

UNIVERSIDADE FEDERAL DE SANTA CATARINA
MATEUS BRUSCO DE FREITAS

**CARACTERIZAÇÃO QUÍMICA E ATIVIDADE DE ULVANAS NA
INDUÇÃO DE RESPOSTAS OXIDATIVAS ASSOCIADAS AO
CONTROLE DE *Alternaria brassicicola* E *Colletotrichum higginsianum*
EM *Arabidopsis thaliana***

Florianópolis
2014

MATEUS BRUSCO DE FREITAS

**CARACTERIZAÇÃO QUÍMICA E ATIVIDADE DE ULVANAS NA
INDUÇÃO DE RESPOSTAS OXIDