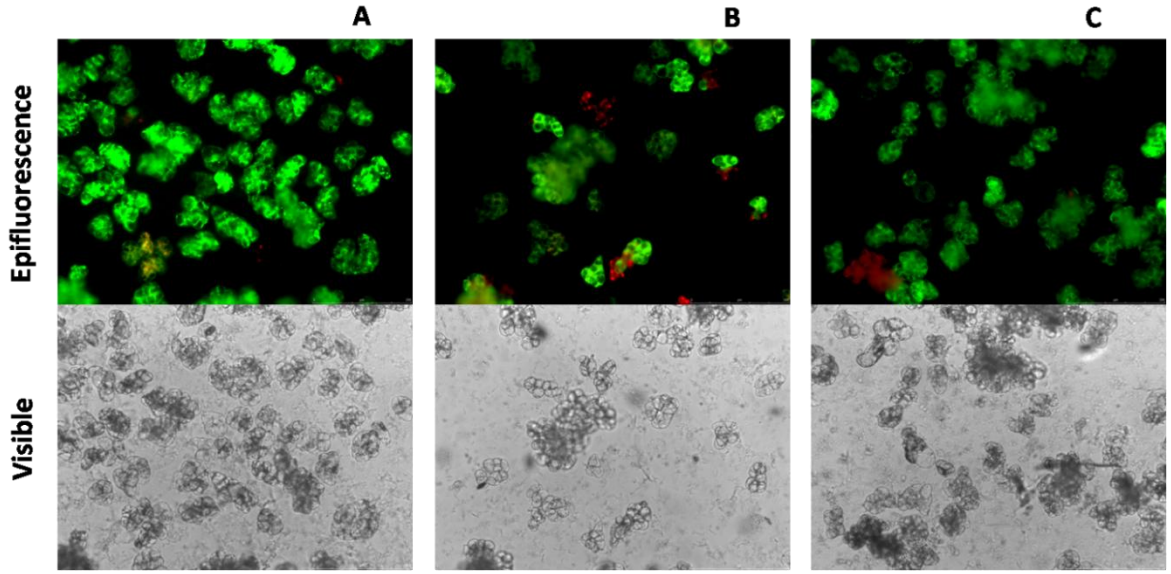


Figure 4.1: Changes on biomass accumulation in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures observed until the end of their exponential growth period. Cell viability measurements for **(C)** HPS and **(D)** *Helos* cultures. Suspension cells were treated with MeJ (100 µM), SA (25 µM), or *C. gloeosporioides* elicitor (Cg). The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

As observed in figure 4.1 (A and B), elicitor treatment led to a significant decrease in biomass accumulation in both cell cultures, from day 9 until the end of the study. Similar results were found for other plant species, such as in *Hyoscyamus muticus* cell suspension cultures treated with a fungal elicitor preparation from *Rhizoctonia solani* [Carvalho *et al.*, 2002]. Treatment with MeJ or SA at the final concentrations of 100 µM and 25 µM, respectively, had no effect on biomass accumulation, when compared to control. The effect of MeJ and SA in biomass accumulation varies greatly between species. Treatment of several *Taxus canadensis* cell lines with the same MeJ final concentration (100 µM) led to a significant decrease in biomass accumulation [Kim *et al.*, 2004], as also observed in *Panax ginseng* cell cultures, treated with MeJ at concentrations ranging from 50 to 400 µM [Thanh *et al.*, 2005]. In the case of our *H. perforatum* cell cultures, concentrations up to 200 µM of MeJ were tested, showing no impact on biomass accumulation. On the other hand, SA concentration values over 40-50 µM were responsible for a stall on growth due to an increased cell death in both HPS and *Helos* cultures (data not shown) while, in *Salvia miltiorrhiza*, SA concentrations up to 500 µM had minor effect on growth

[Chen *et al.*, 1999]. Although the concentrations of 100 µM and 25 µM used for MeJ and SA (respectively) had no direct impact on biomass accumulation, subsequent treatment of these cultures with the elicitor led to a decrease in biomass accumulation, reaching dry-weight values lower than those observed in cultures treated only with the elicitor (Fig. 4.1 – A and B). Furthermore, all the results obtained for biomass accumulation were corroborated by those observed during the time course of the experiment, for cell viability analysis (Fig. 4.1 – C and D and Fig. 4.2), performed as described in chapter 2.2.1.2. An increased cell death was observed in cultures facing the elicitor, especially in those treated with MeJ or SA, prior to elicitation with *C. gloeosporioides* biomass. Similar decreases in biomass accumulation and cell viability are well documented for cell suspension cultures of many plant species, in which oxidative burst and hypersensitive response were detected after elicitation. As an example, suspension cultures of *A. thaliana* elicited with *Fusarium oxysporum* displayed high ROS production, associated with up to 50% increase in cell death, 44 h after treatment [Davies *et al.*, 2006]. These defense mechanisms related to PCD will be studied and discussed in more detail in the following chapter. Treatment with SA (25 µM) showed a transient, negative effect on cell viability (1-2 days after treatment) while MeJ (100 µM) had no effect at all (Fig. 4.1 – C and D). Interestingly, suspension cell cultures from an *H. perforatum* variety from Macedonia, treated with MeJ (100 µM), displayed a significant decrease in cell viability, to values close to 70% [Gadzovska *et al.*, 2007] showing, again, that variability in response to treatment occur not only between plant species, but also within a given species.



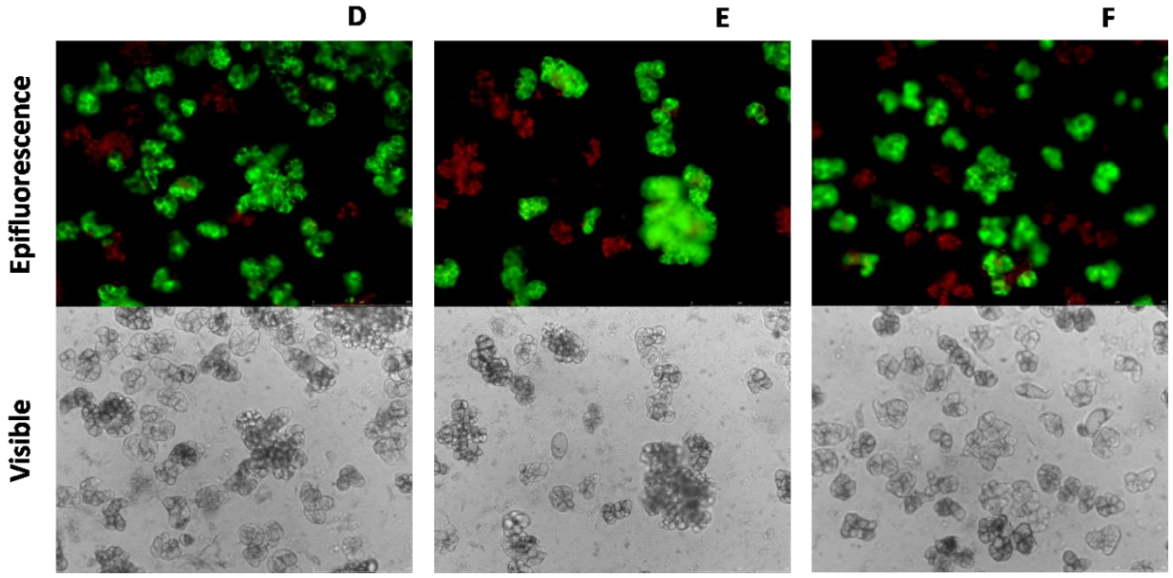


Figure 4.2: Cell viability of *H. perforatum* (*Helos*) suspension cells observed in **(A)** control cultures and 4 days after treatment with **(B)** MeJ, **(C)** SA or **(D)** *C. gloeosporioides*. Cultures pre-treated with **(E)** MeJ or **(F)** SA, prior to pathogen elicitation, are also shown. Viable cells are stained in green (FDA) while non-viable cells are stained in red (PI). All images are representative of the most common results found under the specified treatments.

During the course of this experiment, we also looked for any possible changes in the medium pH values (Fig. 4.3). Regarding this parameter, we can clearly see that treatment with *C. gloeosporioides* elicitor was responsible for lower medium pH values, in both HPS and *Helos* accessions. After the pathogen elicitor treatment, at day 5, pH values lowered significantly and no longer rose, as usually observed in control cultures. Changes in medium pH upon pathogen elicitation vary greatly according to the plant species studied. While for most plant species medium alkalinization is observed, suspension cultures from many species display a significant acidification of the medium [Hagendoorn *et al.*, 1994; Nef-Campa *et al.*, 1994], as in the case of our *H. perforatum* cell cultures. As previously referred in chapter 3, cell death and subsequent release of their content into the medium is known to affect the medium pH, lowering its values. Therefore, cell death could be one reason for the medium pH drop, since a significant decrease in cell viability was observed in both suspension cultures, from the seventh day (24 h after elicitor treatment) until the end of the study (Fig. 4.1 – C and D). Moreover, possible changes in the nutrient uptake may also have a role in this situation and will be discussed later in this chapter. Another mechanism possibly implicated with medium acidification may be the uptake of Fe^{3+} by the cells. Peroxidases have a *Heme* group that requires Fe^{2+} as a cofactor for correct enzymatic activity. Upon pathogen attack, an increase in peroxidase activity is usually observed, associated with lignin synthesis, constituting a common defense mechanism found in plants. It has

been postulated that a Fe^{3+} membrane transporter, also known as *turbo system*, is responsible for the transport of Fe^{3+} into the cell, with consequent release of H^+, acidifying the medium [Bienfait, 1988]. As for the effects of the systemic defense signaling phytohormones, MeJ priming leads to a transient increase in medium pH values for both *H. perforatum* cultures, 24 h after treatment (Fig. 4.3). Despite this increase, the values observed 48 h after priming were similar to those found in control cultures, and remained alike until the end of the study. Treatment with SA alone had no effect on medium pH, when compared to control. Moreover, cells treated with the elicitor, 24 h after MeJ or SA priming, displayed a similar decrease in medium pH values, as observed for elicitor-treated suspension cells (Fig. 4.3).

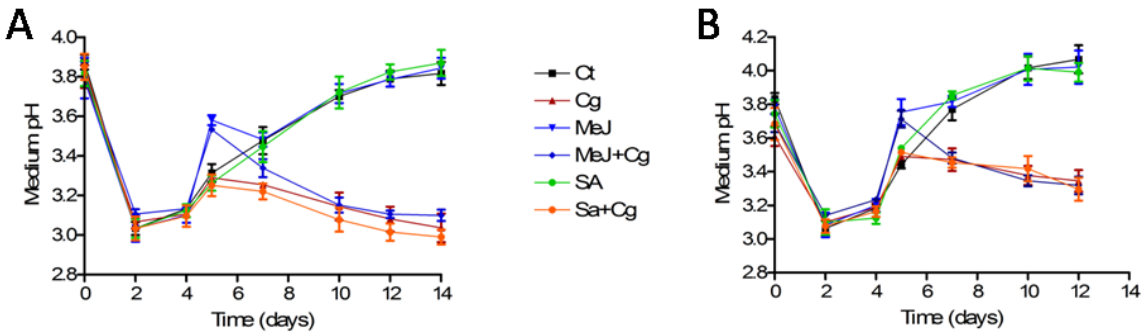


Figure 4.3: Changes on the medium pH values observed in both *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures during their exponential growth period. Suspension cells were treated with MeJ (100 µM), SA (25 µM), or *C. gloeosporioides* elicitor (Cg). The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24 h before the addition of the fungal elicitor.

4.3. Changes on nutrient uptake parameters of elicited *H. perforatum* suspension cells

In these experiments we analyzed the nutrient consumption profiles for the three major nutrient sources. Namely, sugar (sucrose, glucose and fructose), phosphate and nitrogen (nitrate and ammonium) consumption were monitored during the exponential growth period of HPS and *Helos* cell cultures, when these were challenged with the phytohormones (MeJ or SA) and/or the *C. gloeosporioides* elicitor suspension.

4.3.1. Sugar

During the course of this experiment we observed that the sugar concentration in the culture medium was significantly higher after treatment with *C. gloeosporioides*, when compared to the

medium of control cultures. Nonetheless, this information can be misleading since the amount of viable biomass lowered significantly after *C. gloeosporioides* elicitation, as previously described. Therefore, we will only present the final results for sugar consumption, related to the viable biomass available in each experimental set. Figure 4.4 (A and B) shows the total sugar consumption rates for HPS and *Helos*, while figure 4.4 (C and D) shows the relation between the consumption of sugar and cell growth for both cultures (biomass yield per gram of sugar).

From figure 4.4 (A and B) we can see that *C. gloeosporioides* elicitor treatment was responsible for an increase, of approximately 30%, in the rate of sugar consumption, observed between days 7 (24h after treatment) and 10, in both HPS and *Helos* cell cultures. While in some cases a repression in sugar uptake has been reported [Bourque *et al.*, 2002; Amborabé *et al.*, 2008], the most common response of plant cells upon pathogen elicitation is an increase, at least transient, in sugar uptake [Truernit *et al.*, 1996; Fotopoulos *et al.*, 2003; Azevedo *et al.*, 2006]. The increase in sugar consumption by *H. perforatum* suspension cells seems to be a direct consequence of *C. gloeosporioides* elicitation since no significant changes were observed on cultures pre-treated with MeJ or SA, prior to *C. gloeosporioides* elicitation. Moreover, MeJ or SA alone seem to have no significant effect on sugar consumption rates in HPS or *Helos*, although a transient increase in the consumption rate was observed, at day 7, in MeJ-treated HPS cultures.

The differences in sugar consumption become more evident when we analyze their impact on cell growth (Fig. 4.4 – C and D). By observing the results obtained for HPS (Fig. 4.4 – A and C) and *Helos* cultures (Fig. 4.4 – B and D) we can see that, although there was a small increase in sugar consumption after elicitor treatment, this extra sugar was not used for cell growth, during days 7 and 10. Therefore, this carbon source could have been diverted to other cellular needs, such as energy production or biosynthesis of new structures/compounds, probably implicated in *H. perforatum* defense mechanisms. As discussed later in chapters 6 and 7, pathogen elicitation is responsible for a *boost* in xanthone accumulation, in both *H. perforatum* accessions. In fact, the role of sugar in fuelling the biosynthetic pathways of defense-related compounds has already been widely accepted [Herbers *et al.*, 1996; Ehness *et al.*, 1997; Bourbouloux *et al.*, 1998]. While in our case a reduction of 40 - 50% (for HPS and *Helos*, respectively) in biomass yield per gram of sugar was observed after pathogen elicitation, values close to 70% were found in elicited *H. muticus* cell suspension cultures, when compared to control [Carvalho *et al.*, 2002]. Moreover, while pre-treatment with MeJ or SA apparently had no effect on sugar consumption rates (Fig. 4.4 – A and B) in both *H. perforatum* cell cultures

studied, this pre-treatment, prior to *C. gloeosporioides* elicitation, was responsible for a sharper reduction in the conversion of sugar into new biomass, observed between days 7 and 12, approximately (Fig. 4.4 – C and D). The lowest biomass yield per gram of sugar was observed at day 10, when reductions close to 65% - 85% (for HPS and *Helos*, respectively) were found, when compared to control cultures (Fig. 4.4 – C and D). Despite their synergistic effect, MeJ or SA alone had no significant influence on this parameter for HPS cultures (Fig. 4.4 – C) while only a transient decrease was observed for *Helos* cultures, between days 7 and 10 (Fig. 4.4 – D).

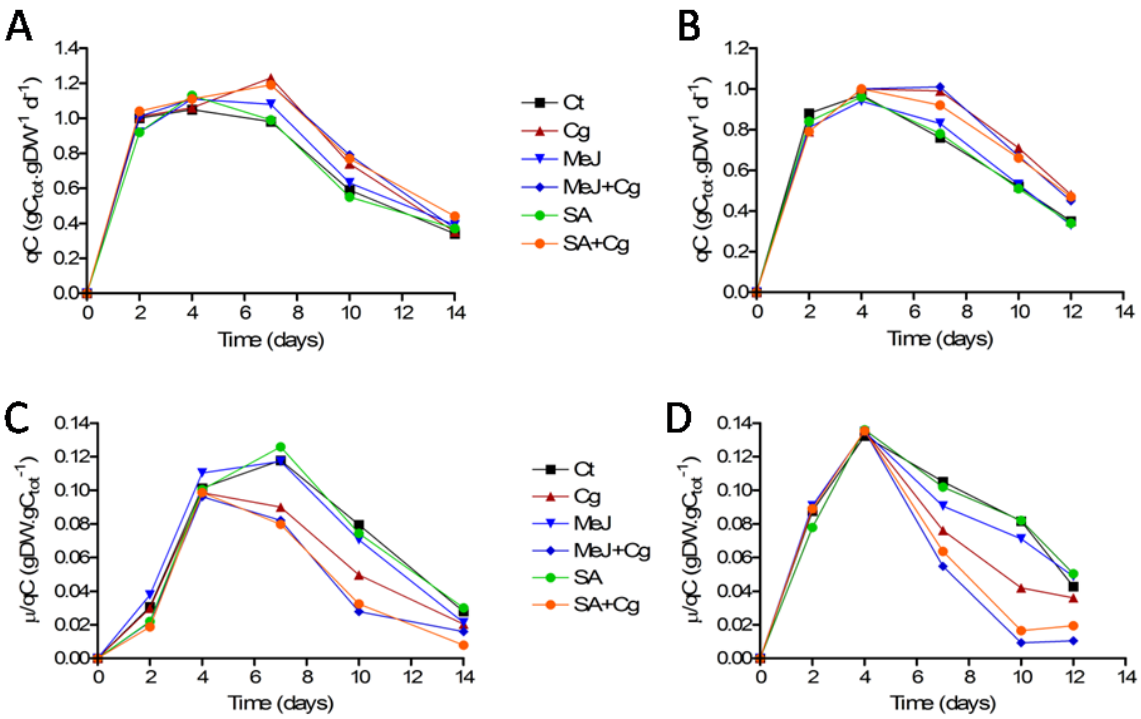


Figure 4.4: Changes on **(A,B)** sugar consumption rates (qC) and **(C,D)** biomass productivity in *H. perforatum* HPS and *Helos* suspension cell cultures, during their growth period. Suspension cells were treated with MeJ (100 μM), SA (25 μM), or *C. gloeosporioides* elicitor (Cg). The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 μM) or SA (25 μM), respectively, 24 h before the addition of the fungal elicitor.

4.3.2. Phosphate

As observed in figure 4.5 (A and B) it seems that, unlike sugar, phosphate consumption by *H. perforatum* suspension cultures was negatively influenced by elicitor treatment, especially from day 10 until the end of the study. Although phosphate uptake inhibition by pathogen elicitation was observed in parsley suspension cells [Strasser *et al.*, 1983], any comparison of these results with those found on

our *H. perforatum* suspensions can be deceivable. As previously discussed (chapter 3.3.2.2), plant cell death and release of its contents is known to be responsible for an increase in the concentration of phosphate in the medium, as observed in *H. perforatum* control cultures, after passing their stationary stages (Chapter 3 – Fig. 3.4). Apparently, a similar conclusion can be drawn from this experiment since a decrease in cell viability (Fig 4.1 – C and D; Fig. 4.2 – D to F) was observed after *C. gloeosporioides* elicitation, with or without prior treatment with MeJ or SA. The release of phosphate from death cells into the medium may constitute a *background noise* strong enough to mask any changes on phosphate consumption rates related to the viable cells available. In a similar way, no useful conclusions can be drawn from the relation between consumption rates and cell growth for cultures treated with the *C. gloeosporioides* elicitor (4.5 – C and D). As for MeJ or SA, it is clear that none of these phytohormones related to systemic defense signaling pathways have any impact, alone, on phosphate consumption rates, in both *H. perforatum* cell cultures studied.

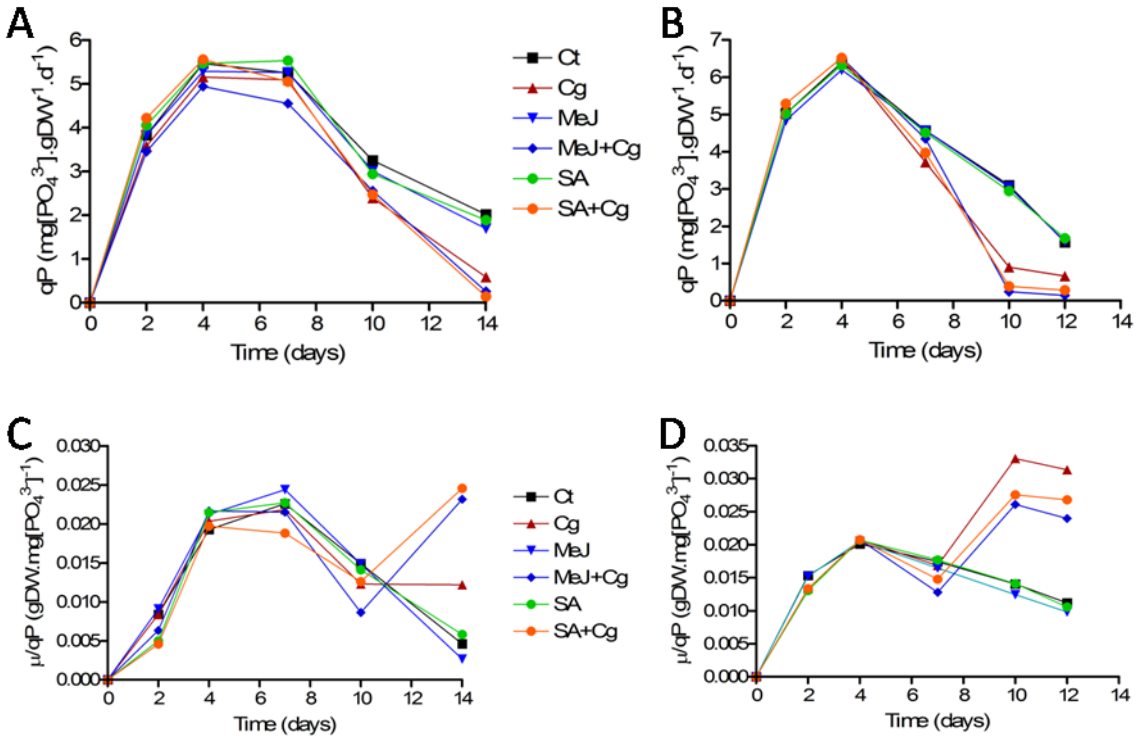
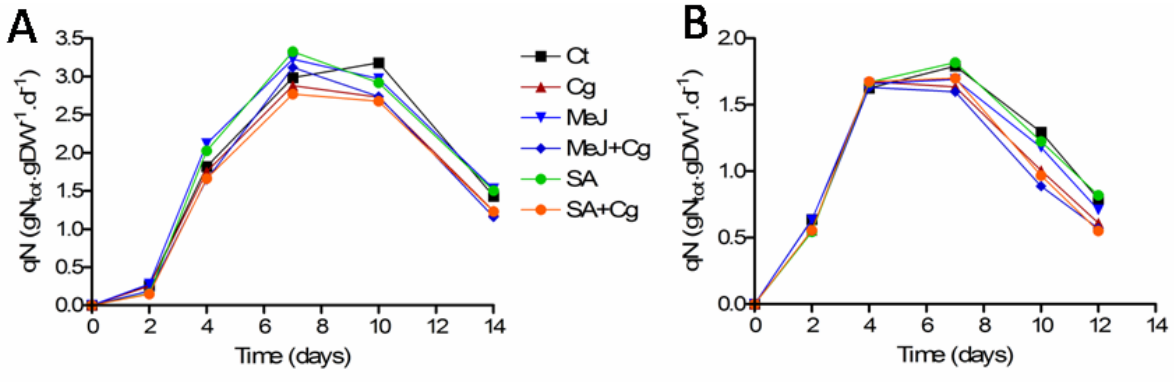


Figure 4.5: Changes on **(A,B)** phosphate consumption rates (qP) and **(C,D)** biomass productivity in *H. perforatum* HPS and *Helos* suspension cell cultures, during their growth period. Suspension cells were treated with MeJ (100 μM), SA (25 μM), or *C. gloeosporioides* elicitor (Cg). The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 μM) or SA (25 μM), respectively, 24 h before the addition of the fungal elicitor.

4.3.3. Nitrate and Ammonium

Besides sugar and phosphate consumption, nitrogen uptake was also evaluated in *H. perforatum* suspension cells, upon elicitation treatments. As observed below, the profiles of total nitrogen (nitrate + ammonium) consumption rates, for HPS and *Helos* cells, show a general decrease in nitrogen consumption due to *C. gloeosporioides* treatment (namely after day 10) and regardless any pre-treatments with the phytohormones MeJ or SA (Fig. 4.6 – A and B). This result may be associated to the increased cell death, observed upon pathogen elicitation. In fact, as discussed later in chapter 5, *H. perforatum* cells developed a hypersensitive response upon treatment with the pathogen elicitor. As referred by some authors, programmed cell death, induced by biotic stress, can be responsible for a decrease in the protein content within the cells (as observed for *P. pinaster* suspension cultures elicited with *B. cinerea* [Azevedo, 2005]) as well as for an efflux of nitrate to the culture medium [Wendehenne *et al.*, 2002]. Furthermore, although no effects were observed in *H. perforatum* suspension cells treated with the phytohormones (Fig. 4.6), MeJ has been found to affect nitrate consumption by decreasing its uptake, as reported in *Brassica napus* plants [Rossato *et al.*, 2002].

As observed in figure 4.6 (C and D), both *H. perforatum* cell cultures also showed a significant reduction in productivity (after day 7), as previously observed for sugar consumption (chapter 4.3.1). While nitrogen consumption was reduced upon pathogen elicitation, some of this nutrient could also have been diverted to defensive processes instead of growth. In fact, a myriad of proteins, known to accumulate upon biotic stress, have already been identified, in many plant models, displaying not only direct defensive functions but also other roles, such as storage (VSPs) [Wendehenne *et al.*, 2002; van Loon *et al.*, 2006].



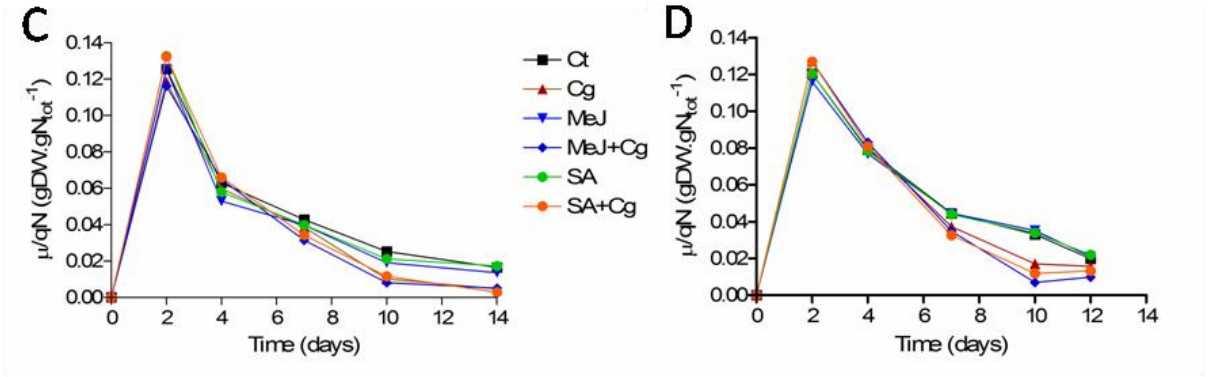


Figure 4.6: Changes on **(A,B)** consumption rates of nitrogen sources (qN_{tot}) and **(C,D)** biomass productivity in *H. perforatum* HPS and *Helos* suspension cell cultures, during their growth period. Suspension cells were treated with MeJ (100 μM), SA (25 μM), or *C. gloeosporioides* elicitor (Cg). The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 μM) or SA (25 μM), respectively, 24 h before the addition of the fungal elicitor.

4.4 Discussion

From the analysis of the previous growth and survival parameters we can conclude that pathogen challenging was responsible for an increased cell death, estimated by the decrease in cell viability but also by the medium pH drop and reduced biomass accumulation, observed after treatment with the *C. gloeosporioides* elicitor preparation. This response is probably associated with plant defense mechanisms such as hypersensitive response (ROS production) and/or an increase in the synthesis of phytoalexins, which can also become toxic for the plant cells [Dmitriev, 2003; Qin *et al.*, 2004; Davies *et al.*, 2006]. In fact, both ROS burst and hypersensitive cell death were observed in *C. gloeosporioides*-elicited cell cultures, as discussed in the next chapter. Moreover, accumulation of secondary metabolites, namely xanthones, occurred in elicited cells, as shown in chapters 6 and 7. Concomitantly, no significant changes on survival parameters were observed due to phytohormone (MeJ or SA) treatment only, much like what will be discussed further, for ROS production and phenylpropanoid metabolism, where these phytohormones had a low or absent impact when used alone. The relevance of these two mechanisms in *H. perforatum* defense against *C. gloeosporioides* will be analyzed in more detail in the following chapters.

Besides these parameters, the increase in sugar consumption, observed upon pathogen elicitation and not directly related to suspension cell growth, may be a clue to the possible shift of carbon sources from primary to secondary metabolism. It is known that a significant proportion of the carbon that enters both glycolytic and tricarboxylic acid (TCA) cycle pathways is channeled to the

synthesis of secondary metabolites [Plaxton, 1996]. Further support for the interaction between sugar consumption and plant defense comes from the fact that cell wall invertases can be induced by viral, bacterial and fungal infections [Kanabus *et al.*, 1986; Herbers *et al.*, 1996]. Moreover, whereas sugars can be directly used as a source of energy or as carbon-skeletons for the synthesis of defensive compounds, they may also be associated to gene expression, including those involved in systemic acquired resistance [Herbers *et al.*, 1996; Bourbouloux *et al.*, 1998].

While useful results from sugar consumption could be observed, the study of phosphate consumption became inconclusive, most likely due to an increased cell death observed upon pathogen elicitation. Consumption of nitrogen sources was also affected by pathogen elicitation. Unlike what was observed for sugar intake, *H. perforatum* suspension cell cultures displayed a significant decrease in nitrogen consumption. As previously referred, the hypersensitive response observed after pathogen treatment could be responsible for an efflux of nitrate, as observed in other plant models. Despite this difference to sugar consumption, a decrease in productivity was also observed, most likely due to a shift of nitrogen sources from growth to storage and/or defensive functions [Rossato *et al.*, 2002; Wendehenne *et al.*, 2002; van Loon *et al.*, 2006].

Considering the effect of the phytohormones on nutrient consumption parameters, we could see that, although SA alone (25 μM) had no effect on any of the parameters analyzed, MeJ (100 μM) was responsible for a transient reduction on biomass yield per gram of sugar, in a pattern closely related to that observed for *C. gloeosporioides*-elicited cultures and, probably, with similar implications in defense mechanisms. In fact, an increase in lignin accumulation was observed, on both *H. perforatum* suspension cell cultures, upon MeJ treatment (Chapter 7). Despite their low effects when used alone, pre-treatment of cultures with MeJ or SA, prior to *C. gloeosporioides* elicitation, potentiated *H. perforatum* cell responses to the pathogen presence in terms of biomass accumulation and cell viability, as well as in biomass yield per gram of sugar consumed. The role of these phytohormones in *H. perforatum* defense *priming* will be discussed along the next chapters.

Chapter 5

The role of Reactive Oxygen Species on *H. perforatum* L. response to elicitation

5. The role of reactive oxygen species on *H. perforatum* response to elicitation.

5.1. Introduction

The importance of Reactive Oxygen Species (ROS) in host defense was originally found in mammalian cells, associated with the respiratory burst of neutrophils [Hancock *et al.*, 2001]. Apart from their direct oxidative damage ability, another role has been widely studied and assigned to ROS, as signaling agents in defense mechanisms. While initial studies were focused on animal cells, soon it became evident that similar defense mechanisms, associated with ROS, existed in plant systems.

In plants, it has been found that ROS play an important role against both biotic and abiotic stresses [Apel *et al.*, 2004; Mittler, 2006]. Moreover, ROS are also responsible for the cross-talk and co-ordination of distinct plant responses [Fujita *et al.*, 2006]. The response to one stress may lead to increased resistance to a second exposure of a similar stress but also to other types of stress. As an example, mild ozone exposure may be responsible for increased resistance to virulent pathogens [Sharma *et al.*, 1996; Hancock *et al.*, 2002].

In terms of biotic interaction, pathogen recognition by plants is known to be responsible for oxidative bursts, the modulation of several defense-related genes and, eventually, hypersensitive cell death [Tenhaken *et al.*, 1995; Resende *et al.*, 2003; Bhattacharjee, 2005]. The increase in ROS accumulation, observed as oxidative bursts, may be due to an increased production but also due to a suppression in their degradation. Plant cells contain many components able to remove such reactive molecules, which include several antioxidant compounds (such as phenolic compounds, ascorbate or carotenes) as well as enzymes (such as catalases, peroxidases or superoxide dismutases). In this chapter we evaluated ROS production in both *H. perforatum* L. suspension cells available upon *C. gloeosporioides* challenging. The effect of SA or MeJ in ROS production was also studied in both pathogen-elicited and non-elicited cultures. Along with ROS production, *H. perforatum* ROS-scavenging capacity (both enzymatic and nonenzymatic) and hypersensitive cell death evidences were also monitored.

5.2. Quantification of intracellular ROS ($O_2^{\cdot-}$) in *H. perforatum* suspension cultures

Reactive oxygen species, such as the superoxide radical ($O_2^{\cdot-}$), are formed during biotic stress conditions, as previously described in chapter 1.4. Intracellular accumulation of superoxide radical was quantified by the XTT method, according to Able (1998), as described in section 2.2.4.1. According to this method, XTT was directly added to the suspension cultures. Interaction of XTT with superoxide radical is responsible for an accumulative/irreversible production of the XTT-reduced form (formazan). Results obtained are depicted in figure 5.1.

Following *H. perforatum* elicitation with *C. gloeosporioides* biomass, a double burst of superoxide radical accumulation was observed, in both HPS and *Helos* cell suspension cultures. The first burst aroused immediately after elicitation (15 min), reaching a peak of accumulation during the first hour (Fig. 5.1). A second burst started more than one hour later, reaching a peak 4 h after *C. gloeosporioides* elicitation, in both *H. perforatum* cell cultures. Despite the response pattern similarity found in both cell cultures, an increased accumulation of superoxide radical was found in *Helos*, reaching values twice as high as those found in HPS (Fig. 5.1). It is although unclear whether this response is the result of an increased accumulation of $O_2^{\cdot-}$ or due to the morphological differences existing between these *H. perforatum* cultures. HPS increased cell aggregation, reported in chapter 3,

could difficult XTT interaction with intracellular O_2^- as well as the release of formazan into the medium. Similar increases in O_2^- levels were found in cell cultures pre-treated with SA and MeJ (at final concentrations of 25 µM and 100 µM, respectively), prior to *C. gloeosporioides* elicitation. Although pre-treatment with these phytohormones had no effect in superoxide radical production in *H. perforatum*, a small increase in O_2^- accumulation was observed when these compounds were used alone. When compared to control cultures, an increase of approximately 2-fold was observed after SA or MeJ treatment, in both *H. perforatum* cultures. Although significant, these values remain residual when compared to the double burst produced by elicitation with the pathogen (Fig. 5.1).

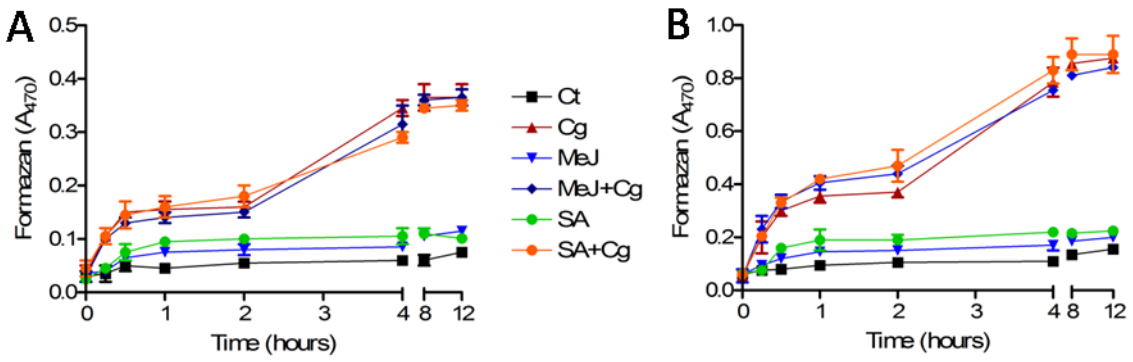


Figure 5.1: Accumulative production of formazan in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures following *C. gloeosporioides* elicitation and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

Intracellular ROS was also monitored recurring to H_2DCFDA, in a method described in chapter 2.2.4.2. This compound was used for assessing the total oxidative stress in both *H. perforatum* suspension cells available. The results, depicted in figure 5.2, are in accordance with those found previously with the XTT-formazan method.

Elicitation of *H. perforatum* cell cultures with *C. gloeosporioides* biomass was responsible for the typical double oxidative burst, found in non-host (type II) and host resistance mechanisms. In HPS, the first peak reached the highest value 30 min after *C. gloeosporioides* elicitation, while in *Helos* it occurred 1 h after pathogen challenge. Moreover, these values were 80% and 130% higher than those found in control cultures of HPS and *Helos*, respectively (Fig. 5.2). The second burst reached its peak 4 h after pathogen challenge, in both HPS and *Helos* cultures. This peak was responsible for an increase of 100% and 300% on the internal ROS accumulation, when compared to HPS and *Helos* control cultures, respectively (Fig. 5.2). In accordance to the XTT method, we can see that the double oxidative

bursts occurred approximately at the same time in both cell cultures and were, again, significantly more intense in *Helos* cultures. In fact, while the basal levels were nearly the same for both cell cultures, the second burst developed by elicited *Helos* cultures was approximately 170% more intense than the corresponding burst found in HPS cultures.

Pre-treatment with SA or MeJ, at the usual concentrations, had no influence on the oxidative bursts observed for *C. gloeosporioides*-elicited cultures. Nonetheless, MeJ or SA alone were responsible for a small, transient increase in the internal ROS accumulation for both *H. perforatum* cell cultures, which lasted approximately 8 - 12 h before basal levels were fully restored.

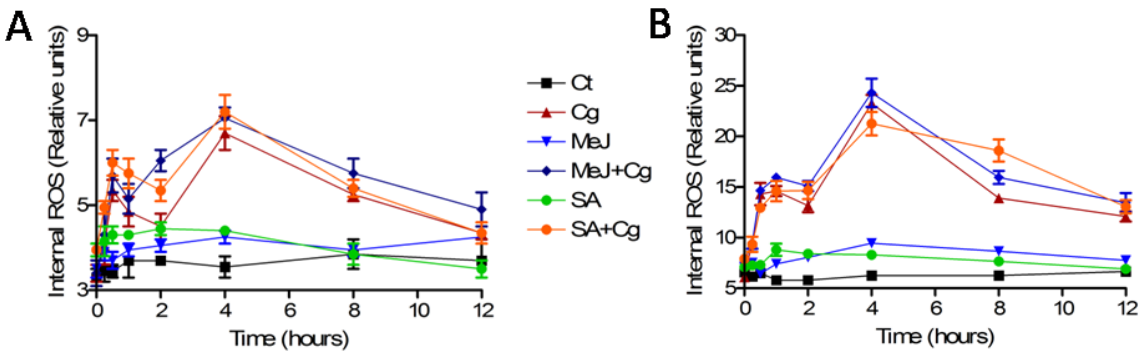


Figure 5.2: Quantification of intracellular ROS by the H_2DCFDA method in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures, following *C. gloeosporioides* elicitation and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

Regardless of the experimental method used, it is clear that both *H. perforatum* suspension cell cultures respond similarly to *C. gloeosporioides* elicitation. This double oxidative burst response shows a rapid and short phase I burst (common also to compatible interactions) that occurs within 1 h [Low *et al.*, 1996; Janisch *et al.*, 2004] and a phase II burst that occurs only in incompatible interactions. Usually, this second burst is more intense and lasts longer than the first one [Wojtaszek *et al.*, 1997], starting approximately 2 h after pathogen recognition, although the kinetics may vary greatly according to the plant and/or the non-host pathogen [Allan *et al.*, 2001].

5.3. Quantification of extracellular ROS (H_2O_2) in *H. perforatum* suspension cultures

Among the several reactive oxygen species produced by plants upon pathogen recognition, hydrogen peroxide (H_2O_2) plays a central role in plant defense. Although also toxic to invading organisms, H_2O_2 is known to act mainly as a signaling molecule in plants, due to its easy diffusion through the cell membrane, unlike other ROS, such as the superoxide radical [Huang *et al.*, 2002]. The central role of H_2O_2 in plant defense is further supported by several studies that associate this molecule with programmed cell death, by activating MAPK cascades, changing Ca^{2+} fluxes or redox status as well as interacting with other signaling molecules like SA or nitric oxide [Levine *et al.*, 1994; Gechev *et al.*, 2005; Torres *et al.*, 2005]. Due to its increased relevance in plant defense, we also investigated any possible changes in H_2O_2 accumulation in *H. perforatum* suspension cultures following MeJ, SA and/or *C. gloeosporioides* treatment. A xylenol-based method was used, as described in chapter 2.2.4.3. Results of H_2O_2 accumulation through time (12 h) are depicted in figure 5.3.

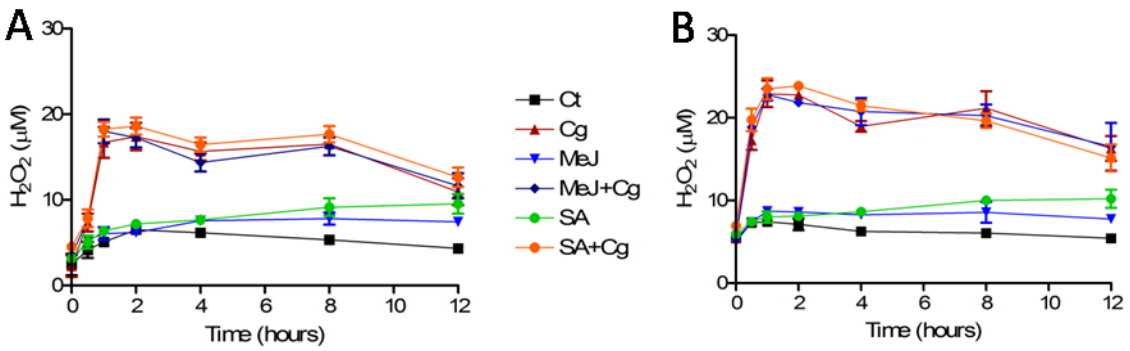


Figure 5.3: Quantification of hydrogen peroxide (H_2O_2) accumulated in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures, following *C. gloeosporioides* elicitation and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

Extracellular accumulation of H_2O_2 was observed on *H. perforatum* cell suspension cultures, under normal growth conditions. Control cultures showed a stable, constant production of this molecule, with concentrations of 5 µM (approximately) found on culture media from both HPS and *Helos* accessions (Fig. 5.3). Considering the innumerous roles and hormonal nature of H_2O_2, its constitutive presence in the media, under normal growth conditions, could be expected and associated to physiological processes other than a direct defensive function [Orozco-Cardenas *et al.*, 1999].

Treatment with SA was responsible for a significant increase in extracellular accumulation of H_2O_2. Both *H. perforatum* accessions showed, 12 h after treatment, an increase of 40 - 60% (approximately) in the extracellular H_2O_2 levels (Fig. 5.3). Nonetheless, values returned to normal 24 h after treatment (data not shown). The cross-talk between SA and H_2O_2 has already been extensively reported. On the one hand, Rao *et al*. (1997) reported the accumulation of H_2O_2 in *Arabidopsis* leaves after plant exposure to exogenous SA. In *Triticum aestivum*, application of SA (1mM) was also responsible for a substantial increase in H_2O_2 content [Agarwal *et al*., 2005]. On the other hand, exogenous application of H_2O_2 induced the accumulation of SA and its direct precursor, benzoic acid, in *N. tabacum* leaves [Leon *et al*., 1995]. A similar pattern in extracellular H_2O_2 accumulation was observed upon MeJ treatment. Although less intense than the one observed for SA treatment, the results suggest that H_2O_2 production in *H. perforatum* may also be influenced by this phytohormone. As reported for other plant models, MeJ treatment has already shown to enhance H_2O_2 generation. Per instance, MeJ concentrations as low as 10 µM were applied to *Eriobotrya japonica* fruits, improving H_2O_2 accumulation [Cao *et al*., 2008].

Elicitation with *C. gloeosporioides* biomass was also responsible for a significant increase in H_2O_2 accumulation levels, readily observable 30 minutes after treatment, on both *H. perforatum* accessions (Fig. 5.3). In both cases, a peak in accumulation was observed 2 h after treatment. While *Helos* showed absolute values higher than those found in HPS (23 µM and 18 µM, respectively), both accessions responded equally, displaying a 3 fold increase (approximately) in H_2O_2 accumulation, when compared to control cells. Suspension cell cultures from other plants developed a similar response to pathogen elicitation. For instance, *A. thaliana*, elicited with a preparation from *Fusarium oxysporum*, displayed a single peak in H_2O_2 accumulation of 20 µM, 60 – 80 minutes after treatment [Bindschedler *et al*., 2006; Davies *et al*., 2006], curiously showing both timing and accumulation values similar to those observed in elicited *H. perforatum* cell cultures. *Phaseolus vulgaris* suspended cells showed a faster response to a *C. lindemuthianum* elicitor preparation, reaching a peak of 15 µM, 10 -15 minutes after treatment [Bindschedler *et al*., 2001]. A single H_2O_2 peak was also observed in *N. tabacum* suspended cells, 30 min after treatment with an elicitor preparation from *P. syringae* [Baker *et al*., 2005] while a double burst response was observed in tobacco leaves upon elicitation with oligogalacturonides [Bellincampi *et al*., 2000]. The results obtained for *H. perforatum* cells are dubious, being difficult to conclude whether these cells display a second peak in extracellular H_2O_2 accumulation

or not. If present, this second peak should occur approximately 8 h after pathogen treatment, displaying similar accumulation levels as those observed for the first peak (Fig. 5.3).

Although the existence of a second peak in *H. perforatum* suspension cells is not clear, we can conclude that, in general, external ROS accumulation occurred in accordance to what was previously observed for the accumulation of internal ROS (chapter 5.2). While a lower response was observed upon SA or MeJ treatment, elicitation with *C. gloeosporioides* biomass was responsible for an intense response, associated with bursts of ROS production, both intra- and extracellularly. Additionally, no significant differences were observed on pathogen-elicited cultures, pre-treated with SA or MeJ (Fig. 5.3), as previously observed for intracellular ROS accumulation (chapter 5.2). Apparently, the recognition of the pathogen elicitor by *H. perforatum* cells seems to play a central role in the establishment and intensity of ROS defensive responses, regardless the presence of the defensive phytohormones SA or MeJ at the site of infection.

5.4. Antioxidant enzymes in *H. perforatum* suspension cultures

Accumulation of ROS, typical of incompatible biotic interactions, may be the result of an increase in their production. Nonetheless other mechanisms, including suppression of degradation, may equally contribute to ROS build-up. Due to their toxicity, living organisms have developed several approaches to scavenge such highly reactive compounds, thus maintaining the ROS balance within the cell. As previously referred, these scavenge mechanisms include nonenzymatic as well as enzymatic means (such as catalases, peroxidases or superoxide dismutases). It is therefore important to study how the activity of *H. perforatum* ROS-scavenging enzymes is affected during pathogen elicitation and/or SA or MeJ treatment.

5.4.1. Superoxide dismutase (SOD)

Superoxide dismutases (E.C. 1.15.1.1) are metaloenzymes responsible for the conversion of the extremely reactive O_2^- into H_2O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). Several isoforms exist, being differentially distributed in the cytosol, peroxisomes, glyoxisomes, mitochondrias or in the chloroplasts [Asghari *et al.*, 2006]. Overall SOD activity was measured according to its ability to inhibit the photochemical reduction of NBT, as described in chapter 2.2.6.2. Results observed for *H. perforatum* protein extracts are shown in figure 5.4.

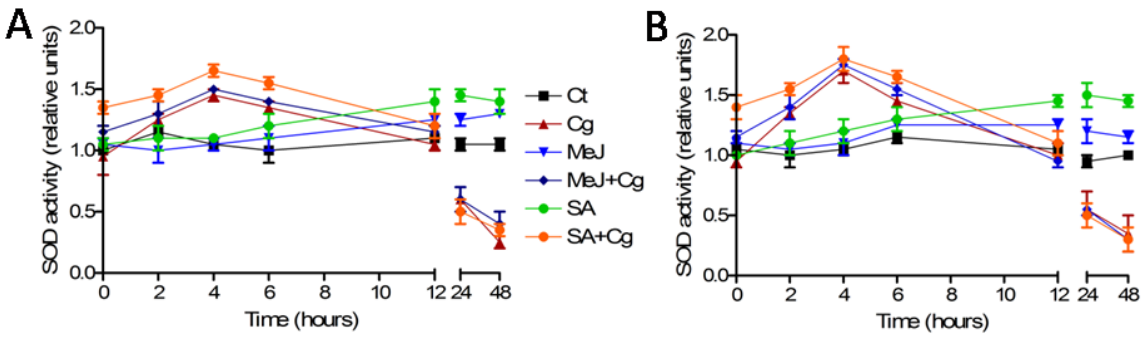


Figure 5.4: Changes on superoxide dismutase (SOD) activity observed in protein extracts from *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cells, following *C. gloeosporioides* elicitation (Cg) and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

As observed in figure 5.4, suspension cells from both *H. perforatum* accessions developed similar response patterns, according to the treatments they were subjected. Treatment with *C. gloeosporioides* biomass was responsible for an initial increase in SOD activity and, 4 h after elicitation, values observed in protein extracts from HPS cells were 40% higher than observed in control samples, while *Helos* displayed an increase of up to 60%. Despite this initial increase, SOD activity returned to basal levels, 12 h after elicitation (approximately). Values kept decreasing thereafter and, 2 days after elicitor treatment, SOD activity on both *H. perforatum* accessions reached values approximately 70% lower than those observed in control samples (Fig. 5.4). Pathogen recognition has also shown to be associated to a decrease in SOD activity from other plant species. When *P. pinaster* suspension cells were elicited with *B. cinerea* spores, a decrease in the activity of several SOD isoforms was observed within 24 h [Azevedo, 2005]. In a similar way, *P. vulgaris* leaves showed reduced SOD activity upon a viral infection [Clarke *et al.*, 2002]. Despite these and other cases, SOD activity seems to vary greatly according to the plant-pathogen model used. Incompatible interactions between *N. tabacum* and *Peronospora tabacina* [Edreva *et al.*, 1991] or between *Coffea arabica* and *Hemileia vastatrix* [Daza *et al.*, 1993] have displayed opposite results, with an increase in SOD activity. Moreover, the complexity and specificity associated to this defense mechanism is further supported by the interaction between *Pennisetum glaucum* and *Sclerospora graminicola*. While SOD activity decreased when a susceptible genotype from *P. glaucum* was inoculated with *S. graminicola*, a resistant genotype showed increased SOD activity upon pathogen recognition [Babitha *et al.*, 2002].

Along with the differential response developed by cells upon pathogen recognition, treatment with SA was also responsible for significant changes (Fig. 5.4). Upon SA treatment, both *H. perforatum*

cells displayed an overall increase in SOD activity. Unlike the transient increase previously observed during *C. gloeosporioides* elicitation, SOD activity levels begun raising only 6 h after SA treatment (approximately) but no longer decreased, reaching a somewhat constant level. As shown in figure 5.4, SOD activity found between the 12th hour and the last day of the study was 40 - 50% higher than the one found in control cells of HPS and *Helos*, respectively. The incremental effect of SA in SOD activity has also been reported in other plants such as *T. aestivum* [Agarwal *et al.*, 2005], *Nicotiana plumbaginifolia* [Bowler *et al.*, 1989] or *A. thaliana* [Rao *et al.*, 1997]. Furthermore, SA was also found to up-regulate a gene encoding a Cu,Zn SOD isozyme in *P. pinaster* [Azevedo *et al.*, 2004]. As observed for SA treatment, changes in SOD activity due to MeJ treatment were qualitatively similar, although displaying minor increases, on both *H. perforatum* accessions studied (Fig. 5.4). As reported for other plants, exogenously applied jasmonates frequently increase SOD activity, as observed in *Helianthus annuus* [Naik *et al.*, 2002] or *Brassica napus* [Comparot *et al.*, 2002]. Nonetheless, some physiological mechanisms are known to interact with this process. For instance, it has been reported that cytokinins, such as benzyladenine, can interfere with jasmonates, thereby blocking its effects on SODs [Naik *et al.*, 2002].

Furthermore, although both *H. perforatum* suspension cells displayed a differential response to SA or MeJ treatment alone, when these pre-treated cells were later elicited with the pathogen biomass, the levels of SOD activity followed a pattern similar to the one shown by cells elicited with *C. gloeosporioides* only (Fig. 5.4). Although the results obtained (Fig. 5.4) show a stronger response in SA pre-treated cells (SA+Cg) during the first 6 – 12h, it is unclear whether the higher activity is associated to a *boost* in response or if that is simply related to the higher basal levels, found on SA-treated cells at the time of elicitation. Despite this difference, SOD activity found 24 – 48h after *C. gloeosporioides* elicitation seems to depend solely on the pathogen, regardless the pre-treatment effects or the presence of any of the phytohormones used.

5.4.2. Catalase (CAT)

Catalases (E.C. 1.11.1.6) play an important role in several plant developmental processes including defense, aging and senescence; consequently, they are under strict temporal and spatial regulation [Magbanua *et al.*, 2007]. These enzymes are responsible for the final step in ROS degradation, converting hydrogen peroxide into harmless water ($2H_2O_2 \rightarrow H_2O + O_2$). In order to study the influence of catalases in *H. perforatum* defense mechanisms, its activity was measured

spectrophotometrically according to Aebi (1984), as described in chapter 2.2.6.2. Results obtained for both *Helos* and HPS suspension cells are shown in figure 5.5.

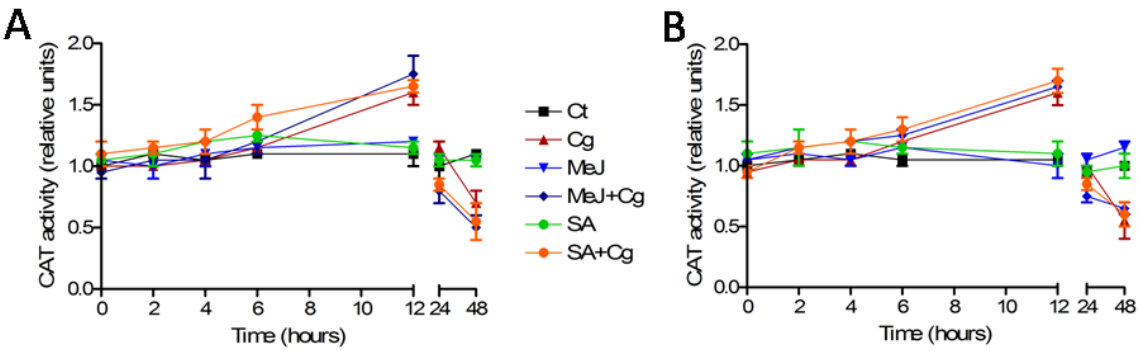


Figure 5.5: Changes on catalase (CAT) activity observed in protein extracts from *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cells, following *C. gloeosporioides* elicitation (Cg) and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

As observed in figure 5.5, elicitation of *H. perforatum* cells with *C. gloeosporioides* biomass was responsible for a transient increase in CAT activity. Although the peak was not fully defined, it is clear that an increase in activity, of at least 60%, occurred on both *H. perforatum* accessions. Despite this initial increase, CAT activity eventually decreased, reaching values lower than those observed in control samples, 24 h after elicitation. Until the end of the study, at the 48th hour, CAT activity in pathogen-elicited cells kept decreasing, reaching values 40 - 50% lower than those found in control samples from HPS and *Helos*, respectively (Fig. 5.5). The decrease in CAT activity upon pathogen elicitation is a widely reported feature. *A. thaliana* cultured cells showed lower CAT activity after elicitation with the phytotoxin fusicoccin [Beffagna *et al.*, 2007] while, for instance, the pathogen *Phytophthora nicotianae* was responsible for the down-regulation of CAT gene expression in *N. tabacum* [Blackman *et al.*, 2008].

Unlike what was previously observed for SOD activity, neither SA nor MeJ treatment were responsible for differential responses. During the time course of the experiment, no changes in CAT activity were observed on suspension cells from both *H. perforatum* accessions used (Fig. 5.5). Furthermore, pre-treatment of cells with these phytohormones, prior to elicitation with the pathogen, did not affect the results previously referred for cells treated with the pathogen only (Fig. 5.5). While pathogen elicitation usually results in a decrease in CAT activity, the effects of SA or MeJ treatments are not so easily generalized, varying among host and pathogen species studied. As observed in *H. perforatum* suspension cells, CAT activity in *N. tabacum* was also not affected by SA treatment [Fodor

et al., 1997]. Nonetheless, SA is usually responsible for a decrease in CAT activity and/or gene expression, as reported for *Hordeum vulgare* [Zeshuang *et al.*, 1997], *Solanum tuberosum* [Panina *et al.*, 2004] or *Oryza sativa* [Shim *et al.*, 2003]. Furthermore, reports exist indicating increases in CAT activity upon SA treatment [Agarwal *et al.*, 2005]. As for MeJ, divergence in results also exists. For instance, while in one study using *A. thaliana* plants a decrease in CAT activity was observed after treatment with MeJ (10 μM) [Maksymiec *et al.*, 2002], a similar study reported a significant increase in CAT activity upon treatment with 100 μM of MeJ [Jung, 2004]. Additionally, the same MeJ concentration (100 μM) effectively induced CAT activity in *Morinda elliptica*, 24h after treatment.

Considering all these variations in response, described for both SOD and CAT activities, it is clear that multiple parameters influence enzymatic ROS-scavenging mechanisms. Pathogen species, host species, phytohormonal concentrations used or timings, all exert significant effects in the fine-tuning of plant defense responses. In fact, the defensive fine-tuning seems to be a continuous process, associated, for example, to shifts in enzymatic activity. Both SOD and CAT activities have been shown to change drastically with time. As reported in *L. esculentum – B. cinerea* interaction, both SOD and CAT activities increase initially, upon pathogen recognition. Nonetheless, this increase is followed by a sharp decrease in enzymatic activities, 2 - 3 days after elicitation, as disease advances in the host [Kuzniak *et al.*, 2005].

The initial, transient increase in ROS-scavenging enzymes activity, observed during *H. perforatum – C. gloeosporioides* interaction, could be associated to a basal or general response to the increase in ROS, accumulated immediately after elicitation by the pathogen. The following decrease in SOD/CAT activities, on the other hand, could be related to a specific, regulated plant response upon recognition of a particular pathogen or simply due to cell integrity/viability losses, as disease advances or HR occurs. Further support in this topic should come, per instance, from gene expression studies, concerning CAT or SOD isozymes from elicited *H. perforatum* cells, as referred in chapter 8.6.

5.5. Antioxidant potential of *H. perforatum* methanolic extracts

Along with the ROS-scavenging enzymes, we also evaluated the scavenging potential of *H. perforatum* crude methanolic extracts. For this purpose, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed, as described in chapter 2.2.7. The results for the antiradicalar activity of methanolic extracts are depicted in figure 5.6.

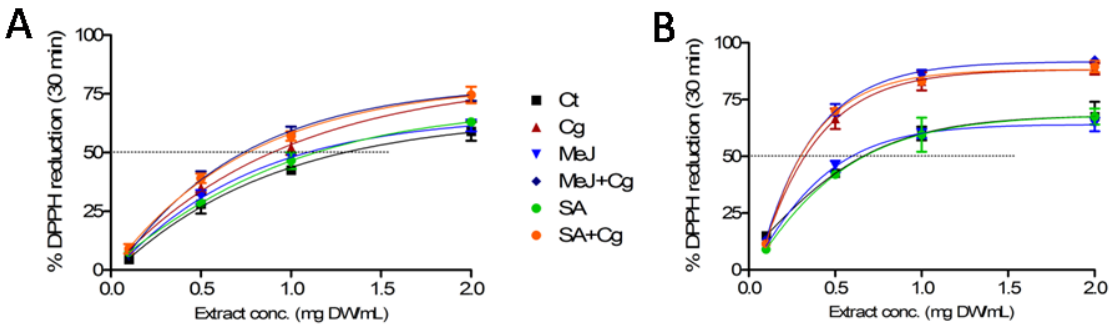


Figure 5.6: Antioxidant potential of methanolic extracts obtained from *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures 48 h after *C. gloeosporioides* elicitation and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

Methanolic extracts from *H. perforatum* suspended cells have shown distinct antioxidant potentials, according to the accession studied as well as the treatments applied. In all six conditions assayed, *Helos* extracts (Fig. 5.6 – B) showed higher antioxidant potential than the corresponding HPS ones (Fig. 5.6 – A). Under normal growth conditions, *Helos* achieved an EC_{50} value of 0.65 mg DW/mL (Fig. 5.6 – B) while, for HPS extracts, this value was only achieved at concentrations 2 fold higher of, approximately, 1.31 mg DW/mL. Identical results were observed after SA or MeJ treatment (for 48 h), on both *H. perforatum* accessions. Nonetheless, extracts from treated HPS cells showed a tendency for higher ROS-scavenging potential than control cells, reaching EC_{50} values of 1.13 mg DW/mL (approximately), for both SA or MeJ treatment (Fig. 5.6 – A).

Unlike the small (or absent) effects of MeJ or SA treatments, elicitation with *C. gloeosporioides* biomass was responsible for a substantial increase in the antioxidant potential of *H. perforatum* extracts. As previously reported, elicitation of *Helos* cells with *A. tumefaciens* was responsible for an increase in the ROS-scavenging properties of methanolic extracts [Franklin *et al.*, 2009]. Concomitantly, 48 h after *C. gloeosporioides* treatment, EC_{50} values had dropped to 0.90 and 0.33 mg DW/mL in HPS and *Helos*, respectively (Fig. 5.6). Furthermore, pre-treatment of *Helos* cells with SA or MeJ, prior to pathogen elicitation, had no differential effect on the extracts' antioxidant potential (Fig. 5.6 – B). On the other hand, a decrease in the EC_{50} values was observed in HPS cells pre-treated with SA (0.76 mg DW/mL) or MeJ (0.74 mg DW/mL), prior to pathogen elicitation (Fig. 5.6 – A).

The increase in antioxidant properties from *H. perforatum* methanolic extracts, observed upon *C. gloeosporioides* elicitation, should be directly associated to the significant changes in its composition, namely, the increase in xanthone production, described in the following chapters. As reported by some

authors, xanthones were found to effectively suppress ROS accumulation [Pinto *et al.*, 2005; Foti *et al.*, 2005]. Furthermore, some major xanthones isolated from *A. tumefaciens*-elicited *H. perforatum* cells, but equally present in *C. gloeosporioides*-elicited cells, had proven to display antioxidant properties [Franklin *et al.*, 2009].

5.6. Lipid peroxidation in *H. perforatum* suspension cultures

Free radicals are responsible for the damage of a myriad of cellular components such as DNA, proteins and lipids. Due to the results previously reported for ROS accumulation and scavenging, we decided to study its possible implications in lipid peroxidation of *H. perforatum* suspension cells, by quantifying the formation of MDA-TBA complexes (chapter 2.2.5). Figure 5.7 displays the results obtained for both HPS and *Helos* cell suspension cultures, elicited with *C. gloeosporioides* biomass and/or the phytohormones SA and MeJ.

Lipid peroxidation is a biological process often associated with ROS production and hypersensitive cell death [Göbel *et al.*, 2003]. Both plants and animals have evolved enzymatic and nonenzymatic mechanisms for the production of lipid peroxidation products [Sattler *et al.*, 2006]. Although being a common, constant process in living organisms, lipid peroxidation levels can increase dramatically during stress situations, both biotic and abiotic [Baryla *et al.*, 2000; Thoma *et al.*, 2003; Mithöfer *et al.*, 2004]. Pathogen recognition in incompatible interactions, for example, is responsible for oxidative stress conditions. The ROS produced interact with cell membrane components, resulting in the peroxidation of polyunsaturated fatty acids (PUFAs) in membrane lipids, therefore disrupting the selective permeability of the lipid bilayer. In the process, some lipid peroxidation products, such as jasmonates, can be formed, acting as signaling phytohormones, related to several plant defense mechanisms [Sattler *et al.*, 2006].

As depicted below in figure 5.7, elicitation of *H. perforatum* cultures with *C. gloeosporioides* biomass was responsible for a steady increase in lipid peroxidation, starting 6 h after treatment. The increase observed during this time may be associated with the increase in ROS production described earlier (chapters 5.2 and 5.3), which occurred within the first 8 h after cell suspension elicitation. Despite the initial increase in ROS levels, values observed 12 h after treatment were lower (in *Helos*) or no longer distinct from control cells (in HPS) (chapters 5.2 and 5.3). Nonetheless, lipid peroxidation levels kept rising (up to 100% in *Helos* and 40% in HPS) until the end of the study, 48 h after *C.*

gloeosporioides treatment. This suggests that other mechanisms, distinct from direct ROS contact, may be associated with the increase in lipid peroxidation. In fact, enzymes such as peroxidases or lipoxygenases may also be implicated in the conversion of unsaturated fatty acids into lipid peroxides [Mithöfer *et al.*, 2004]. Additionally, other nonenzymatic mechanisms may be acting. Lipid peroxyl radicals, formed earlier by interaction with ROS, are known to interact with neighboring PUFAs, propagating a chain reaction of lipid peroxidation through the membranes [Sattler *et al.*, 2006].

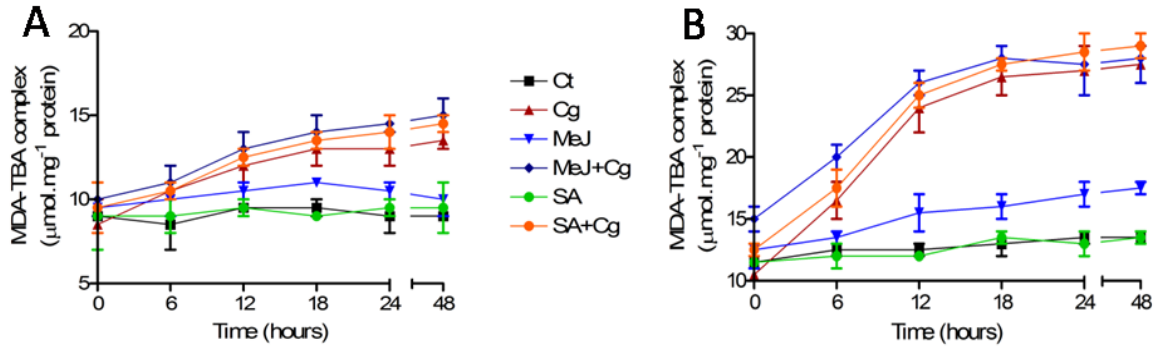


Figure 5.7: Lipid peroxidation in *H. perforatum* **(A)** HPS and **(B)** *Helos* suspension cell cultures, following *C. gloeosporioides* elicitation and/or phytohormone (SA or MeJ) treatment. The samples indicated as MeJ+Cg and SA+Cg represent cells primed with MeJ (100 µM) or SA (25 µM), respectively, 24h before the addition of the fungal elicitor.

As previously observed for ROS production (chapters 5.2 and 5.3), pre-treatment of *H. perforatum* cultures with SA or MeJ, prior to *C. gloeosporioides* elicitation, had no effect on lipid peroxidation. However, when MeJ was used alone, a minor increase in lipid peroxidation was observed on both cell cultures (Fig. 5.7). This small increase could, at first, be related to the equally small increase in ROS levels observed before (Figs. 5.1 and 5.2). Nonetheless, SA alone had similar effects in ROS accumulation but no significant change on lipid peroxidation was observed (Fig. 5.7). It is therefore possible that other mechanisms, associated with jasmonate signaling, play a role in this situation. Véronési *et al.* showed that MeJ at high concentrations (800 µM) was responsible for lipoxygenase induction in tobacco cell suspension cultures [Véronési *et al.*, 1996; Dubery *et al.*, 2000]. Furthermore, Ali *et al.* (2006) have shown that lipid peroxidation increased significantly when *Panax ginseng* roots were treated with MeJ, while SA (both at 200 µM) had no effect in MDA production. In the case of our study, lower concentrations of MeJ were used (100 µM) but similar plant response mechanisms could be involved. Further studies of lipoxygenase or allene oxide synthase activity/expression (for example) in *H. perforatum* suspension cell cultures should be carried out in order to bring new clues to the possible relevance of these results.

5.7. Evidences on Hypersensitive Response in *H. perforatum* suspension cultures: TUNEL assay.

Upon attempted infection and pathogen recognition, plants usually deploy a plethora of defensive reactions, including the previously described ROS build-up. These reactions are often accompanied by activation of mitogen-activated protein kinases, and the up- and down-regulation of gene expression, often leading to programmed cell death (PCD) processes, such as Hypersensitive Response (HR), at the infection site [Hancock *et al.*, 2002].

Considering the results previously shown along this chapter, we decided to evaluate the possibility of cell apoptosis occurrence by TUNEL, in order to better understand the effects of all the physiological changes taking place in *H. perforatum* cells upon elicitation. The TUNEL (*Terminal Transferase dUTP Nick End Labeling*) assay method is described in chapter 2.2.9 and the results are depicted in figure 5.8.

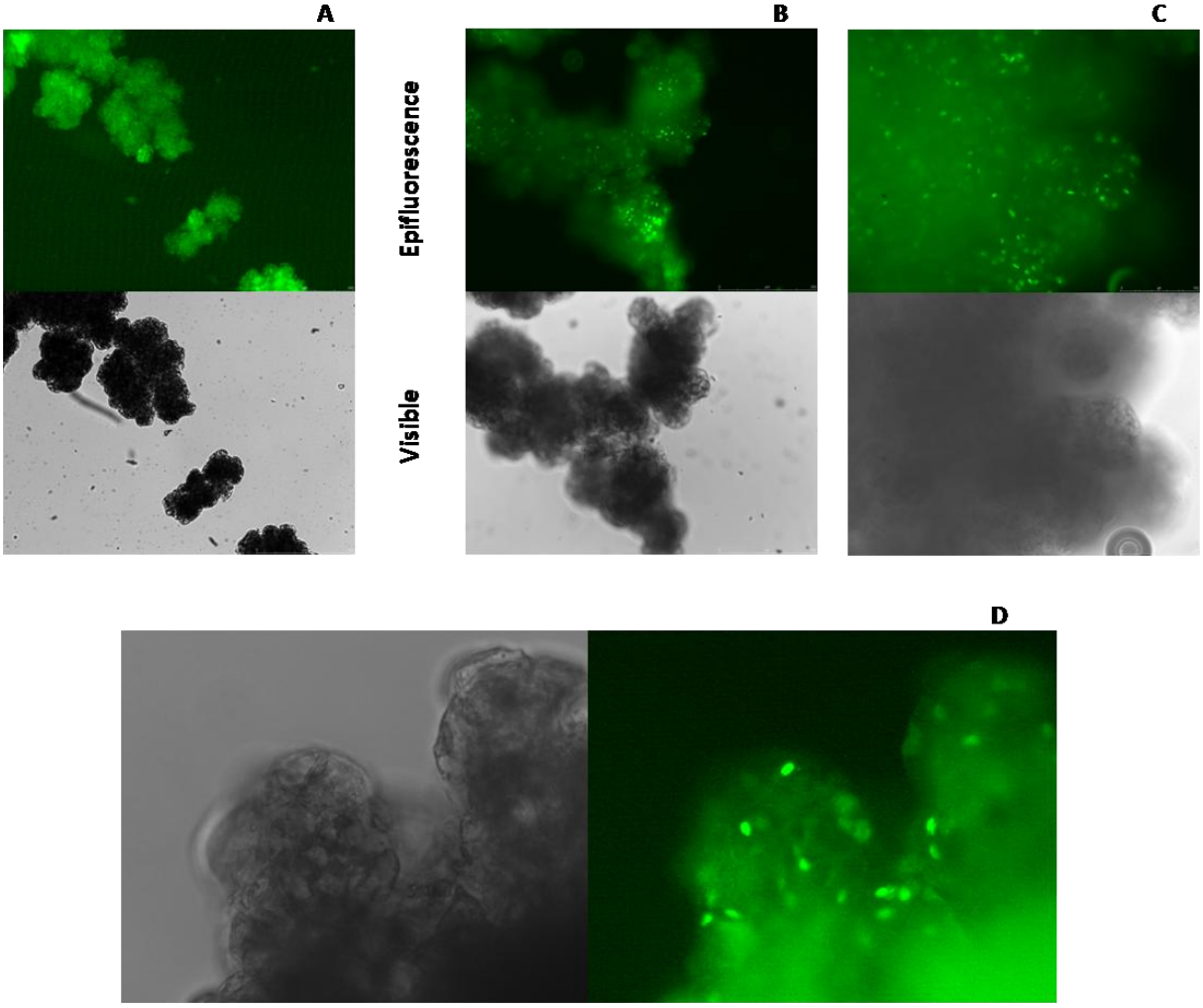


Figure 5.8: TUNEL assay performed in *H. perforatum* HPS suspension cell cultures. **(A)** Control cells exhibiting autofluorescence. **(B)** *C. gloeosporioides*-elicited cells, collected 18 h after treatment. **(C)** SA pre-treated cells, collected 18h after *C. gloeosporioides* elicitation. **(D)** Cells presenting apoptosis exhibited intense nuclei fluorescence. Nonfluorescent nuclei and autofluorescence indicated the absence of DNA cleavage.

As a typical phenomenon from apoptotic cells, DNA cleavage results in several 3'-hydroxyl termini, target sites for the action of an enzyme (TdT) that catalyzes the addition of a fluorescent-tagged nucleotide. As observed in figure 5.8, HPS control cells developed autofluorescence, most likely associated to cell wall components, while TUNEL-positive nuclei were very few (Fig. 5.8 – A). *C. gloeosporioides* treatment, on the contrary, was responsible for a significant increase in nuclei fluorescence, indicating that DNA cleavage was taking place 18 h after elicitation (Fig. 5.8 – B and D). Pre-treatment of HPS cells with SA, prior to pathogen elicitation, led to similar results (Fig. 5.8 – C) while SA alone had no significant effects (data not shown), showing similar proportions of TUNEL-positive nuclei than observed in control cells.

The results obtained by TUNEL assay come in accordance with some evidences, previously observed in chapter 4. A decrease in biomass accumulation and cell viability, as well as phosphate leakage into the medium, indicated that programmed cell death in *H. perforatum* suspension cultures should be underway, following elicitation with the pathogen.

5.8. Discussion

Pathogen challenge often leads to a variety of defensive responses from plants. One of the earliest reactions include the accumulation of reactive oxygen species, such as superoxide or hydrogen peroxide which may, eventually, lead to hypersensitive response at the infection site [Hancock *et al.*, 2002; Bhattacharjee, 2005]. Upon *C. gloeosporioides* elicitation, *H. perforatum* suspension cells developed a classic, incompatible response, described in many other plant-pathogen interaction models.

Production and accumulation of ROS has been one of the most studied defense mechanisms, not only in plants, but also in animal models, especially in phagocytic cells [Gelderman *et al.*, 2007]. In both *H. perforatum* accessions studied, pathogen recognition was responsible for an immediate ROS build-up, both internally and extracellularly. A typical two-phase burst was observed for internal ROS accumulation, reaching peaks 1 and 4 hours after *C. gloeosporioides* elicitation (chapter 5.2). These

responses, associated with an increase in ROS accumulation, are not present in type I nonhost resistance but are common to both host (gene-for-gene) and type II nonhost resistance, as mentioned in chapter 1.4. Furthermore, in incompatible interactions two ROS bursts are observed, being the second one more intense and prolonged, often considered as an indicator of recognition and mediated response against the invading pathogen [Lamb *et al.*, 1997; Azevedo, 2005].

Besides the increase in ROS accumulation, *C. gloeosporioides* elicitation was also responsible for the decrease in enzymatic ROS-scavenging potential. Protein extracts from both *H. perforatum* accessions showed reduced SOD and CAT activities, already observable 24 h after elicitation and decreasing thereafter. Similar decreases have been observed in many other plant-pathogen models, as discussed in chapter 5.4. Under most circumstances, these enzymes act as control agents, preventing the accumulation of ROS, inevitable by-products from basal plant metabolism, as referred in chapter 1.4. In fact, SOD and CAT activities showed a significant (but transient) increase during the first hours after pathogen elicitation, perhaps driven by a fast, basal response to increased ROS levels and not by a fine-tuned response to a specific, recognized invader. While apparently contradictory, suppression of ROS-scavenging enzymes activity during oxidative bursts is a necessary step towards an efficient and fast accumulation of reactive species, aimed for a local hypersensitive response, thus restraining the pathogen spread and protecting the plant as a whole, at the expenses of restricted tissues.

While enzymatic ROS scavenging means had been suppressed during *H. perforatum* – *C. gloeosporioides* interaction, one non-enzymatic mechanism had shown to improve upon pathogen recognition. Methanolic extracts from pathogen-elicited *H. perforatum* cells had shown an increased antioxidant potential, when compared to control cultures (chapter 5.5). While the differential regulation of enzymatic/non enzymatic scavenging mechanisms may seem conflicting, the compounds present on these extracts may have been synthesized to display other roles, more relevant in defense (such as antimicrobial activity), than providing antioxidant protection to *H. perforatum* cells. As discussed in detail in the following chapters, these extracts contain great amounts of one particular class of secondary metabolites, xanthones. Although able to contribute for the maintenance of ROS homeostasis, these compounds have shown to be scarce to circumvent the damaging effects of the increased accumulation of ROS observed and discussed below.

The combined effects of an overwhelming production of ROS and suppression of enzymatic ROS-scavenging means (despite the increase in scavenging potential from methanolic extracts) were responsible for an increased lipid peroxidation (chapter 5.6), observed in cell suspension cultures from

both *H. perforatum* accessions studied. While ROS accumulated during the double oxidative burst could account for the initial increase in lipid peroxidation, their values decreased within 12 h but lipid peroxidation kept increasing thereafter. Thus, other mechanisms (perhaps signaled by the previously accumulated ROS) could be responsible for the propagation of this plant response, as discussed in chapter 5.6. Impaired membrane structure and function may contribute to plant cell necrosis/apoptosis observed during hypersensitivity [Dubery *et al.*, 2000]. Therefore the increased lipid peroxidation, as well as the DNA cleavage observed by the TUNEL labeling, may be associated to the decrease in cell viability, biomass accumulation and phosphate leakage, previously discussed in chapter 4, as evidences of programmed cell death in *C. gloeosporioides*-elicited *H. perforatum* cell cultures. As an extreme PCD reaction, the hypersensitive response is performed by the plant cells in order to restrict pathogen growth, being especially effective against biotrophic invaders. On the other hand, its advantages against necrotrophic pathogens are still a matter of debate since HR is thought, in fact, to favor the invading pathogen by providing it with new entry points in the local environment [Kliebenstein *et al.*, 2008].

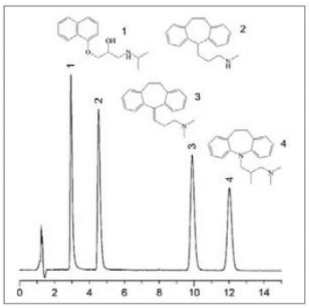
While the leading responses were observed upon pathogen elicitation, treatment of *H. perforatum* suspension cell cultures with the phytohormones SA or MeJ also led to significant changes in ROS accumulation and scavenging. A small, transient increase in intracellular ROS was observed, as well as a later, yet significant, increase in H_2O_2 accumulation in the culture medium (chapters 5.2 and 5.3). Although other ROS-scavenging enzymes (such as peroxidases) were not studied, it is curious to note how these phytohormones may have influenced the increase in extracellular H_2O_2 levels, by improving SOD activity without affecting degradation by CAT (chapter 5.4). Furthermore, while neither SA nor MeJ influenced the antioxidant potential from *H. perforatum* methanolic extracts (chapter 5.5), lipid peroxidation was affected by MeJ only (chapter 5.6). Since the last decade, SA and jasmonates have been generally considered as antagonizing hormones, with considerable data supporting it [Niki *et al.*, 1998; Fidantsef *et al.*, 1999; van Wees *et al.*, 1999]. Nonetheless, increasing studies suggests that the cross-talk between these two hormones is more complex, with greater subtlety than simple antagonism [Mur *et al.*, 2006; Liu *et al.*, 2008]. Several aspects, such as hormone concentration, may influence the overall plant response. Per instance, studies in *A. thaliana* and *N. tabacum* suggested that, while antagonistic effects are dose dependent, synergistic effects are observed when SA and jasmonates are used at low concentrations, whereas high doses are responsible for cell death [Mur *et al.*, 2006], as observed on both *H. perforatum* accessions, when SA concentrations higher than 50 µM were tested (unpublished results). Furthermore, it has also been suggested that some forms of cell

death require both jasmonate and SA signaling [Asai *et al.*, 2000]. Although related to distinct defense signaling pathways, both phytohormones do not have necessarily to influence distinct aspects of plant defense. In our experimental model, both SA and MeJ had a similar, yet minor influence in ROS production and scavenging. Considering the always growing list of known variables influencing and interacting in plant defense, it would be naïve to point one single variable as responsible for one specific plant response without further, deeper studies. For this purpose, the use of *H. perforatum* cultures should be avoided and surrogated by better characterized and flexible plant models, such as *A. thaliana*. Regardless the individual roles of SA or MeJ in plant defense modulation, it is clear that *C. gloeosporioides* recognition was the prime source of response and no significant effects were observed by pre-treatment of *H. perforatum* cultures with these phytohormones, at the concentrations used.

While response patterns were similar in both accessions, *Helos* showed a significantly higher accumulation of ROS, especially within the cell, when compared to elicited HPS suspension cultures. Considering their direct high toxicity (besides their signaling properties), it is possible that increased accumulation of ROS, observed in *Helos*, could account for its higher tolerance to *C. gloeosporioides* infection *in vivo*. Despite the simplistic nature of our experimental model, the defensive mechanisms developed by *H. perforatum* suspension cells are in accordance to those found in other plant models, studied both *in vitro* and *in vivo*. Nonetheless, further studies at the plant level should be carried out before drawing definite conclusions for this plant-pathogen model.

Chapter 6

Induction of phenolic compounds in *H. perforatum* L. cells by *C. gloeosporioides* elicitation



6. Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation.

6.1 Introduction

Hypericum perforatum L. (St. John's wort) is a medicinal plant used all over the world. Extract of HP is widely used to treat mild to moderate depression. The efficacy of the extract has been supported by some pharmacological and clinical studies [Erdelmeier *et al.*, 2000, Izzo *et al.*, 2003; Butterweck, 2003], attracting the interest of pharmaceutical industries. Presently, HP is one of the leading medicinal herbs sold both in EU and in USA [Erdelmeier *et al.*, 2000].

The growing demand for HP-derived products and their phytochemical consistency lead the producers to utilize the biomass of cultivated plants instead of wild collection. Nowadays, HP cultivation covers several hundred hectares in Europe [Gaudin *et al.*, 2003]. Most of these plants are grown organically, so they are highly exposed to pathogens. One of the main problems concerning the long-term cultivation of HP is the fungal disease anthracnose, caused by *Colletotrichum gloeosporioides* [Gaudin *et al.*, 2003]. This pathogen is responsible for heavy losses in HP plantations by lowering yield

and modifying the chemical composition of the plant extracts. Several efforts have been taken to obtain HP plants resistant to anthracnose. Nevertheless, little is known about the defence responses of this plant against pathogen attack. Differential accumulation of hyperforin and hypericin after elicitation of *H. perforatum* plantlets with *C. gloeosporioides* was reported [Sirvent *et al.*, 2002].

Plant cell cultures of several species have been utilized successfully as models to study the biochemical changes related to plant defence responses against pathogens [Hagemeier *et al.*, 1999; Conrath *et al.*, 2002; Hahlbrock *et al.*, 2003]. This system is relatively easy to manipulate and provides a better control of external factors that can interfere with the metabolic activities and thus advantageous over *in vivo* plant–pathogen interaction.

Here, we report the utilization of HP cell suspension cultures as a tool to study the defence responses related to phenolic metabolism against *C. gloeosporioides* attack.

6.2. Phenolic profiles of non-elicited and elicited *H. perforatum* cultures. Identification of HPS cell cultures major compounds.

Fig. 6.1 (A) shows a typical HPLC profile of the phenolics produced by HPS cell cultures (control). A major group of compounds were putatively identified as xanthone derivatives with 1,3,6,7 oxygenation pattern based on their characteristic UV spectra previously defined [Dias *et al.*, 2000 and 2001]. A major xanthone (compound X1) was putatively identified as mangiferin. HPLC–MS/MS analysis of this compound gave a molecular ion *m/z* $[M - H]^-$ of 421.5 and major $-MS^2$ fragments at *m/z* 331.0 $[M - H - 90]^-$ and 301.2 $[M - H - 120]^-$, losses characteristics of C-hexosyl compounds [Cuyckens *et al.*, 2001]. HPLC–DAD–MS/MS comparison analysis with a commercial standard of mangiferin (Extrasynthèse, Genay, France) confirmed this identification. Xanthones X7 and X10 were identified as 1,3,7-trihydroxy-6-methoxy-8-prenylxanthone (molecular ion *m/z* $[M - H]^-$ of 341.5) and *γ*-mangostin (molecular ion *m/z* $[M - H]^-$ of 395.5), respectively, by HPLC–DAD and HPLC–MS–MS comparisons with pure compounds previously isolated from *Hypericum androsaemum* cell cultures [Dias *et al.*, 2000]. Several other minor compounds were classified as 1,3,6,7-xanthone derivatives by HPLC–DAD–MS/MS analysis, but not fully identified.

A second set of phenolics (F2–F4) produced by HPS cell cultures were identified as flavonols based on their characteristic UV spectra. An intense deprotonated ion of the aglycone (*m/z* 300.2) was observed for these compounds indicating that they are quercetin derivatives. Compounds F2 and F3

contained similar deprotonated molecular ion *m/z* at 463.6 and a major $-MS^2$ fragment at *m/z* 301.0, corresponding to the loss of a hexoside residue from quercetin aglycone. Commercial standards (Extrasynthèse, Genay, France) were used to confirm the compound F2 as hyperosid and F3 as isoquercetrin. Compound F4 shared similarity with F2 and F3 in UV-spectra indicating its quercetin 3-derivative nature. It has a deprotonated molecular ion at *m/z* 505.2 and $-MS^2$ fragments at *m/z* of 462.7 $[M - H - 42]^-$ (quercetin 3-hexoside), that resulted from the loss of an acetyl group, and *m/z* of 301.0 (quercetin aglycone). According to its UV and mass spectra, this compound could be an acetyl derivative of hyperoside or isoquercetrin, a compound recently identified in *H. perforatum* plants [Silva *et al.*, 2005].

HPS cells primed with MeJ produced several compounds that were not detected in the control cells (Fig. 6.1 – B, compounds F1, F5–F10). Compound F1 has shown a similar UV and MS–MS spectra (*m/z* of 463.8 and 301.0) to those of compounds F2 and F3, indicating that it is a quercetin-3-hexose derivative. Compounds F5–F10 were identified as flavone derivatives. Compounds F5 and F6 have a deprotonated molecular ion at *m/z* 447.9, $-MS^2$ fragment at *m/z* of 285.7 (aglycone) and a similar UV spectra (267 and 339 nm). They were characterized tentatively as 6- and/or 8-OH-apigenin-7-hexosides based on their UV and MS–MS spectra. Compound F9 was identified as luteolin-*C*-prenyl by HPLC–DAD–MS/MS after comparison with the pure compound, previously isolated from *H. androsaemum* cell cultures [Dias *et al.*, 1998]. Compounds F7, F8 and F10 were also assigned as luteolin derivatives due to their UV spectra (255, 277 and 346 nm) and the presence of an intense $-MS^2$ fragment at *m/z* of 285.9. Cells primed only with SA did not produce any new compounds.

Several compounds were produced *de novo* in HPS cultures when elicited with *C. gloeosporioides* biomass (Fig. 6.1 – C) mainly in those cells previously primed with MeJ (Fig. 6.1 – D). On perusal of the UV spectra, the compounds were identified as 1,3,6,7 xanthone derivatives. Similar HPLC profiles were obtained when the cultures were first primed with SA and then elicited with CG extract (results not shown). Compound X2 was identified as 1,3,6,7-tetrahydroxyxanthone aglicone (single intense molecular ion *m/z* $[M - H]^-$ of 259.9). Compound X3 was putatively identified as mangiferin-*C*-prenyl. HPLC–MS/MS analysis of this compound gave a molecular ion *m/z* $[M - H]^-$ of 489.6 and major $-MS^2$ fragments at *m/z* 399.1 $[M - H - 90]^-$ and 369.2 $[M - H - 120]^-$, which are losses characteristics of C-hexosyl compounds [Cuyckens *et al.*, 2001]. Compound X4 gave a molecular ion *m/z* $[M - H]^-$ of 517.7, major $-MS^2$ fragments at *m/z* of 365.0 and an intense fragment at *m/z* of 257.1 characterized it as a dimer of 1,3,6,7-tetrahydroxyxanthone. Compounds X5 and X6 had an UV spectra characteristic of

1,3,6,7-oxygenated xanthones and molecular ion *m/z* [M - H]⁻ of 326.9. Consequently, these compounds were identified as 1,3,6,7-tetrahydroxyxanthone-*C*-prenyl isomers. Compounds X8, X9 and X11 were putatively identified as isomers of *γ*-mangostin (1,3,6,7-tetrahydroxyxanthone-*C*-bis-prenyl), since they have a similar molecular ion *m/z* [M - H] of 395.4 but different UV spectra and retention times. Several minor compounds were also identified as 1,3,6,7-tetrahydroxyxanthone derivatives but their identification was not fully accomplished.

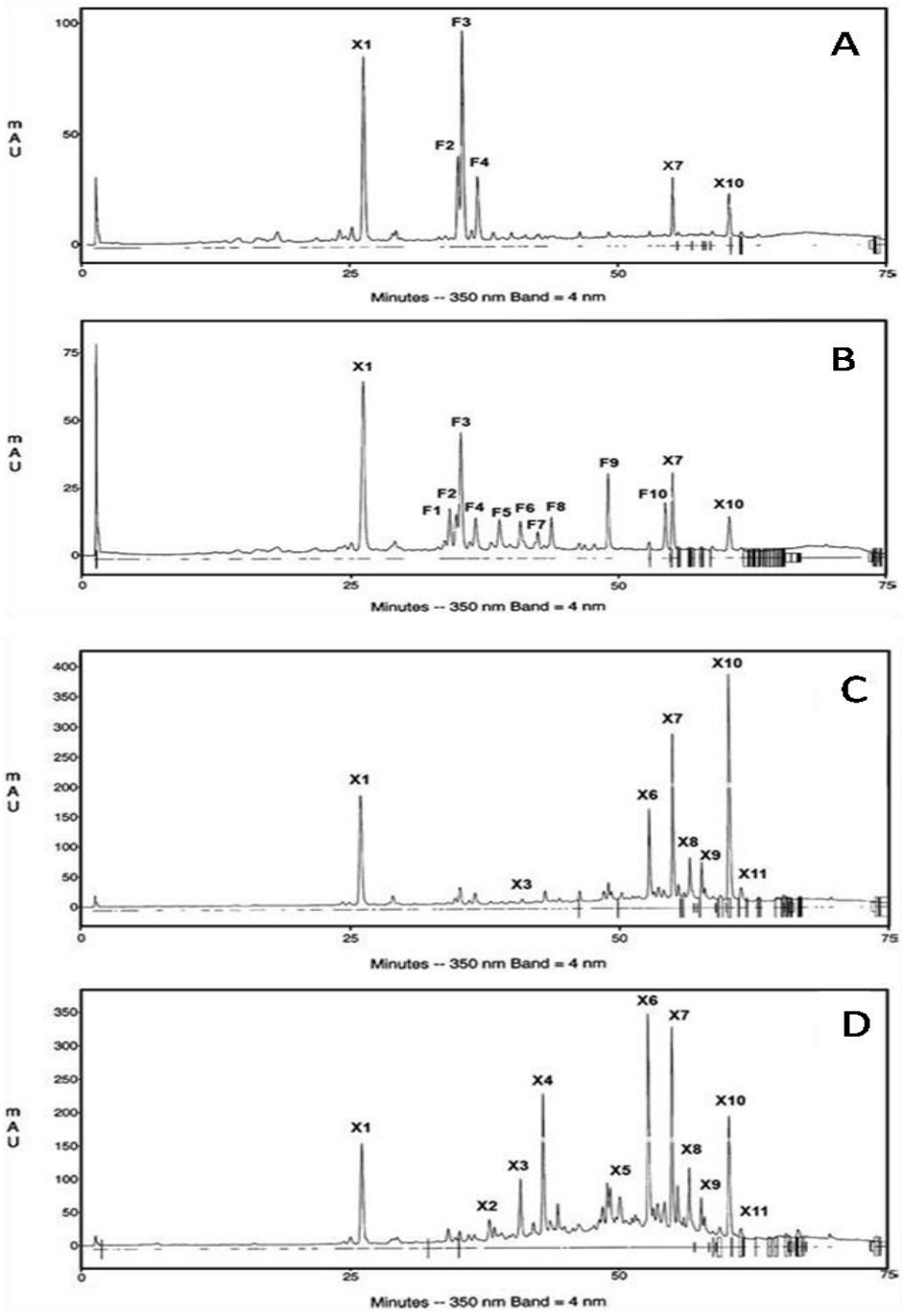


Figure 6.1: HPLC–DAD chromatograms of methanolic extracts from *H. perforatum* HPS cells, at the 7th day of growth: **(A)** control samples; **(B)** MeJ-treated cultures; **(C)** cultures elicited with *C. gloeosporioides* elicitor; **(D)** cultures primed with MeJ, prior to *C. gloeosporioides* elicitation. Compounds: **X1**– mangiferin; **X2**– 1,3,6,7-tetrahydroxyxanthone; **X3**– mangiferin-*C*-prenyl; **X4**– 1,3,6,7-tetrahydroxyxanthone dimer; **X5**, **X6**– 1,3,6,7-tetrahydroxyxanthone-*C*-prenyl isomers; **X7**– 1,3,7-trihydroxy-6-methoxy-8-prenylxanthone; **X8**, **X9**, **X11**– isomers of *γ*-mangostin; **X10**– *γ*-mangostin (1,3,6,7-tetrahydroxyxanthone-bis-prenyl). **F1**– quercetin-3-hexose derivative; **F2**– hyperosid; **F3**– isoquercetrin; **F4**– acetyl quercetin-3-hexoside; **F5** and **F6**– glycoside apigenin derivatives; **F7**, **F8** and **F10**– luteolin derivatives; **F9**– luteolin-*C*-prenyl.

6.3. Differential accumulation of phenolics due to MeJ or SA priming and/or *C. gloeosporioides* elicitation.

The accumulation of major phenolic groups, 24 h after of *C. gloeosporioides* elicitation of seven days old HPS cell suspension cultures is shown in Fig 6.2. HPS suspension cultures treated with the fungal elicitor showed a significant increase (seven fold) in xanthone accumulation. This burst was due to an increased production of usual xanthones (like X7 and X10) as well as the synthesis of new ones (Fig. 6.1 – C). This effect was particularly noticeable when HPS cells were primed with MeJ prior to elicitation with *C. gloeosporioides* biomass (Fig. 6.1 – D). In this condition, total xanthone content in HPS increased approximately twelve times when compared to the control (Fig. 6.2 – A). Addition of SA alone to HPS cultures also increased xanthone accumulation significantly ($P<0.05$).

Flavonoids accumulation in HPS suspension cultures also changed considerably after priming and elicitation procedures (Fig. 6.2). Flavonoids were not detected in cells exposed only to the fungal elicitor. Accumulation of these compounds decreased in the cells primed with MeJ or SA, followed by *C. gloeosporioides* elicitation ($P<0.05$). HPS suspension cultures exposed only to MeJ produced a new class of flavonoids, the flavones (Fig. 6.2). Flavones represent a significant proportion (approx. 40%) of the total flavonoids accumulated in those cells. Interestingly, in this condition, the total accumulation of flavonoids (flavonols plus flavones) did not change significantly from the control ($P>0.05$).

In spite of the significant changes in the accumulation of different phenolic classes, the total amount of phenolics produced by MeJ and SA-elicited cells was not statistically different to that of the control. However, HPS cells elicited with *C. gloeosporioides* biomass produced a higher amount of phenols, mainly due to the increase in xanthone accumulation (Fig. 6.2).

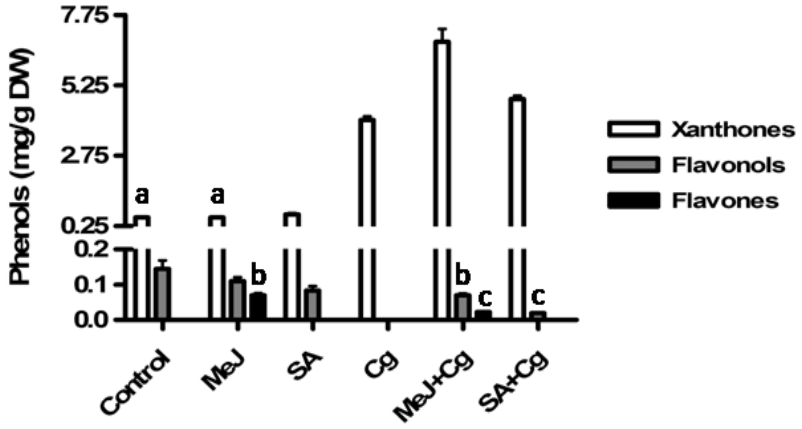


Figure 6.2: Total phenols produced by cell cultures of *H. perforatum* (accession HPS), at the 7th day of growth, 24 h after fungal elicitor addition. Cultures were treated with MeJ (100 µM), SA (25 µM) or *C. gloeosporioides* elicitor (Cg). The bars indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates, from two independent experiences. All the values are statistically different ($P<0.05$) except those signalized with the same letter.

6.4. Time course changes of phenolic accumulation in cells due to MeJ and SA priming and CG elicitation.

The elicitation process also induced significant changes in the phenols accumulated throughout the culture period by the cells (Fig. 6.3). HPLC analysis of the cell culture medium did not show any traces of phenolics, at any stage of culture growth. Apparently, the phenolics produced were either accumulated or metabolized intracellularly. Similar results have been described for the accumulation of xanthones in *Centaurium* species [Beerhues *et al.*, 1995].

In HPS cell suspension cultures elicited only with MeJ, xanthone accumulation remained constant throughout the culture period (Fig. 6.3 – A). However, cells elicited with SA only showed a significant increase in xanthone production during growth period ($P<0.05$). Xanthone level of HPS cells primed with MeJ reached the maximum 24 h after the addition of *C. gloeosporioides* elicitor (Fig. 6.3 – A). Cell suspensions treated with the fungal elicitor, with or without SA priming, attained the highest xanthone accumulation 72 h after elicitor addition. After day 9, the amount of xanthones started decreasing gradually. By the day 12, control cultures and cells elicited with *C. gloeosporioides* biomass produced the same amount of xanthones. However, cells primed with MeJ and SA prior to fungal elicitor addition retained a higher xanthone level even after 12 days (Fig. 6.3 – A).

MeJ and SA addition to the suspension significantly decreased the accumulation of flavonols in HPS suspension cultures, at the end of the culture period (Fig. 6.3 - B). Flavonols accumulation in HPS suspension cultures was negatively affected by the addition of *C. gloeosporioides* elicitor, resulting in their rapid disappearance throughout the culture time. It is interesting to mention that this disappearance coincided with the onset of xanthone production (Fig. 6.3).

Addition of MeJ to HPS cells induced the synthesis of a new group of phenolic compounds, the flavones, which accumulated during the first 48 h after MeJ addition and subsequently declined (Fig. 6.3 – C). Addition of the fungal elicitor to those cells resulted in a fall of flavone levels until they could no longer be detected, 72 h after elicitation.

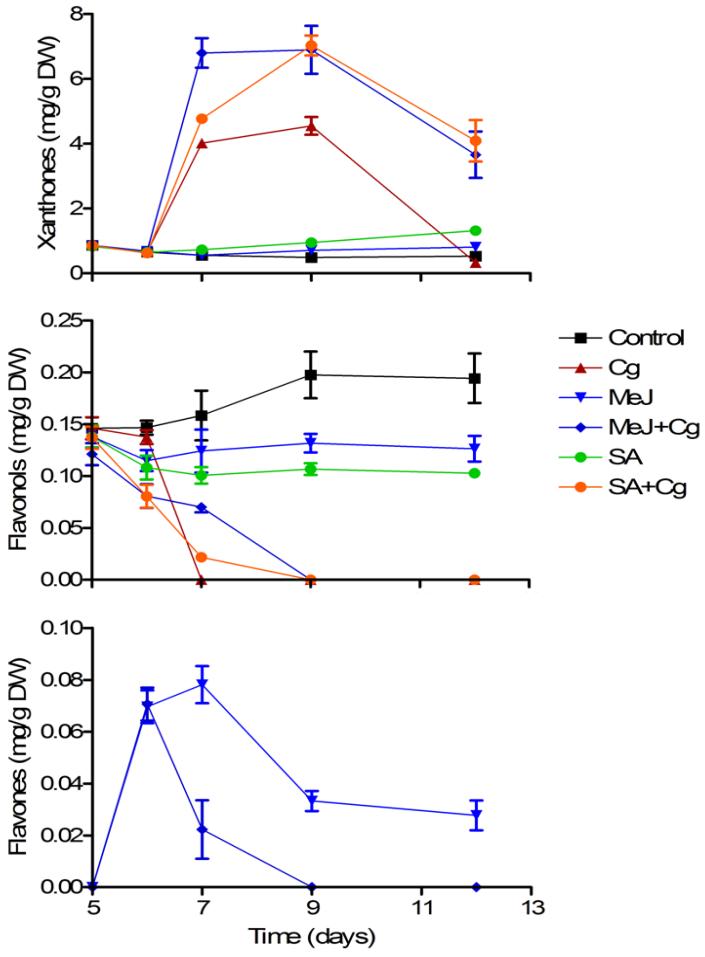


Figure 6.3: Accumulation of main phenolic compounds by cell suspension cultures of *H. perforatum* (accession HPS), throughout growth period, treated with MeJ (100 µM), SA (25 µM), or *C. gloeosporioides* elicitor (Cg). The lines indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates, from two independent experiences.

6.5. Discussion

In the presence of a pathogen, plants develop a vast array of metabolic defense responses sequentially activated in a complex multicomponent network that may be local and/or systemic [Hahlbrock *et al.*, 2003]. Defense responses to pathogen infection include the production of several secondary metabolites such as phenolics [Dixon *et al.*, 1995; Dixon, 2001; Tan *et al.*, 2004].

In the present study, *H. perforatum* (HPS) cell suspension cultures developed a differential phenolic response upon *C. gloeosporioides* elicitation which includes an increase in xanthone accumulation and the production of new constituents (Fig. 6.1 – C). Beerhues *et al.* (1995) already observed an increase in the accumulation of new xanthones in the cultures of *Centaurium* sp., upon elicitation with yeast but not with the cell-wall preparations of various pathogenic fungi. Xanthones are known for their pharmacological activities [Hostettmann *et al.*, 1989] such as antibacterial and anti-fungal [Beerhues *et al.*, 2000; Braz-Filho, 1999]. In addition, xanthones produced by *H. androsaemum* cell cultures have shown to inhibit the growth of *Candida utillis* and *Saccharomyces cerevisae* [Dias, 2003].

H. perforatum (HPS) cell cultures elicited with fungal biomass accumulated a significant amount of mangostin, a xanthone known to display anti-fungal activity [Hostettmann *et al.*, 1989]. Moreover, the majority of the xanthones accumulated after elicitation of both suspension cell cultures have a non-polar nature, which renders higher antimicrobial activity to the compounds. Therefore, the increase in xanthone accumulation observed in HPS cells can be described as a defense response triggered by some of the components present in the fungal elicitor. It is known that several fungal products such as proteins, glycoproteins or oligosaccharides can trigger the defense mechanisms in plants [Dmitriev, 2003].

The increase of xanthone accumulation observed in HPS cultures after treatment with the fungal elicitor could be the reason why flavonols became undetectable after elicitation (Fig. 6.1 – C and D). It is known that xanthones and flavonoids are biosynthetically related compounds, sharing a *pool* of precursors [Schröder, 1997; Dias, 2003; Liu *et al.*, 2003]. Those precursors could have been shifted for the xanthones biosynthesis, in detriment to the flavonoid pathway, resulting in a too low flavonol production to be detectable by HPLC.

Plant defense can be triggered by local recognition of pathogens but more effective responses include systemic signaling pathways [Conrath *et al.*, 2002]. Two of the most important compounds

having this ability are Salicylic Acid (SA) and Jasmonic Acid (JA). Systemic responses include those dependent on SA signaling and are named Systemic Acquired Resistance [Dempsey *et al.*, 1999]. The Induced Systemic Resistance is known to be dependent on JA [Feys *et al.*, 2000]. SA, JA and its derivatives, like MeJ, have been used as inducers in plants and were found to stimulate their secondary metabolism [Hahlbrock *et al.*, 2003; Thomma *et al.*, 2000]. For this reason we evaluated the possible effects of those molecules on the phenolic composition of *H. perforatum* cell suspensions. An overall increase in xanthone production and a quick response of *H. perforatum* HPS cells to the *C. gloeosporioides* elicitor (Figs. 6.2 and 6.3) after the pre-treatment with MeJ or SA suggests that these molecules *primed* HPS cells defensive mechanisms. This faculty is known for a long time but progress in understanding is still scarce [Conrath *et al.*, 2002]. The ability of jasmonate to *boost* plant defenses against fungal pathogens has already been reported [Thomma *et al.*, 2000]. The mechanism of action of SA and MeJ (one of several "jasmonates" known) is still a matter of debate [Felton *et al.*, 2000]. These two compounds seem to act independently via antagonistic pathways, giving rise to different plant responses. Nevertheless, a clear dichotomy does not always exist. In our case, both SA and MeJ were able to induce the priming for increased xanthone accumulation in HPS cell suspensions, as a response to the fungal elicitation, at different levels. However, significant differences were observed (Fig. 6.2 and 6.3). SA was able to increment the production of xanthones by itself, whereas MeJ alone did not interfere significantly in xanthone biosynthesis but resulted in a selective accumulation of flavones. The physiological significance of this pattern is not clear but might indicate that SA and MeJ stimulate different pathways in *H. perforatum* cells, independently of the result being the same: priming of the xanthone biosynthesis due to fungal elicitation.

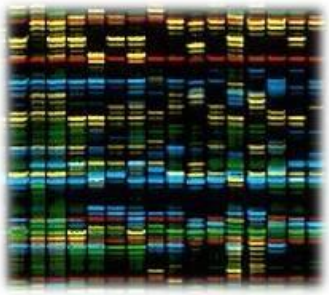
The use of JA and SA in *H. perforatum* cell suspension cultures was already reported [Walker *et al.*, 2002]. The authors have observed that the utilization of JA originated an increase in hypericin production in *H. perforatum* cultures. On the contrary, this response was not observed when SA or a pathogen extract was used. The elicitation of *H. perforatum* plantlets with SA, MeJ and *C. gloeosporioides* resulted in a differential accumulation of hyperforin and hypericin, depending on the treatments [Sirvent *et al.*, 2002]. Moreover, hypericin proved to inhibit the growth of *C. gloeosporioides*. Thus hyperforin and hypericin could be considered as presumptive phytoanticipins of *H. perforatum*. In our case, we did not observe the accumulation of either hypericin or hyperforin in suspension cell cultures after elicitation with *C. gloeosporioides*, in agreement with previous studies [Dias, 2003]. This

could be due to the fact that compounds like hypericins are accumulated in specialized tissues (glands) and not in cells of an undifferentiated state, as previously described in chapter 1.1.

In conclusion, the accumulation of xanthones in *H. perforatum* (HPS) cultures was strongly induced by *C. gloeosporioides* elicitation especially when primed with SA and, namely, MeJ. The results indicate that these compounds could act as defense-related compounds in HPS cells, and eventually in *in vivo* plants. *H. perforatum* plants do not accumulate xanthones in significant amounts at the aerial parts, with the exception of mangiferin [Kitanov *et al.*, 1998]. Nevertheless, we detected other xanthones in the biomass of *H. perforatum* plants occasionally (unpublished results). Taking in account the results presented here, this could be due to a biotic stress suffered by the plants in field conditions. Further studies are needed to validate this thesis. Additionally, isolation of the major xanthones produced by *H. perforatum* cells due to the elicitation process are ongoing, in order to test their potential activity against *C. gloeosporioides*.

Chapter 7

Differential responses from two *H. perforatum* L. accessions to *C. gloeosporioides* challenging



7. Differential responses of two *H. perforatum* accessions to *C. gloeosporioides* challenging.

7.1 Introduction

Hypericum perforatum L. is a perennial shrub distributed all over the world that has been described as a medicinal plant since ancient times. The increasing demand for *H. perforatum* biomass, for commercial purposes, can only be attended by field cultivation of selected HP accessions. The main drawback of mass production of *Hypericum perforatum* is contamination by *Colletotrichum gloeosporioides*, a fungus known to cause anthracnose on several commercially valuable plant species, as previously described in chapter 1.2. To our knowledge, no HP accession seems to be fully resistant to *C. gloeosporioides*. Nevertheless, the level of susceptibility (as well as the chemical composition) may vary between distinct *H. perforatum* accessions. One particular accession developed in Denmark, *Helos*, is referred as displaying an increased tolerance to anthracnose infection [Pundt *et al.*, 2005; Hammer *et al.*, 2007] but the mechanisms associated with this resistance are still unclear.

One of the known plant responses to biotic stress includes the accumulation of phenolic compounds, as previously described in chapter 6. These compounds play an important role in plant defense either by acting as constitutive phytoanticipins or newly-formed phytoalexins, produced upon pathogen recognition [VanEtten *et al.*, 1994]. A broad range of metabolites, derived from the phenylpropanoid pathway, have already been identified in many plant species and are usually divided into 3 broad classes, as described in chapter 1.3.4. These greatly diverse compounds are known to display a multitude of biological activities, not only against biotic stresses, but also against abiotic ones, such as UV radiation. Nonetheless, most secondary metabolites have relatively broad-spectrum activities against pathogens and do not constitute targeted responses to specific pathogens [Dixon, 2001; Azevedo, 2005].

While pathogen recognition may be responsible for the triggering of phenolic compounds accumulation in plants, several molecules are also known to modulate this defensive process. Salicylic acid (SA) and jasmonates (such as methyl-jasmonate) are phytohormones related to two plant systemic resistance mechanisms. The relevance of SA and MeJ in the modulation of Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR), respectively, has been pointed by many authors in the past decade [Gaffney *et al.*, 1993; Shulaev *et al.*, 1995; Pieterse *et al.*, 1999; Durrant *et al.*, 2004]. Nonetheless, the full extent of these systemic signaling pathways and the depth of their cross-talking, aiming for the fine-tuning of plant defenses, is still a matter of debate [Bostock, 2005; Adie *et al.*, 2007].

Here we evaluated some differences in phenylpropanoid metabolism, occurring between cell suspension cultures from two *H. perforatum* accessions, HPS and *Helos*, which display distinct susceptibilities to anthracnose *in vivo*. While chapter 6 was focused in the study of accumulation and identification of soluble phenolic compounds in elicited HPS suspension cells, this chapter presents new results related to changes in *H. perforatum* phenylpropanoid metabolism upon elicitation. In order to bring new clues about the differences on resistance to *C. gloeosporioides* infection, displayed by the two *H. perforatum* accessions available, other parameters were evaluated. Along with soluble phenolics, lignin accumulation, PAL enzymatic activity and expression of some key phenylpropanoid pathway enzymes were also monitored.

7.2 Accumulation of phenolic compounds upon elicitation. Differences between two *H. perforatum* accessions.

One of the known metabolic changes found in plants, related to pathogen recognition, is the production of soluble phenolic compounds, as described before (chapter 1.4). To complement the results previously found for *H. perforatum* HPS accession (chapter 6), a similar experiment was carried out with suspension cell cultures from the less anthracnose-susceptible accession *Helos*. Cells were elicited with *C. gloeosporioides* biomass and/or the phytohormones SA and MeJ, as described in chapter 2.1.9. In order to better compare the results obtained for HPS [Conceição *et al.*, 2006] and *Helos* cell cultures, figure 7.1 shows the accumulation of phenolics observed 72h after *C. gloeosporioides* elicitation. Moreover, time course changes in soluble phenolic compounds accumulation, observed under all treatments described, are shown in figure 7.2.

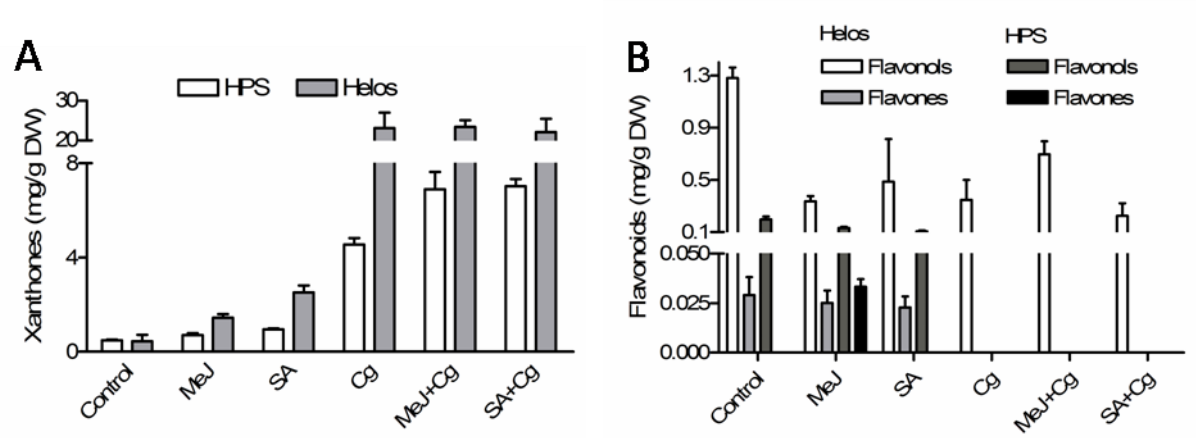


Figure 7.1: Total phenols produced by cell cultures of *H. perforatum* HPS and *Helos*, at the 9th day of growth, 72 h after fungal elicitor addition. **(A)** Accumulation of xanthones. **(B)** Accumulation of flavonoids (flavonols and flavones). Cultures were treated with MeJ (100 µM), SA (25 µM), or *C. gloeosporioides* elicitor (Cg). The bars indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates, from two independent experiences.

Accumulation of xanthones in HPS reached a peak 72 h after *C. gloeosporioides* elicitation, as previously described (Fig. 6.3 – A). Concomitantly, *Helos*, the *H. perforatum* accession less susceptible to anthracnose infection showed, 72 h after fungal elicitation, an increased amount of xanthones, reaching values 45x higher than those observed in control samples (Fig. 7.1 – A). While both *H. perforatum* accessions accumulate similar amounts of xanthones in normal growth conditions (approximately 0.5 mg/g DW), the burst observed in elicited *Helos* cultures was significantly stronger

(up to 6x) than the one observed in HPS cultures. Moreover, while the amount of xanthones accumulated in HPS eventually returned to levels found on control cultures, accumulation in *Helos* cultures kept rising until the end of the study, reaching values 50x higher than those observed in control samples (Fig. 7.2 – A).

Unlike what was observed for HPS cell suspension cultures, pre-treatment of *Helos* cultures with SA or MeJ, prior to *C. gloeosporioides* elicitation, made no significant changes in xanthone accumulation throughout the exponential growth period, leading to values similar to those observed in cultures treated only with the *C. gloeosporioides* elicitor preparation (Figs. 7.1 – A and 7.2 – A). On the contrary, treatment with SA or MeJ alone led to a small but significant increase in xanthone accumulation, as also observed for HPS cell suspension cultures (Figs. 7.1 – A and 7.2 – A). Although showing a pattern similar to HPS, the amount of xanthones accumulated in *Helos*, due to MeJ or SA treatment, were 3x and 6x higher (respectively) than observed in control samples (Fig 7.1 – A).

Xanthones were, quantitatively, the most prominent group of phenolic compounds identified on *H. perforatum* suspension cells. Nonetheless, a differential accumulation of flavonoids (flavonols and flavones) was also observed due to the treatments that both *H. perforatum* accessions were subjected.

Accumulation of flavonols in *Helos* cell cultures was negatively affected by *C. gloeosporioides* elicitation. As previously observed in HPS cultures, flavonols could no longer be detected in elicited *Helos* cells, at the end of exponential growth (Fig. 7.2 – B). Moreover, pre-treatment of *Helos* cultures with SA or MeJ, prior to pathogen elicitation, led to similar results. Accumulation of flavonols also decreased significantly after treatment with MeJ or SA alone, in a pattern similar to that found in HPS cell cultures (Figs. 7.1 – B and 7.2 – B). A sharp decrease was observed until the 9th day, stabilizing thereafter. At the end of the exponential growth period, values were approximately 4x lower than those found in control samples (Fig. 7.2 – B). Despite this decrease, the total amount of flavonols accumulated in *Helos* was still 4x higher than observed in HPS cultures (Figs 6.3 – B and 7.2 – B).

Flavones were produced *de novo* by HPS cultures only after treatment with MeJ (Fig 6.3 – C). In *Helos* cell cultures, however, these compounds accumulate in normal growth conditions (Fig. 7.2 – C). Despite the decrease in accumulation observed in *Helos* control cells during the exponential growth period (Figure 7.2 – C), flavone levels build up later on, during stationary stage, reaching the original values (data not shown). Elicitation of *Helos* cells with *C. gloeosporioides* biomass, with or without prior treatment with SA or MeJ, led to the irreversible decrease in flavones available and, 24 h after treatment, these compounds could no longer be detected by HPLC-DAD. Furthermore, treatment of

Helos suspension cells with MeJ or SA alone also led to a transient decrease in flavones accumulation, observed between days 6 and 7 and recovering to levels similar to control thereafter (Fig. 7.2 – C).

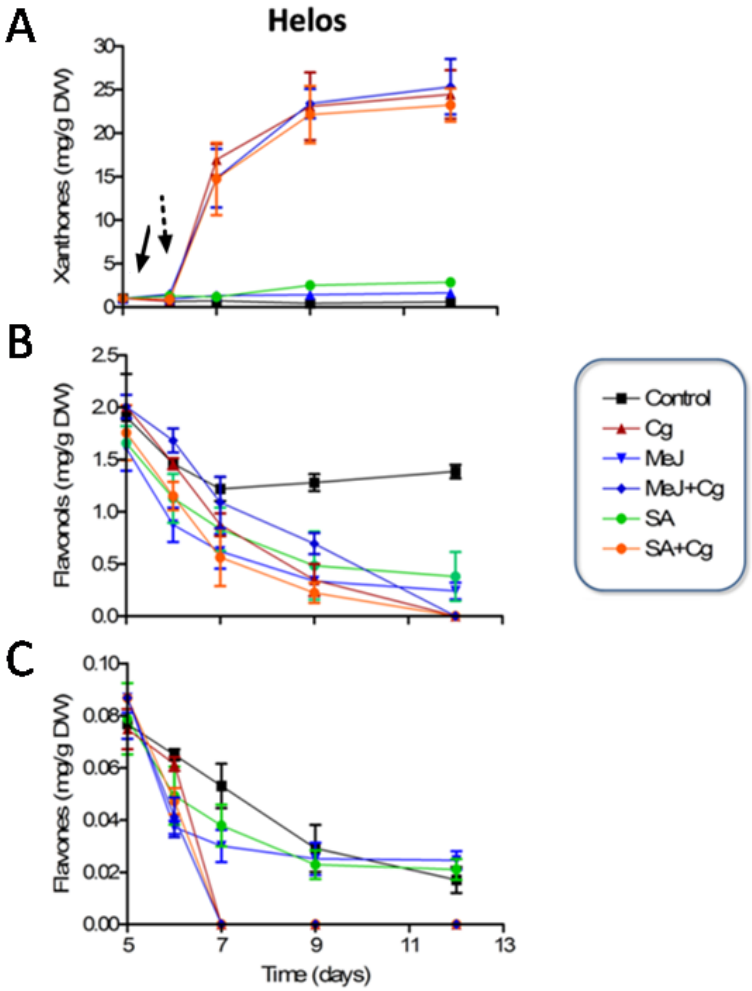


Figure 7.2: Accumulation of main phenolic compounds by cell suspension cultures of *H. perforatum* (var. *Helos*), throughout the growth period, treated with MeJ (100 µM), SA (25 µM), or *C. gloeosporioides* elicitor (Cg). Namely, accumulation of **(A)** xanthones, **(B)** flavonols and **(C)** flavones was observed. The lines indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates, from two independent experiences. All the values are statistically different ($P<0.05$). The straight arrow indicates the addition of SA or MeJ, while the dotted arrow indicates addition of fungal elicitor.

7.3. Changes on lignin accumulation

Apart from soluble phenolics, we also studied possible variations on the lignin content of *H. perforatum* cell suspension cultures. Lignin plays an important role in plant defense, reinforcing the plant cell-wall against pathogen penetration, as previously described in chapters 1.2 and 1.4. The

variations found in both HPS and *Helos* accessions are depicted in figure 7.3. Unlike what was observed for soluble phenolics, elicitation with *C. gloeosporioides* biomass was not responsible for an increase in lignin deposition in the cell-wall. This result is contrary to what is usually observed in pathogen-elicited cell suspension cultures from most plant species [Smith *et al.*, 1997; Mandal *et al.*, 2007; Egea *et al.*, 2001]. In fact, while elicitation of *Helos* cultures had no effect in lignin accumulation (Fig. 7.3 – B), HPS cultures treated with the pathogen elicitor showed a significant decrease in its contents (Fig. 7.3 – A). Although unusual, similar results were observed, per instance, in *P. pinaster* suspension cells elicited with *B. cinerea* spores [Azevedo, 2005].

SA-treatment of both suspension cell cultures had no significant effect in lignin accumulation. On the other hand, MeJ was responsible for a significant increase in lignin deposition, on both *H. perforatum* cell cultures, reaching values up to 100% higher at end of the study. In fact, MeJ-treated HPS cultures showed a peak in lignin deposition at day 9 of 3x (approximately) when compared to control cultures (Fig. 7.3 – A). Concomitantly, *Helos* cultures displayed 2x more lignin accumulation at that day, due to MeJ-treatment (Fig. 7.3 – B). Finally, when MeJ-treated cultures were later faced with the pathogen elicitor, a gradual decrease on lignin accumulation was observed. Nonetheless, these values were still significantly higher than those observed on cultures elicited with *C. gloeosporioides* only, throughout most of the period of time studied.

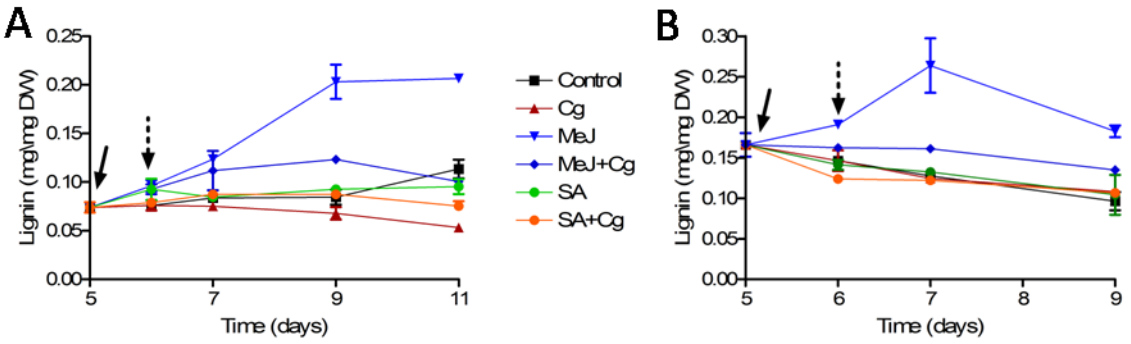


Figure 7.3: Accumulation of lignin on the cell-wall of cell suspension cultures from *H. perforatum* **(A)** HPS and **(B)** *Helos*, during exponential growth phase. Suspension cultures were treated with MeJ (100 µM), SA (25 µM) and/or *C. gloeosporioides* elicitor (Cg). The lines indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of three independent replicates, from three independent experiences. The straight arrow indicates the addition of SA or MeJ, while the dotted arrow indicates addition of fungal elicitor.

7.4. Phenylpropanoid pathway and elicitation: The effect of *C. gloeosporioides* in some key phenylpropanoid pathway enzymes.

The differential accumulation of phenolic compounds (both lignin and soluble phenolics), observed upon phytohormonal priming and/or pathogen elicitation, was further studied by accessing the gene expression of some key phenylpropanoid pathway enzymes. Namely, expression of the three general phenylpropanoid pathway enzymes (PAL, C4H and 4-CL) was assayed, as well as the PKS enzymes in the entry point of flavonoid biosynthesis (CHS) and BPS, an enzyme directly related to xanthone biosynthesis in *Hypericum*. Furthermore, PAL enzymatic activity was assayed as well. Figure 7.4 represents a simplified view of the general pathway and the following branches studied (lignin, flavonoid and xanthone biosynthetic pathways).

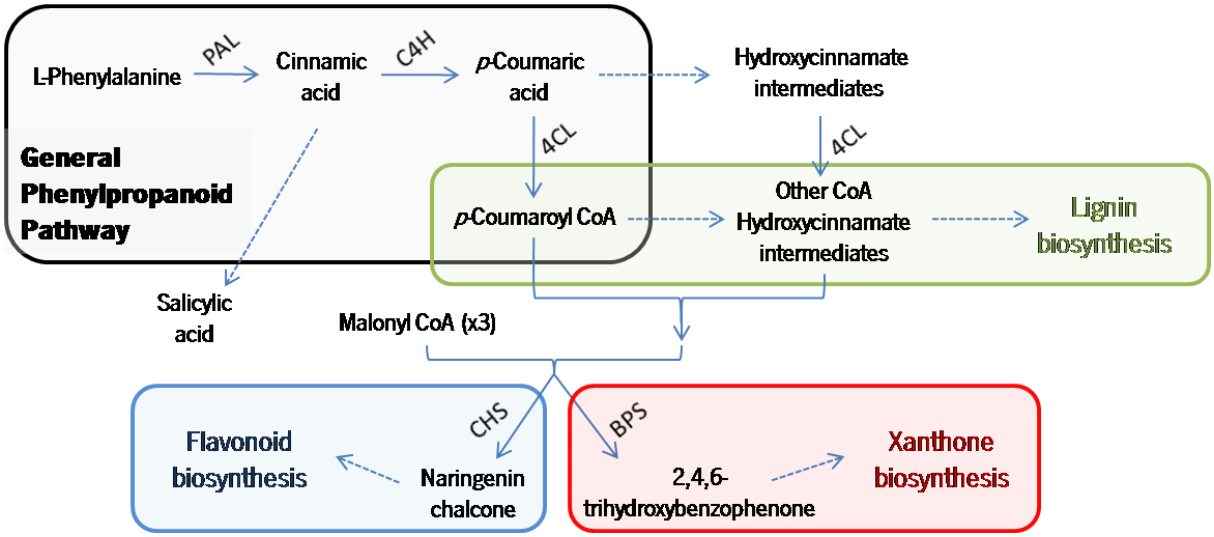


Figure 7.4: Schematic view of the phenylpropanoid pathway. Dotted arrows represent reactions not studied and carried out by one or more enzymes. **PAL** – phenylalanine ammonia-lyase; **CHS** – Chalcone synthase; **C4H** – Cinnamate 4-hydroxylase; **BPS** – Benzophenone synthase and **4-CL** – 4-coumarate:CoA ligase.

7.4.1. Phenylalanine ammonia-lyase (PAL) enzymatic activity.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the entry point of the phenylpropanoid pathway, connecting both primary and secondary metabolism, as previously discussed in chapter 1.3. This enzyme catalyzes the deamination of the aminoacid L-phenylalanine into cinnamic acid, as depicted in figure 7.4. Due to its relevance in phenylpropanoid metabolism, the enzymatic activity of PAL was assayed using *H. perforatum* crude protein extracts, as described in chapter 2.2.7.1. Figure

7.5 shows the results obtained, for all experimental conditions previously described, in both HPS and *Helos* cell suspension cultures.

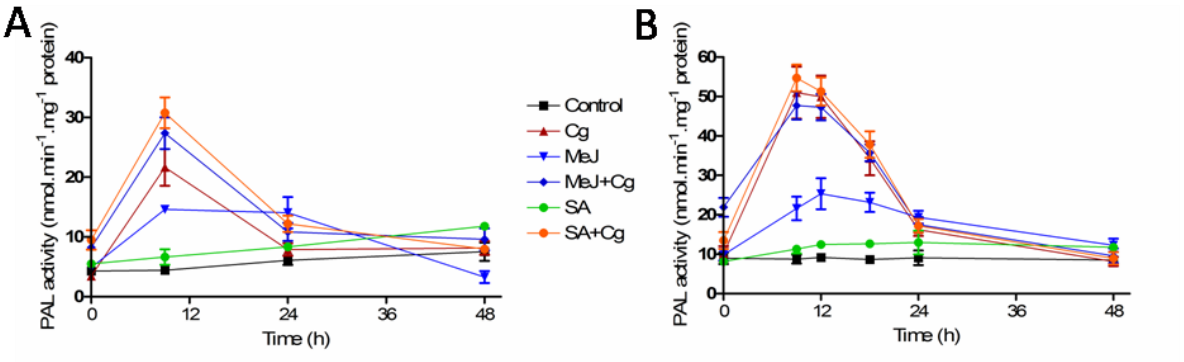


Figure 7.5: Activity of phenylalanine ammonia-lyase (PAL) in cell suspension cultures from *H. perforatum* **(A)** HPS and **(B)** *Helos*. Suspension cultures were treated with MeJ (100 µM), SA (25 µM) or *C. gloeosporioides* elicitor (Cg). The lines indicated as MeJ+Cg and SA+Cg correspond to cells primed with MeJ (100 µM) or SA (25 µM), respectively, before the addition of the fungal elicitor.

From figure 7.5 we can assume that both *H. perforatum* accessions display similar changes in PAL activity. These results are in accordance with what was previously observed, regarding phenolic compounds accumulation. Namely, an increased PAL activity was found in cultures elicited with *C. gloeosporioides* biomass, reaching a peak 9 h after treatment, in both cell suspension cultures (Fig. 7.5). Moreover, PAL activity in *Helos* cultures remained at the highest levels, for up to 12 h (Fig. 7.5 – B). During this time, activity was approximately 4 to 5 times higher than observed in control samples, in HPS and *Helos*, respectively. The relatively fast PAL response, observed upon pathogen recognition, is a common feature found in many plant models. *Cistanche deserticola* suspension cells achieved a peak in PAL activity 24 h after yeast extract elicitation [Cheng *et al.*, 2005b] while in *Manihot esculenta* and *Medicago sativa* suspended cells the peak was observed 15 h and 8 h after elicitation, respectively [Gómez-Vásquez *et al.*, 2004; Ni *et al.*, 1996]. Following that peak in PAL activity, an equally sharp decrease was observed and values obtained 48h after elicitation, for both *H. perforatum* accessions, were similar to their corresponding control extracts. The fast decrease in PAL activity (within 1 - 3 days) was also observed in the cell cultures from the plants referred above.

Pre-treatment of *Helos* cultures with MeJ or SA, prior to *C. gloeosporioides* elicitation, led results similar to those described for cultures treated with the elicitor only (Fig. 7.5 – B). In contrast, protein extracts from HPS cultures, pre-treated with SA or MeJ, showed a significantly higher activity 9h

after pathogen elicitation (up to 50% and 30%, respectively), when compared to cultures elicited with the pathogen extract only (Fig. 7.5 – A).

Protein extracts from both *H. perforatum* accessions, treated with MeJ only, also showed a significant increase in PAL activity. Although not as prominent as previously found for *C. gloeosporioides*-elicited cultures, the peak in activity (also observed 9 – 12 h after treatment) reached values 2 to 3 fold higher than in extracts from control cells of *Helos* and HPS, respectively. The peak in PAL activity was no longer observed 48 h later, with values returning to basal levels (in *Helos*) or even reaching significantly lower levels, as for HPS (Fig. 7.5). A similar response was observed in *Lithospermum erythrorhizon* cultured cells. While PAL activity reached a peak 8 h after yeast elicitor treatment (decreasing in 3 days), activity in MeJ-treated cultures also increased sharply within hours but kept at the highest levels until the end of the study, 3 days later [Tsuruga *et al.*, 2006]. Furthermore, in a similar study, *H. perforatum* cell suspensions were shown to increase PAL activity by 6 fold, 24 h after treatment with MeJ (at the same final concentration) decreasing thereafter. Despite the similarity of this study, values remained significantly higher than in control cultures, for more than 20 days [Gadzovska *et al.*, 2007]. Treatment with SA only also led to a small but constant increase in PAL activity, starting 9 h after treatment. The activity was approximately 20% - 30% higher than in extracts from HPS and *Helos* control cells (respectively) and was observed until the end of the study, 48 h after SA-treatment (Fig. 7.5). *Vitis vinifera* berry tissues, treated with SA to a final concentration of 150 µM, showed similar results, as a 2 fold increase in PAL activity was observed 1 to 3 hours after treatment, decreasing thereafter [Wen *et al.*, 2005].

Despite the similarities observed for both *H. perforatum* accessions, extracts obtained from *Helos* suspension cells have shown a tendency for higher PAL activity, when compared to HPS extracts. Under normal growth conditions, PAL activity was significantly higher in *Helos*, the only exception occurring at the end of the study, when activity was similar. Furthermore, the peak observed at 9h reached PAL activity values 2.5 times higher in *Helos* cultures elicited with *C. gloeosporioides* only. Pre-treatment of HPS cultures with MeJ or SA have shown to increase PAL activity. Nonetheless, values found in *Helos* were still 60% higher, regardless of MeJ or SA priming, prior to pathogen elicitation. Accordingly, peak activity observed 9 h after MeJ treatment was higher (2 fold) in *Helos* extracts than in the matching HPS protein extracts.

7.4.2 Identification of genes of interest from phenylpropanoid pathway enzymes.

The results obtained for PAL activity in crude protein extracts were further complemented with studies on gene expression. For this purpose, homologous probes for the enzymes of interest (referred in figure 7.4) were produced. In order to obtain the cDNA for *H. perforatum* PAL, a heterologous probe from *Digitalis lanata* was used to screen the *H. perforatum* cDNA library, available in the lab. The probe was radiolabeled as described in chapter 2.3.8, the library screening was carried out as described in chapter 2.3.3 and resolved in autoradiographic films (chapter 2.3.8.4). After several attempts, no positive clones were obtained, probably due to low copy number of *Pal* clones in the cDNA library or low similarity between *D. lanata Pal* fragment and *H. perforatum Pal* nucleotide sequence.

As a new approach, a homologous *Pal* probe was synthesized by PCR (chapter 2.3.11), after reverse transcription (chapter 2.3.10) of *H. perforatum* mRNA (obtained as described in chapter 2.3.1.2). Since no *Pal* sequences for *Hypericum* species were available in GenBank databases, degenerated primers (Table 7.1) were designed for a conserved region, deduced from the alignment of several nucleotide sequences from other dicots. PCR was carried out as previously described, the expected fragments were isolated from agarose gel (chapter 2.3.9.2), cloned in a suitable vector (chapter 2.3.13; Table 7.1) and used to transform *E. coli* DH5-α cells (chapter 2.3.14). After sequencing, the fragment was subjected to BLAST analysis, confirming the high homology to *Pal* sequences from other plants. The *Pal* fragment obtained was then used as a homologous probe to screen the *H. perforatum* cDNA library (without success) and to perform northern blotting assays, as described below (chapter 7.4.3). A similar RT-PCR approach was used to obtain homologous probes for the genes coding C4H and 4CL enzymes. Due to the variety of 4CL sequences expected (as described later), *4cl* degenerated primers were designed considering the enzymes' nucleotide sequence coding the substrate binding pocket and the highly conserved flanking motifs, namely, Box-I and Box-II (Table 7.1) [Stuible *et al.*, 2001]. This approach resulted in two distinct *4cl* fragments, showing increased homology to *A. thaliana 4cl1* (fragment 4cl "1") or *A. thaliana 4cl3* (fragment 4cl "2").

While RT-PCR was used to obtain homologous probes for *Pal, C4h* and *4cl*, the library screening approach was successfully used for obtaining the *H. perforatum* cDNA of CHS, by using a *H. androsaemum* heterologous probe available in the lab. To obtain the *H. perforatum Bps* probe, an RT-PCR was performed using primers designed according to the only *Bps* sequence available in GenBank, from a closely related species, *H. androsaemum*. The following cloning steps were performed as

described for *Pal* and, after sequencing confirmation, one positive clone was successfully used for screening the cDNA library. Furthermore, *H. perforatum* cDNA sequence for BPS was submitted to GenBank (acc. nº EF507429.1). All 5 homologous probes obtained were then used in gene expression studies, as described in the following chapters.

Table 7.1: Homologous probes obtained from *H. perforatum* mRNA or cDNA library.

Enzyme	**Cloning vector**	**Origin**	**Primers** (5' – 3')
PAL	pGEM-T Easy (Promega)	RT-PCR	Fw: CCDYTDCARAARCCWRAACAA Rv: CDCCYTTDAABCCRTAATC
C4H	pCR 2.1 (TOPO - Invitrogen)		Fw: AYGARGACAAYGTTCTTTAC Rv: CGATCRTGGAGGTTCA
4-CL "1" 4-CL "2"	pKS- II (Bluescript - Stratagene)		Fw: CCGGGATCCGACRGGNKTNCCXAAAGGRGTSATG Rv: CGCTCTAGAGCCNCKDATGCARATYTCACC
CHS	pJET1.2 (Fermentas)	cDNA library screening	X
BPS	pDONR (Gateway - Invitrogen)		X

7.4.3 Effect of C. gloeosporioides *elicitation and MeJ priming in phenylpropanoid pathway gene expression.*

The expression analysis for all phenylpropanoid pathway enzymes were performed by Northern blotting (as described in chapters 2.3.7 and 2.3.8) using total RNA (chapter 2.3.1.1) obtained from *H. perforatum* cell suspension cultures. Figure 7.6 shows the results for *Pal* expression in *Helos* suspension cells, grown under normal conditions or elicited with *C. gloeosporioides* biomass. As observed, *Pal* expression was very low and barely detectable by northern blotting, under normal growth conditions (Fig. 7.6). On the other hand, *C. gloeosporioides*-elicited cultures displayed an increase in *Pal* expression, starting 3 h after pathogen recognition. Although *Pal* upregulation seems to occur continuously until the end of the study (12 h), this result can be misleading. In fact, a peak in *Pal* expression was observed 8h after elicitation, as shown later, in figure 7.7.

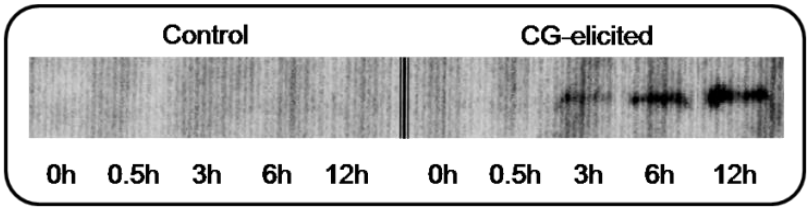


Figure 7.6: Northern blotting results for the expression of phenylalanine ammonia-lyase (PAL) in *H. perforatum Helos* cell suspension cultures grown under normal conditions or elicited with *C. gloeosporioides* biomass.

Considering the result obtained for *Pal* transcription, a new assay was performed in order to study the expression of genes coding for all previously referred phenylpropanoid pathway enzymes. During the laboratory work, HPS suspension cells had proven to be easier to handle than *Helos*, especially when samples from longer elicitation periods were needed. Upon elicitation, the integrity of *Helos* RNA decreases continuously, compromising the production of proper northern membranes. Therefore, HPS cells were preferably used for the expression studies shown below. Figure 7.7 shows the changes in expression of the genes of interest, obtained from HPS cells elicited with *C. gloeosporioides*, with or without prior treatment with MeJ.

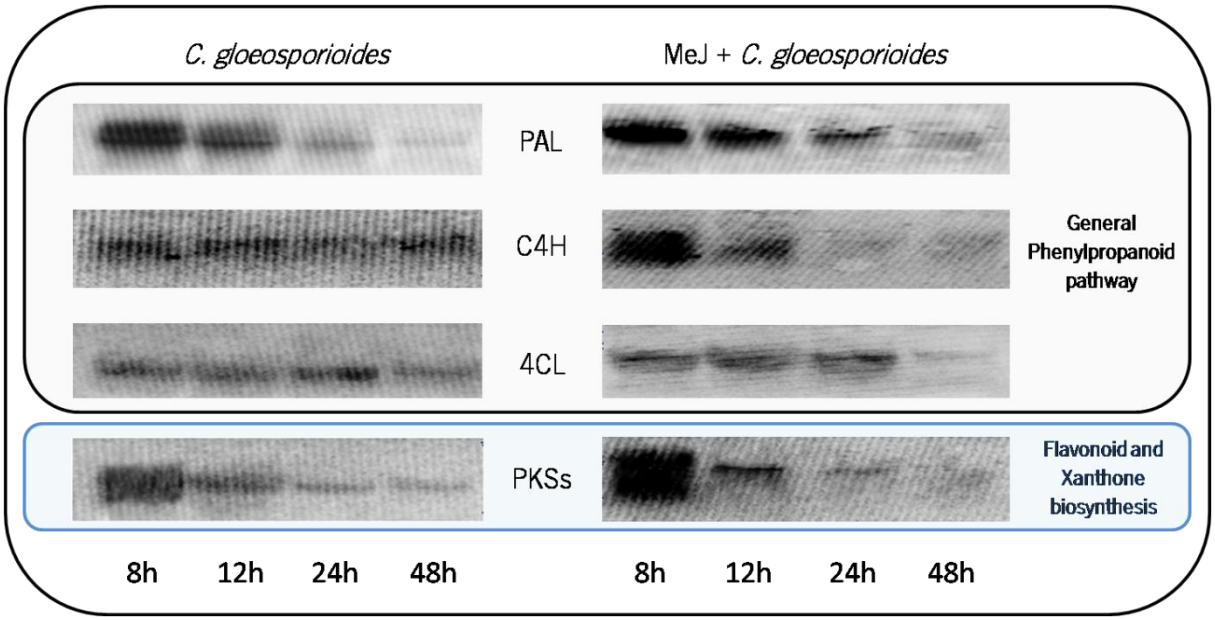


Figure 7.7: Gene expression of some key phenylpropanoid pathway enzymes found in *H. perforatum*, HPS accession. **PAL** – phenylalanine ammonia-lyase; **C4H** – Cinnamate 4-hydroxylase; **4-CL** – 4-coumarate:CoA ligase and the **PKS**s from *H.perforatum* (BPS – Benzophenone synthase and CHS – Chalcone synthase).

As depicted, and with the exception of *4cl*, expression of all other genes was higher in cells primed with MeJ (prior to *C. gloeosporioides* elicitation) than in cells treated with the pathogen only (Fig. 7.7). This result confirms what was previously found for phenolic compounds accumulation and PAL enzymatic activity, suggesting that upregulation of genes is necessary for an increased phenolic accumulation in elicited *H. perforatum* cells. Furthermore, and although no data exists for the shortest elicitation periods, the timing of the responses was similar for all upregulated genes, with the highest transcription levels found not later than 8 h after pathogen recognition, decreasing thereafter. This result comes in accordance with the general idea that organized metabolic channeling occurs in several

biosynthetic pathways, being a common feature in secondary metabolism [Hartmann, 2007]. Enzymes found in the beginning of the phenylpropanoid pathway (such as PAL), or at a "relatively distant" point (like CHS), may form multienzyme complexes. As referred in chapter 1.3, these complexes provide a better control on the synthesis of secondary metabolites, such as phenolic compounds. Therefore, it is reasonable to consider that a co-ordinated expression should be observed, for genes coding these closely related, phenylpropanoid pathway enzymes [Winkel, 2004].

As shown in figure 7.7, it is clear that *Pal* transcript levels reached a peak no later than 8 h after pathogen recognition, regardless of prior MeJ priming. This result confirms what was previously observed for PAL activity, using protein extracts from *C. gloeosporioides*-elicited cells (Fig. 7.5 – A). The highest levels of activity (9 h after elicitation) were preceded by upregulation or the corresponding gene, while the decrease in enzymatic activity was equally accompanied by lower levels of transcripts.

Transcription of *C4h*, on the other hand, showed no change upon pathogen elicitation. Nonetheless, priming of HPS cells with MeJ (24 h before *C. gloeosporioides* elicitation) was responsible for the upregulation of the gene, as observed for *Pal* and the *Pks*'s genes. C4H is the second enzyme found in the general phenylpropanoid pathway and catalyzes the conversion of cinnamate into *p*-coumarate (Fig. 7.4). Therefore, C4H competes for a substrate also required for SA biosynthesis [Schoch *et al.*, 2002]. Although the pathway for SA synthesis is not fully understood, studies with inhibitors of C4H, such as piperonylic acid (PIP) [Schalk *et al.*, 1998], have shown to promote the accumulation of SA in tobacco suspension cells [Chong *et al.*, 2001; Schoch *et al.*, 2002]. The close relation between C4H and the phytohormone SA makes it a strong candidate for regulation by jasmonates, therefore contributing for the cross-talk between the two systemic defense pathways. In fact, *C4h* expression is known to be upregulated by wounding [Mizutani *et al.*, 1998; Reymond *et al.*, 1998], a stress condition often associated with jasmonate signaling, as described in chapter 1.4.

4CL, the last enzyme of the general phenylpropanoid pathway, catalyzes the formation of CoA thiol esters of the distinct hydroxycinnamates (Figs. 1.12 and 7.4), therefore playing a pivotal role at a divergence point of phenylpropanoid metabolism, into several major branch pathways, such as flavonoids or xanthone biosynthesis. Associated with the relatively broad variety of substrates, this enzyme presents many distinct isoforms, with variable affinity to the hydroxycinnamates available. The divergence in 4CL isoforms (4 disctinct ones, at least), and its branching position in the general pathway, may be also associated with distinct physiological roles [Hu *et al.*, 1998]. In fact, it has been hypothesized that some 4CL isoforms should channel metabolism through flavonoids biosynthesis

(such as *A. thaliana* 4CL3) while others are associated with lignin production (such as *A. thaliana* 4CL1 and 2) [Ehlting *et al.*, 1999; Stuible *et al.*, 2000; Fofana *et al.*, 2005; Davies *et al.*, 2006]. As previously referred in table 7.1, PCR fragments for two distinct 4CL isoforms were obtained. *H. perforatum 4cl* "1" fragment was chosen for this work due to its theoretically closer association to lignin biosynthesis. As a result, and while *Pal* and *C4h* were strongly upregulated (upon one or both treatments that suspended cells were subjected), *4cl* transcript levels did not change significantly upon elicitor treatment (Fig. 7.7). Moreover, cells previously primed with MeJ showed only a minor increase in *4cl* expression, especially when compared to all other genes studied. These results resemble what was previously observed for lignin accumulation (Fig. 7.3), suggesting that, while precursor depletion could have played the central role in the decrease of flavonoids accumulation (as discussed below), the levels found for lignin production, on the other hand, could also be associated to the levels of *4cl* transcription observed. Additional work on the expression of other 4CL isoforms from *H. perforatum* should be carried out, in order to confirm these results.

Furthermore, in a parallel study, the remaining *4cl* fragment obtained (4cl "2") was used as a probe in the study of *Agrobacterium tumefaciens* interaction with *H. perforatum* suspended cells. In this work, an increased accumulation of *4cl* transcripts was observed, starting 4h after *A. tumefaciens* elicitation and reaching a peak after 12h, decreasing thereafter [Franklin *et al.*, 2008]. Although being a distinct experiment, the transcription levels of *Pal* followed a similar pattern of co-expression as described here, for *C. gloeosporioides* elicitation. Despite the resemblance of these experiments, further studies comparing the expression levels of both *4cl* gene fragments available, especially in suspension cultures treated only with MeJ, should be carried out in order to draw reasonably accurate conclusions about the role of 4CL isoforms in *H. perforatum* defense against *C. gloeosporioides* infection.

Along with the general phenylpropanoid pathway enzymes, we studied the gene expression of the PKSs responsible for the branching point between flavonoids biosynthetic pathway (CHS), as well as xanthone biosynthesis (BPS) in *H. perforatum*. Due to the relevance of xanthones in this work, and the close connection existing between BPS and CHS, their study will be discussed in more detail below.

7.4.4. Benzophenone synthase and Chalcone synthase: branch point in flavonoids/xanthone biosynthesis

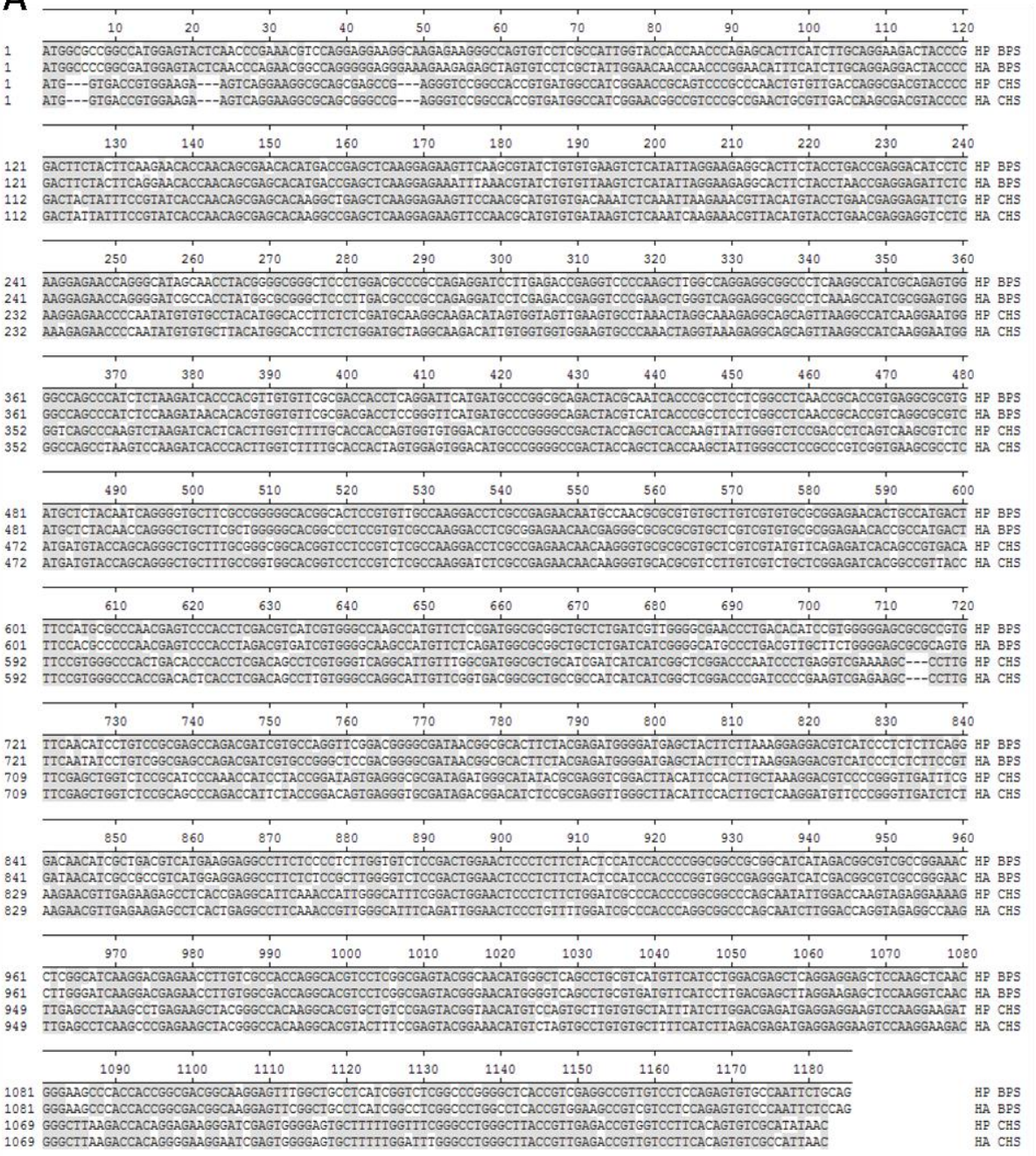
Both BPS and CHS are polyketide synthases (PKSs). These enzymes catalyze an early step in the biosynthesis of polyketides, an extremely broad group of compounds produced by bacteria, fungi, plants and animals. Divided into three classes, PKSs types I and II consist of many subunits and active sites. On the other hand, type III PKSs (which include BPS and CHS) are structurally simpler enzymes, catalyzing the chain elongation between a starter unit (usually an aromatic CoA) and acetyl units (from malonyl-CoA) [Tsai, 2004]. The remarkable functional diversity, found between these enzymes, derives from small differences in the active site, which greatly influence the selection of substrates as well as their further modifications [Abe *et al.*, 2005].

CHS was the first type III PKS enzyme to be discovered, being a ubiquitous enzyme in higher plants. This enzyme provides the first committed step in flavonoid biosynthesis, catalyzing the sequential decarboxylative addition of three acetate units (from malonyl-CoA) to a *p*-coumaroyl-CoA starter molecule, derived from the general phenylpropanoid pathway [Austin *et al.*, 2003], as described in chapter 1.3. BPS, on the other hand, is an enzyme associated to xanthone biosynthesis that employs the same mechanism of reaction as CHS but uses benzoyl-CoA, a minor substrate for other plant PKSs, as the preferred starter molecule [Benye *et al.*, 2003].

Although BPS and CHS differ in the starter substrates, these, in their turn, arise from the same earlier precursors in the general phenylpropanoid pathway [Austin *et al.*, 2003], as shown in figure 7.4. As a result, aside from flavonoid biosynthesis, intermediates and products from the general phenylpropanoid pathway are diverted to synthesize other classes of compounds, such as coumarins, lignin precursors [Austin *et al.*, 2003] or, as described in this work, xanthones.

Despite their functional diversity, the nucleotide sequence and aminoacidic composition of these two PKSs show increased similarity. Figure 7.8 shows the nucleotide and aminoacidic alignments of CHSs and BPSs from *H. perforatum* and a closely related species, *H. androsaemum*.

A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1 ATG---GTGACCGTGGAAGA---AGTCAGGAAGGCGCAGCGGGCCG---AGGGTCCGGCCACCGTGATGGCCATCGGAACGGCCGTCCCGCCGAACTGCGTTGACCAAGCGACGTACCCC HA CHS
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121 GACTTCTACTTCAGGAACACCAACAGCGAGCACATGACCGAGCTCAAGGAGAAATTTAAACGTATCTGTGTTAAGTCTCATATTAGGAAGAGGCACTTCTACCTAACCGAGGAGATTCTC HA BPS
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250 260 270 280 290 300 310 320 330 340 350 360
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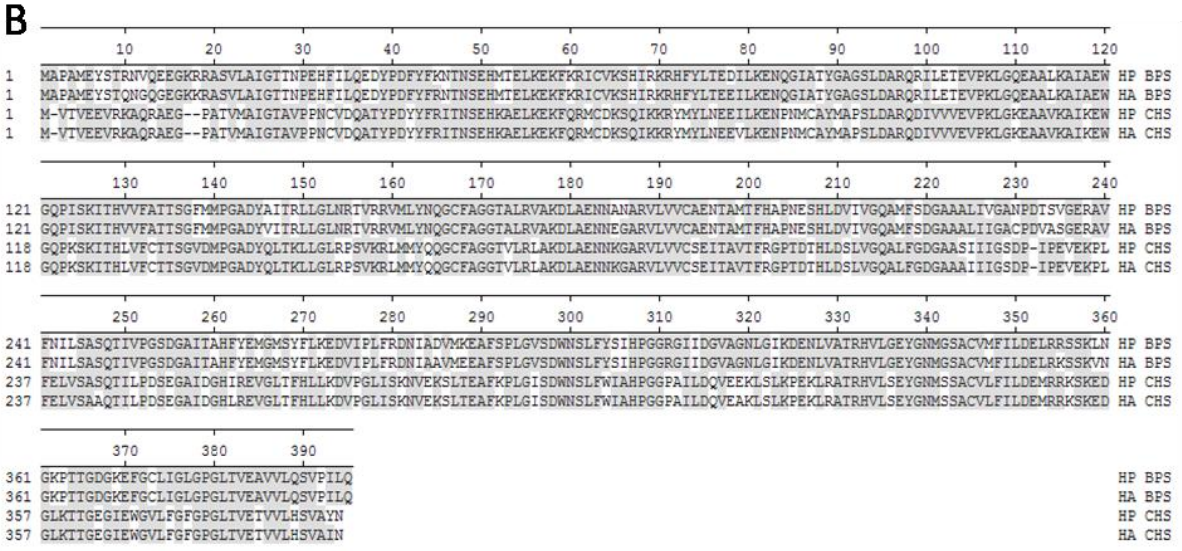

Figure 7.8: Two PKSs, BPS and CHS, from *H. perforatum* (HP) and *H. androsaemum* (HA). **(A)** Nucleotide and (**B**) deduced amino acid sequence alignments. The single letter code is used for amino acid depiction. Residues that match the consensus exactly are shown with solid gray shade. Coding sequences were analyzed using ClustalW and maximum-likelihood algorithms. GenBank accession numbers were the following: HP BPS (EF507429.1), HA BPS (AF352395.1), HP CHS (AF461105.1), HA CHS (AF315345.1).

As observed in figure 7.8, BPS genes from both *Hypericum* species are highly conserved, as expected. Analyzing in detail (Fig. 7.9), a 95% identity was observed between both BPSs while, for CHS genes, an equally high identity was found (98%). Furthermore, the similarity between these two PKS enzymes was also high, displaying values close to 60%.

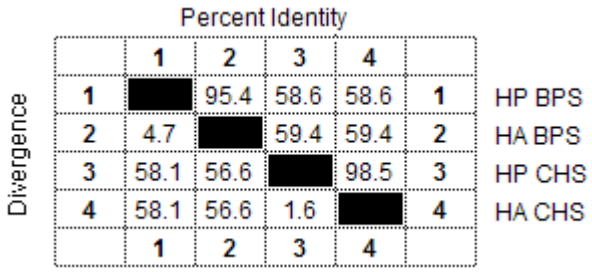

Figure 7.9: Nucleotide sequence identity found between the CHS and BPS present in two related *Hypericum* species, namely, *H. perforatum* (HP) and *H. androsaemum* (HA).

Due to the similarity observed between CHS and BPS nucleotide sequences (Fig. 7.8 – A and 7.9), it has not been possible to study gene expression of these enzymes individually by northern blotting. No radiolabeled probes could be specific enough to study one particular PKS gene without binding also to the mRNA from the other PKS. Furthermore, not only both CHS and BPS genes are alike in their coding sequences but also their mRNAs are similar in their sizes (data not shown). This fact

rendered it impossible to distinguish their specific bands in the membrane precisely and, therefore, study the changes in radiolabelling intensity for each enzyme individually.

Taken together, transcription of *Pks* genes in *H. perforatum* was upregulated by elicitor treatment, as previously observed for *Pal*. Moreover, HPS cultures pre-treated with MeJ showed higher levels of transcription then cultures elicited with *C. gloeosporioides* only (Fig. 7.7).

It is not clear whether the decrease in flavonols accumulation, observed upon *C. gloeosporioides* treatment, was due only to a depletion of a *pool* of precursors (common also to xanthones) or due to a decrease in *Chs* transcript levels. Nonetheless, the increased PKSs transcripts, accumulated upon pathogen elicitation, could be mostly due to an increase in *Bps* expression since a direct relation between xanthone accumulation and the level of PKSs genes expression could be found, as shown in figure 7.10. As observed, basal gene expression is present but barely detectable in *Helos* control cultures, as well as in those treated with MeJ only, resembling the lower levels of xanthones accumulated under these situations, in cell cultures. On the other hand, treatment with *C. gloeosporioides* biomass is responsible for the increase in gene transcription, starting 6h after elicitation. Priming with MeJ, prior to pathogen elicitation, led to the highest levels of transcripts available (Fig. 7.10), in accordance to what was observed for xanthone accumulation, under this condition.

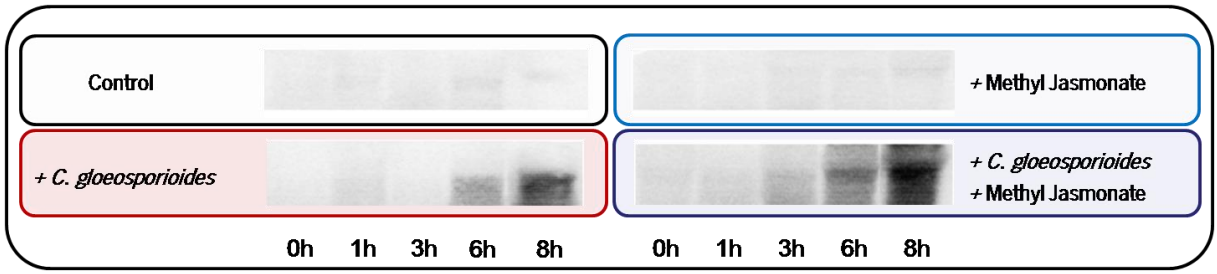

Figure 7.10: PKSs (BPS/CHS) gene expression in *H. perforatum Helos* cell suspension cultures.

Despite the similarities in the pattern of responses found between HPS and *Helos* cultures, PKSs gene expression was relatively higher in *Helos* than in HPS suspension cells. As observed from figure 7.11, when both suspended cells were exposed to the pathogen elicitor, *Helos* cell cultures showed a stronger (and perhaps faster) response in terms of gene expression. Therefore, an increased synthesis of BPS by *Helos* the cells could possibly account for the higher amounts of xanthones that accumulate it this *H. perforatum* accession.

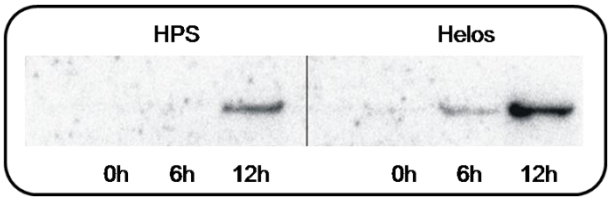

Figure 7.11: PKSs (BPS/CHS) expression in *H. perforatum* HPS and *Helos* cell suspension cultures, elicited with *C. gloeosporioides* only.

7.5 Discussion

The relevance of plant phenolic compounds as agents against *Colletotrichum* infection has been reported for some time [Conceição *et al.*, 2006b]. For instance, *C. lagenarium* is known to cause enhanced incorporation of cell wall-associated phenolics in cucumber [Dean *et al.*, 1987] and epicatechin is involved in the resistance of unripe avocado fruits to *C. gloeosporioides* [Wattad *et al.*, 1994], while a reduction in the phenolic content of leaves of water lilies has been associated to a higher disease severity, caused by *C. nymphae* [Vergeer *et al.*, 1997].

Among these phenolic responses against *Colletotrichum* infection is *H. perforatum* increased accumulation of xanthones [Conceição *et al.*, 2006]. As previously referred in chapter 6, cell suspension cultures from an anthracnose-susceptible accession (HPS) are known to differentially accumulate three major classes of soluble phenolic compounds (xanthones, flavonols and flavones) upon *C. gloeosporioides* elicitation. As discussed, these classes include several compounds that display antimicrobial activities [Conceição *et al.*, 2006]. *H. perforatum* cell suspension cultures from the less anthracnose-susceptible accession, *Helos*, follow a pattern of soluble phenolic compounds accumulation similar to HPS, upon the treatments applied. A general increase in xanthone accumulation occurs, starting 24h after *C. gloeosporioides* elicitation. Meanwhile, the depletion in the *pool* of precursors, shifted towards xanthone biosynthesis, may be responsible for the decrease in flavonoids accumulation, as previously discussed for HPS cultures [Conceição *et al.*, 2006 and 2006b]. Furthermore, a small but significant increase in xanthone accumulation, observed in cultures treated only with SA or MeJ, is another common aspect to these *H. perforatum* accessions, which may also be related to the minute decrease in flavonols accumulation observed. The effect of jasmonates in *H. perforatum* suspension cultures has also been evaluated in other labs. For instance, jasmonic acid was also responsible for a differential accumulation of phenolic compounds [Gadzovska *et al.*, 2007]. Namely, while flavonols and other phenolic compounds were found in higher amounts, anthocyanins,

for example, were negatively affected by jasmonate treatment. Despite some divergence in the results (increase/decrease in flavonols), both works support the idea of a metabolic channeling of phenylpropanoid precursors in *H. perforatum* suspension cells, according to the treatments applied.

As occurred with HPS, *Helos* newly synthesized xanthones have a non-polar nature, probably incrementing its efficacy as antimicrobial compounds [Conceição *et al.*, 2006]. The antimicrobial proprieties of *H. perforatum* xanthones were recently studied in our lab. The whole soluble phenolic extract, as well as one major xanthone present in *Helos* suspension cells, paxanthone, proved to effectively inhibit bacterial growth [Franklin *et al.*, 2007 and 2008]. Although effective against bacteria, the efficacy against *C. gloeosporioides* is still not resolved. Nonetheless, extracts from *H. perforatum* plants have proven to inhibit *C. gloeosporioides* growth *in vitro*, most likely due to their content in hypericin and/or hyperforin [Sirvent *et al.*, 2002].

Despite the general similarity, the level of response is clearly distinct between the two *H. perforatum* accessions. HPS and *Helos* cells accumulate similar amounts of xanthones in normal growth conditions. Nonetheless, upon pathogen recognition, the xanthone levels found in *Helos* cultures raise to values 6x higher than observed in HPS cells, at the peak of their xanthone accumulation. The increased accumulation of xanthones in *Helos* is corroborated by the higher levels of *Pks* transcription, observed upon *C. gloeosporioides* elicitation of both *H. perforatum* accessions, as shown in figure 7.11. Additionally, while the levels of xanthones produced in HPS could be incremented by SA or MeJ pre-treatment (prior to elicitor treatment), *Helos* suspension cultures did not respond differentially to phytohormonal priming. Xanthones accumulated at the same levels as in cultures elicited only with *C. gloeosporioides*. This result could mean that *Helos* not only accumulates more xanthones than HPS, but also do it without any need for externally applied priming compounds. In other words, pathogen recognition could be enough to trigger the maximum phenolic response from *Helos*, readily depleting the *pool* of precursors previously referred.

Differences in soluble phenolic accumulation between HPS and *Helos* are not solely related to xanthones. Flavonoids accumulation in both accessions is clearly distinct. *Helos* control cells accumulate approximately 10x more flavonols than the corresponding HPS cultures. Furthermore, upon the several treatments applied, their values were still higher (after MeJ or SA treatment) or lasted longer before becoming undetectable (after *C. gloeosporioides* treatment). Another significant difference observed between these two *H. perforatum* accessions was the accumulation of flavones. While flavones were only detectable in HPS after MeJ treatment, these compounds were present, in *Helos*, at

normal growth conditions. The overall difference in soluble phenolic compounds accumulation, found between the two *H. perforatum* accessions, is summarized in figure 7.12. For several decades, the high phenolic contents of given plants have been correlated to their increased resistance to infection against many pathogens [Sztejnberg *et al.*, 1983; Luzzatto *et al.*, 2007], as previously described, although some exceptions exist [Ranger *et al.*, 2007]. Therefore, it seems plausible to assume that the significantly higher amount of soluble phenolic compounds, accumulated by *Helos*, could be related to an increased tolerance of this *H. perforatum* accession to *C. gloeosporioides* infection *in vivo*. Further studies on the plant level, regarding the quantity and antimicrobial properties of both HPS and *Helos* soluble phenolics should be carried out, in order to complement this hypothesis.

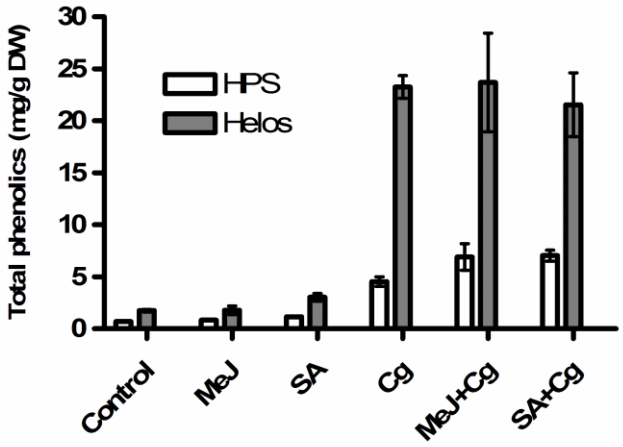

Figure 7.12: Accumulation of soluble phenolic compounds in cell suspension cultures of *H. perforatum* HPS and *Helos*, at the 9th day of growth. Values include xanthones and flavonoids produced.

Along with soluble phenolics, our study also focused on the accumulation of lignin, another class of metabolites derived from the phenylpropanoid pathway. Although pathogen recognition is often associated to increased lignin accumulation in plants, a significant reduction in lignin levels was observed upon *C. gloeosporioides* elicitation of HPS cell cultures. As previously predicted for the decrease in flavonoids accumulation, it could be the case that a depletion of phenolic compound precursors, channeled for xanthone biosynthesis, is directly responsible for this result. References exist for the connection between lignin pathway and other phenylpropanoid pathway branches. Per instance, suppression of the lignin pathway in *A. thaliana* led to the redirection of the metabolic flux through flavonoids biosynthesis [Besseau *et al.*, 2007], while inhibition of 4CL led to the accumulation of benzoic acids in *Vanilla planifolia* suspension cells [Funk *et al.*, 1990]. Although this hypothesis could be true (at first) in the case of *H. perforatum*, a similar study with *P. pinaster* suspension cells showed

no increase in accumulation of soluble phenolics, as well as lignin, upon *B. cinerea* elicitation. Therefore, other mechanisms, such as gene expression, are most likely responsible for this result. Early expression studies have shown that transcription of a *4cl* gene, coding for a 4CL isoform associated to lignin biosynthesis, was not upregulated by pathogen elicitation. Plant cell penetration is known to be a typical infection mechanism found on biotrophic fungi, being uncommon in necrotrophs [Lucas, 1998]. Concomitantly, cell-wall reinforcement is usually found to occur against biotrophic pathogens. Considering that *C. gloeosporioides* nutrition model is quite complex, recurring to both strategies at distinct situations (as described in chapter 1.2), it is not easy to predict how a specific host will respond to the presence of this particular pathogen. Nonetheless, from Fig. 7.3 we can predict that both *H. perforatum* accessions showed a typical response against necrotrophic pathogens, upon *C. gloeosporioides* elicitation. Further support comes from the fact that *H. perforatum* cells were responsive to MeJ but not to SA. It is known that, in general, SA is effective against biotrophs while jasmonate signaling is generally effective against necrotrophic pathogens [Glazebrook, 2005].

The experimental sets used along the work, mimicking distinct plant-microbe interaction situations, may provide other conclusions/hypothesis as well. The initial contact with the invading pathogen was responsible for the metabolic channeling towards the production of active phytoalexins (namely, xanthones) instead of being used in the reinforcement of passive, physical barriers, by lignin deposition. It's still not clear whether soluble phenolics were produced for their antimicrobial properties. Nonetheless, it seems that *H. perforatum* tissues in the close vicinities of the attempted infection (mimicked by cells elicited with *C. gloeosporioides* only) tend to develop a more *definite* and *drastic* approach against contamination while distant parts of the plant, signalized for the spreading pathogen (mimicked by MeJ-treated cultures), shifted its phenolic metabolism towards a *less intense* physical barrier reinforcement.

The results observed for the accumulation of phenolic compounds were further complemented by the study of PAL activity. This parameter shows great resemblance to what was previously found for phenolic compounds accumulation, in both *H. perforatum* cell suspension cultures. An increase in PAL activity was observed when cells accumulated either soluble phenolics or lignin. Additionally, a significant difference was observed between both *H. perforatum* accessions, as PAL activity in *Helos* was predominantly higher. In general, these results support the idea of PAL as being a key enzyme in phenylpropanoid metabolism. Another important aspect in PAL activity is its tight regulation, in order to control the costly production of secondary metabolites. This idea is further supported by the close

relation existing between PAL activity and regulation of transcription levels of the corresponding *Pal* genes. Despite the tight regulation, it seems that the increase in PAL activity is a common feature to a broad range of distinct stresses, both biotic and abiotic. Root cultures from *Lotus corniculatus*, for instance, displayed a peak in PAL activity 8h after treatment with the abiotic stress elicitor glutathione (GSH), with values returning to basal levels, 24h after treatment [Robbins *et al.*, 1991], much like what was observed in this study.

Regardless of the stress origin, PAL activity is known to be closely associated to other phenylpropanoid pathway enzymes, developing multienzyme complexes. These complexes are thought to optimize the biosynthesis of compounds based on substrates or intermediates that may occur in low concentrations, or that may be potentially dangerous to the plant [Winkel, 2004]. Therefore, the expression of their corresponding genes should also be directly correlated and fine-tuned, thus developing an optimal response against a specific stress. In fact, expression of some phenylpropanoid pathway enzymes studied here followed a closely related, co-expression pattern, supporting the idea that these enzymes act "as a whole" (and not independently) towards the production of distinct phenolic compounds. Nonetheless, changes in gene expression can still occur, playing a pivotal role in the regulation of biosynthetic activity of some branch pathways, as observed in the decrease of lignin production and *4cl* expression, upon *C. gloeosporioides* elicitation.

Chapter 8

Final Considerations

8. Final Considerations and Future Perspectives

8.1. *H. perforatum* defense mechanisms: Setting up an *in vitro* model

Despite their sessile nature, plants thrive virtually in all ecosystems, adapted to extreme environmental conditions and corresponding abiotic stresses. Along with these challenges, plants also have to cope with biotic stresses from a multitude of highly diverse pathogens and herbivores. Therefore, their ubiquitous nature is only possible due to an intricate and effective defense system, able to repel most organisms, continuously attempting to invade them. From their co-evolution with pathogens, plants developed three general defense approaches. Pre-formed, constitutive defenses act as the first barriers, protecting plants from most surrounding organisms. Other defenses are induced only upon threat recognition, acting locally and/or systemically. Despite the distinction, all mechanisms seem to act in consonance, developing a "tailor-made" response to a particular stress. Along the work presented in this thesis, some of these basic defense mechanisms, developed by *H. perforatum* against infection by the fungus *C. gloeosporioides*, were evaluated.

For this purpose, an elicitation model composed of *H. perforatum* cell suspension batch cultures from two accessions, distinct in their susceptibility to anthracnose infection *in vivo*, was established and characterized, as described in chapter 3. Relevant growth and survival parameters, including consumption of some major nutrients, were considered. Both cell suspension cultures displayed somewhat similar values for these parameters, under normal growth conditions, enabling easier assessment and comparison of responses to further elicitation treatments. In both tissue cultures, an initial lag period preceded the exponential growth phase that occurred until sugar depletion. Sugar was a major nutrient limiting growth, unlike what was observed for phosphate, ammonium or nitrate. After sugar depletion, a stationary stage was observed and followed by a gradual increase in cell death. These growth stages, observed on both *H. perforatum* cultures, resembled what is usually reported for cell suspension batch cultures from other plants, validating both *in vitro* cultures for the following studies on *H. perforatum* defense mechanisms (though keeping always in mind the "artificial" nature of this biological system).

Following culture establishment and characterization, the elicitation model was designed, taking advantage that suspended cells usually respond in consonance. This response is often associated to the elicited cells only, unlike what is seen in *all-plant* models. In these models, differential responses existing between elicited cells and those neighboring the infection site are often disregarded [Azevedo, 2005]. Furthermore, our model included not only elicitation by the pathogen but also by two of the most relevant phytohormones associated to systemic plant defense against biotic stress, namely, methyl-jasmonate and salicylic acid. As previously described in chapter 4, these hormones were used alone or prior to *C. gloeosporioides* elicitation, contributing for the understanding of systemic signaling that occurs between locally infected and distant *H. perforatum* cells.

8.2. *C. gloeosporioides* elicitation

Elicitation of *H. perforatum* suspension cells with a preparation from the pathogen *C. gloeosporioides* was responsible for several physiological responses, as summarized in figure 8.1. First of all, an increase in sugar consumption (per viable cell) was observed (chapter 4). The additional sugar consumed, not directly associated to cell growth and division, may have been channeled to other biological processes. Protein synthesis, production and accumulation of diverse metabolites or other energy-related expenses are known to require sugar as a carbon and energy source. In *H. perforatum*

suspension cultures, an increase in the synthesis of some secondary metabolites, namely xanthones, was observed upon pathogen recognition (chapters 6 and 7). Although confirmation studies are still needed, this could be an example of the connection between primary and secondary metabolisms in *H. perforatum* cells. Phosphate levels in the medium even increased upon treatment, most likely due to the decrease in cell viability and concomitant cell disruption, observed in this condition. While sugar consumption increased, the intake of nitrogen sources (nitrate + ammonium), was negatively affected by pathogen elicitation. As referred in chapter 4, a decrease in nitrate uptake, as well as its efflux, are common features found in cells during programmed cell death [Wendehenne *et al.*, 2002], corroborating the results for hypersensitive response showed on chapter 5. Furthermore, a reduction in productivity was observed. As previously referred for sugar consumption, it is possible that a shift of nitrogen sources from growth to defense and/or storage could have occurred upon pathogen elicitation.

The decrease in cell viability and biomass accumulation (chapter 4) was in accordance with the increased lipid peroxidation, DNA cleavage and cell death, due to hypersensitive response (HR), reported in chapter 5. Upon *C. gloeosporioides* elicitation, a double oxidative burst, required for an effective HR [Yakimova *et al.*, 2005], was observed on both *H. perforatum* accessions. Curiously, *Helos* cells displayed significantly higher levels of internal ROS, which could account for the increased lipid peroxidation levels and cell death observed in this *H. perforatum* accession, when compared to HPS cells. On both accessions, the increased accumulation of ROS, upon *C. gloeosporioides* elicitation, was associated to a decrease in superoxide dismutase (SOD) and catalase (CAT) enzymatic activities, observed 24 h after treatment. It is clear that the coordination of these ROS-scavenging mechanisms is also involved in the oxidative burst development, by ensuring the control of ROS levels [Hancock *et al.*, 2002]. In fact, it has been suggested that hypersensitive response can only occur due to the inhibition of ROS-scavenging mechanisms, therefore allowing ROS to build up to the levels required for programmed cell death development [Apel *et al.*, 2004]. While a decrease in some enzymatic ROS-scavenging means was observed, methanolic extracts obtained from pathogen-elicited *H. perforatum* cells displayed significantly higher antiradicalar properties (up to 2 fold in *Helos*) than extracts from control cultures. As referred in chapter 5, xanthones isolated from elicited *H. perforatum* cells (such as paxanthone) have proven to display an increased antioxidant potential [Franklin *et al.*, 2008b]. Despite the *boost* in xanthone production observed upon elicitation, the resulting increase in non-enzymatic antioxidant properties has proven to be insufficient to control the overwhelming production of ROS,

responsible for the increased cell death observed in pathogen-elicited cell cultures from both *H. perforatum* accessions.

As discussed in chapters 6 and 7, the *boost* in xanthone biosynthesis, more evident in *Helos* suspension cells, could be associated to a role in defense due to their known antimicrobial properties. Acting as phytoalexins, these xanthones could prevent the spread of the fungal disease within the plant. The possible implications of *H. perforatum* xanthones as anti-*C. gloeosporioides* compounds will be discussed below, in chapter 8.6. The burst in xanthone production could also be responsible for the decrease in accumulation of other phenolic compounds, namely, flavonoids and lignin (chapters 6 and 7). While up-regulation of genes coding for the PKSs (CHS and/or BPS) was observed upon elicitation, the decrease in the level of flavonoids could be due to the depletion of a common *pool* of precursors, shared between xanthones and flavonoids biosynthetic pathways or due to down-regulation of *Chs* transcription, as observed for the reduction of lignin levels in the cell wall, where no increase in gene expression (*4cl*) was observed, as previously discussed in chapter 7.

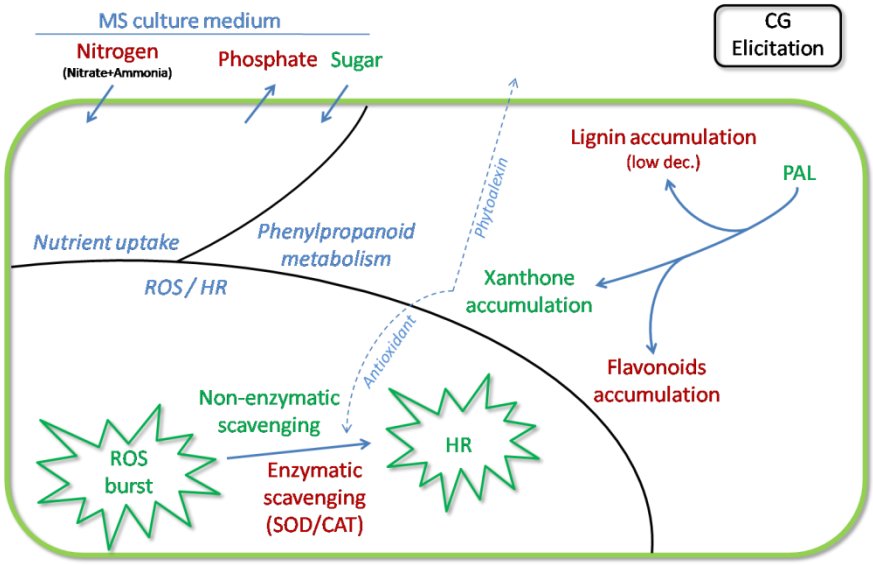

Figure 8.1: Schematic representation of the main physiological changes observed in *H. perforatum* suspension cells treated with an elicitor preparation from *C. gloeosporioides* (CG). Green color represents increased responses while red color represents decreased or absent responses. Responses similar to control conditions are shown in black.

8.3. The influence of signaling molecules: SA and MeJ

The elicitor preparation was responsible for several physiological changes in *H. perforatum* suspension cells, common to many other plant models. On the other hand, treatment of cell cultures

with SA or MeJ, key phytohormones associated to Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) in plants, did not result in such marked responses. Nonetheless, the most relevant results are summarized below, in figure 8.2.

Treatment of *H. perforatum* cell cultures with MeJ, at a final concentration of 100 µM, was responsible for a significant (yet transient) increase is sugar consumption, while no changes were observed for consumption of other major nutrients (chapter 4). In accordance with the previously hypothesized connection between sugar consumption and xanthone biosynthesis (chapters 4 and 8.2), the increased carbon uptake observed upon MeJ treatment could be associated to the concomitant increase in lignin biosynthesis (chapter 7). Further studies on sugar transport and metabolism should be carried out before taking definite conclusions on this subject, as discussed below, in chapter 8.7.

As previously referred, responses developed by suspension cell cultures treated only with the phytohormones could resemble those found in plant tissues distant from the infection site, but *warned* for an invasion attempt (chapter 4). It is curious that, only upon MeJ signaling, a substantial increase in lignin deposition in the cell wall was observed, while elicitation with a pathogen preparation showed absent or opposite results. These differential responses could be associated to distinct plant strategies, according to the *proximity* of the invader, as discussed in chapter 7. Furthermore, the increase in lignin deposition was accompanied by a small raise in xanthone accumulation and a decline in flavonoids accumulation, bringing more evidence about the extensively reported channeling/shifting of phenylpropanoid precursors and the fine-tuning of plant responses upon systemic signaling [Liang *et al.*, 2006; Hendrawati *et al.*, 2006].

Besides its effects in phenylpropanoid metabolism, MeJ was also responsible for a small increase in ROS accumulation within *H. perforatum* cells. Nonetheless this was a transient, single burst response. Working in parallel, some ROS-scavenging mechanisms had also improved. Non-enzymatic scavenging means improved marginally, due to the small accumulation of xanthones, while a significant increase in SOD activity was also observed (chapter 5). Despite the transient changes in ROS homeostasis, no HR reaction was triggered in *H. perforatum* cells.

As observed for MeJ treatment, *H. perforatum* suspension cell cultures treated with SA, at a final concentration of 25 µM, developed rather similar responses, for most parameters evaluated (Fig. 8.2). In fact, the only tangible differences between SA and MeJ treatments occurred in sugar consumption and lignin biosynthesis, where the absence of significant changes were observed, when compared to control conditions. Accumulation of xanthones and flavonoids, as well as ROS production

and scavenging, followed patterns similar to those found in MeJ-treated cultures. As discussed in chapters 5 and 6, a clear dichotomy between the two systemic signaling pathways is not always observed and increasing reports confirm that, per instance, SA treatment may lead to the accumulation JA in the cells [Salzman *et al.*, 2005]. Furthermore, several variables, such as the plant species or concentration of the phytohormones used, may influence the plant metabolism, culminating in a concerted defensive response [Asai *et al.*, 2000; Mur *et al.*, 2006; Liu *et al.*, 2008].

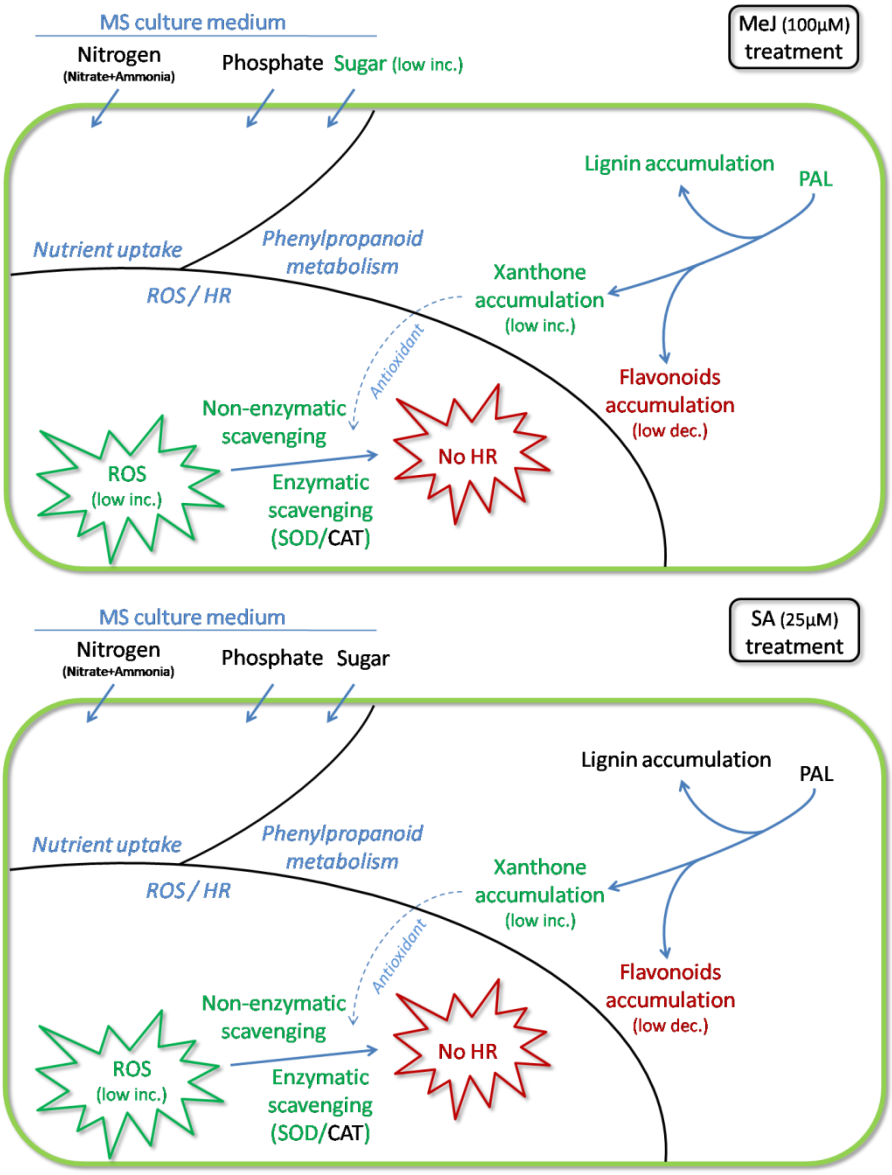

Figure 8.2: Schematic representation of the main physiological changes observed in *H. perforatum* suspension cells treated with MeJ (100 µM) or SA (25 µM). Green color represents increased responses while red color represents decreased or absent responses. Responses similar to control conditions are shown in black.

8.4. Priming of *H. perforatum* cells for defense: Effects of MeJ or SA pre-treatment, prior to elicitation

While treatment of *H. perforatum* suspension cells only with the phytohormones didn't confer marked, definite responses for most parameters studied, it doesn't mean that some physiological changes, aiming for an improved defense, couldn't be taking place within the plant cell. In fact, as observed for xanthone production in HPS, pre-treatment with MeJ, prior to *C. gloeosporioides* elicitation, led to an improved *boost* in accumulation, when compared to cells treated with the pathogen elicitor only (chapter 6). Concomitantly, sugar consumption had also increased on elicited cells pre-treated with the phytohormones (chapter 4). Finally, while no significant changes were observed for ROS accumulation and scavenging, a tendency for increased cell death was observed in elicited cells, pre-treated with SA and MeJ, as reported in chapters 4 and 5. Therefore, while treatment with the phytohormones didn't render noticeable effects in *H. perforatum* metabolism, these compounds could be responsible for signaling several defensive mechanisms against the upcoming pathogen, resulting in improved response, upon recognition of the threat. One example of this signaling cascade in *H. perforatum* cells could come from H_2O_2 accumulation. As referred in chapter 5, SOD activity increased upon phytohormonal treatment while CAT activity was not significantly affected. These responses could have possibly accounted, during the following hours, for the increase in extracellular H_2O_2 accumulation, observed when cultures were then elicited with the pathogen preparation. Considering the hormonal nature of H_2O_2 in plants [Dempsey *et al.*, 1999], an amplification of plant defense signals could be taking place, inducing an improved resistance. Further studies of H_2O_2 metabolism in *H. perforatum* should help clarifiying this hypothesis.

It has been suggested that ISR is associated to resistance against necrotrophs while SAR provides resistance to biotrophs [Thomma *et al.*, 1998; Ton *et al.*, 2002]. *H. perforatum* suspension cells responded similarly after pathogen elicitation, regardless the systemic signaling pathway induced. Therefore, through the experiments shown in this thesis, it is not possible to point the most relevant systemic resistance pathway for this plant-pathogen model, especially considering the dual nature of *C. gloeosporioides*, a fungus that displays both nutrient acquisition models. Furthermore, increased evidence suggests an intense cross-talking and coordination exists between both systemic defense pathways, in order to *organize* and prepare the plant metabolism against a wide range of potential biotic and abiotic stresses [van Wees *et al.*, 2000; Spoel *et al.*, 2003; Salzman *et al.*, 2005].

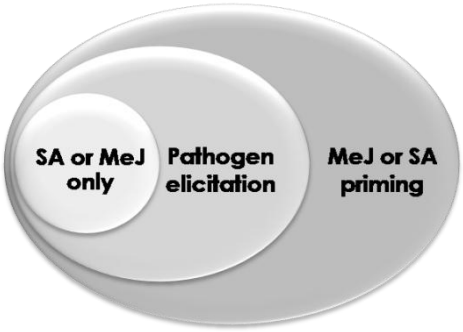

Figure 8.3: Priming of *H. perforatum* suspension cells with MeJ (100 µM) and SA (25 µM) prior to pathogen elicitation may lead to some improved responses.

8.5. *H. perforatum* suspension cells: Drawbacks of their use in secondary metabolism studies

While some classes of secondary metabolites from *H. perforatum* were present and differentially accumulated upon *C. gloeosporioides* elicitation, at least one important group of secondary metabolites found in *Hypericum* species, the naphtodianthrones (which includes compounds like hypericin, described in chapter 1.3), was not present in cell cultures from both accessions studied, as previously discussed [Conceição *et al.*, 2006]. Despite their absence, an increase in hypericin accumulation was found in other *H. perforatum* suspension cell cultures upon, per instance, jasmonate treatment [Gadzovska *et al.*, 2007]. Most authors support the idea that undifferentiated tissues, such as suspension cells, cannot accumulate hypericins [Pasqua *et al.*, 2003]. Nonetheless, other authors suggest that cell aggregation, as well as certain growth conditions, play an important role in the production of some classes of secondary metabolites [Bais *et al.*, 2002] by promoting a certain degree of differentiation to the previously undifferentiated suspension cultures [Gadzovska *et al.*, 2005]. Despite the ongoing debate about tissue culture differentiation, it is clear that caution should be taken before generalizing results obtained from suspension cell cultures. Although technically convenient, some studies may be compromised by its use. In fact, hypericins were found to accumulate at high amounts in pathogen-elicited *H. perforatum* plants and, therefore, could also play a role in plant defense [Sirvent *et al.*, 2002; Sirvent *et al.*, 2003; Çirak *et al.*, 2005]. For those reasons, the use of plants *in vivo* should be regarded as the following, definite step in the study of *H. perforatum* secondary metabolism and its relevance in *C. gloeosporioides* infection.

8.6. *H. perforatum* accessions vs. *C. gloeosporioides*

While disadvantages may be pointed from the use of undifferentiated tissue cultures, some results obtained with this *tool* may give early insights about the mechanisms underlying the reduced susceptibility observed in plants from *H. perforatum Helos* accession *in vivo*. While suspension cultures from both *H. perforatum* accessions were able to recognize the pathogen, developing a resistance response, *Helos* cells showed higher levels of ROS during the double oxidative burst. The increased production of ROS may be associated to the stronger HR developed by these cells. Accordingly, *Helos* cultures also accumulated greater amounts of xanthones than HPS upon elicitation. The *boost* in xanthone biosynthesis was responsible for a contradictory (yet ineffective) increase in antiradicalar properties from methanolic extracts, as previously referred in chapter 5. Nonetheless, xanthones are known to play a dual function in defense against biotic stress, acting also as antimicrobial compounds [Franklin *et al.*, 2008]. At first, the non-polar nature of the newly-synthesized xanthones [Conceição *et al.*, 2006] sounded as promising anti-*C. gloeosporioides* compounds. However, fungal growth was only slightly reduced by crude methanolic extracts (unpublished data). On the other hand, both crude methanolic extract and isolated xanthones (namely, paxanthone) displayed antibacterial activity against *A. tumefaciens* and *A. rhizogenes* [Franklin *et al.*, 2008]. While keeping in mind the *in vitro* nature of our experimental model, it seems that, whereas *H. perforatum* defense mechanisms were effective against pathogens like *Agrobacterium*, providing antimicrobial compounds as well as protecting plant cells from lethal ROS damage, the same may not occur for the interaction with *C. gloeosporioides*, resulting in *H. perforatum* infection, as usually seen *in vivo*. In fact, methanolic extracts from *H. perforatum* plants have also proven to be poorly effective against *C. gloeosporioides* spore germination, reducing it by 15%, approximately [Silva *et al.*, 2008], although displaying antimicrobial activity for other fungi [Lu *et al.*, 2002]. On the other hand, some authors suggest that hypericins and/or hyperforins may stall *C. gloeosporioides* growth [Sirvent *et al.*, 2002]. Further studies on *C. gloeosporioides* growth and spore germination should be carried in order to clarify this situation.

Therefore, in *H. perforatum* cultures, the basal (or non-host type II) resistance was able to prevent bacterial infection (incompatible interaction), without damaging the plant cells [Franklin *et al.*, 2008]. *C. gloeosporioides*, on the other hand, is known to be virulent to *H. perforatum in vivo*, while responsible for increased cell death by HR, as observed *in vitro* (host resistance). Thus, *H. perforatum* and *C. gloeosporioides* also appear to display an incompatible interaction. Upon elicitation with the pathogen (*C. gloeosporioides* biomass) or non-pathogenic organisms (*Agrobacterium*), *H. perforatum*

cells displayed qualitatively similar responses. As referred in chapter 1.4, distinction between host and non-host type II resistance is rather difficult since both may use similar defense mechanisms, such as HR and phytoalexins synthesis [Heath, 2001; Nimchuk *et al.*, 2003; Peart *et al.*, 2002]. *H. perforatum* responses triggered by *C. gloeosporioides* elicitation were stronger, leading not only to higher xanthone accumulation but eventually culminating in cell death by HR. Although host resistance is known to be faster and/or more intense than non-host [Mysore *et al.*, 2004], we can't assume that the difference in response intensity found between *Agrobacterium* and *Colletotrichum* is due to these distinct mechanisms since some parameters, like the inoculum concentration, were found to interfere with the intensity of the response [Franklin *et al.*, 2008]. Regardless the resistance model used by the plant, and considering that *C. gloeosporioides* may use both biotrophic and necrotrophic nutrition models, the development of HR by *H. perforatum* could indeed be more profitable for the pathogen than for the plant, by providing clear access to nutrients from the death plant cells, therefore explaining the success of *C. gloeosporioides* as a pathogenic fungus, able to infect a broad range of hosts.

8.7. Future Perspectives

Considering the results presented and discussed in this thesis, some suggestions can be outlined, in order to continue this research project, aiming to a better understanding on *H. perforatum* defensive responses against *C. gloeosporioides*.

In general, the most promising results presented here should be confirmed *in vivo*, on fully differentiated *H. perforatum* plants, whenever possible. As previously referred, cell suspension cultures can be seen as an interesting *tool* for basic research, due to its easy manipulation and control, but also because these cultures usually display concerted responses to a given stimulus. Nonetheless, it is clear that some cell culture responses may fail to resemble those found *in vivo*. Furthermore, while the experiments regarding systemic resistance pathways described in this thesis were performed with exogenously applied phytohormones, the presence/relevance of endogenous jasmonates and salicylic acid should also be considered. Therefore, it could be interesting to access the levels of these phytohormones in *H. perforatum* upon pathogen recognition, as well as the effects of defense signaling pathway inhibition.

Other research lines could be considered, regarding the results shown in each chapter, individually:

As described in chapter 4, an increase in sugar consumption was observed upon pathogen elicitation. It could be interesting to confirm this result by studying both sugar uptake and gene expression of the corresponding transporters, when suspension cells are faced with the pathogen preparation. Regarding this research line, a cDNA sequence coding for one monosaccharide transporter (MST) from *H. perforatum* was already obtained. Furthermore, tracking sugar metabolism using, for example, radiolabeled glucose, could be employed in order to find out whether the sugar uptaken upon *C. gloeosporioides* elicitation is channeled to xanthone/lignin biosynthesis or used in other physiological processes.

The results described in chapter 5 could be complemented by gene expression analysis of enzymes associated to ROS scavenging, such as catalases, superoxide dismutases or peroxidases. For this purpose, the cDNA sequence coding for a catalase from *H. perforatum* was already obtained. Further in-gel activity assays, after Native PAGE of *H. perforatum* protein extracts, could also complement the results obtained by accessing the presence and/or activity of distinct enzyme isoforms, when available. Activities of catalase and superoxide dismutase isoforms, for example, are known to be differentially regulated according to the stimulus the plant is subjected [Frugoli *et al.*, 1996; Azevedo 2005]. Besides ROS-scavenging, ROS production could also be complemented by studies on activity/expression of key enzymes, upon pathogen elicitation. For instance, the role of NADPH oxidase in the development of the oxidative bursts, as well as the roles of MAPK or calcium channels in signal transduction could also be evaluated by using specific inhibitors like DPI (NADPH oxidases inhibitor), staurosporine (MAPK inhibitor) or $LaCl_3$ (calcium channel inhibitor).

The work presented in chapters 6 and 7 could be further supported by activity assays for other key phenylpropanoid pathway enzymes, such as BPS, CHS or 4-CL. Moreover, new expression studies, focused on enzymes directly associated to flavonol, flavone and lignin biosynthesis should be performed. This approach could help understanding whether gene regulation plays a role in the decrease in accumulation of these phenolic compounds (observed upon pathogen recognition) or if the depletion in the *pool* of precursors, channeled to xanthone synthesis, is the only mechanism responsible for this result. It could also be interesting to analyze the monomeric composition of lignin. Besides the differences observed in overall lignin accumulation upon *C. gloeosporioides* elicitation or MeJ treatment, the ratio between the monomeric units that compose the lignin polymers are known to play a significant role in cell wall strengthening, by regulating its crosslinking level. Another research line can be suggested, associated to new 4-CL isoforms. It could be interesting to search and characterize,

in *H. perforatum*, the 4-CL isoform associated to benzoyl-CoA synthesis, a main precursor in xanthone biosynthesis. Furthermore, the differential expression of all isoforms should be accessed, for the conditions studied, in order to better understand the channeling of phenylpropanoid precursors upon biotic stress and systemic defense signaling.

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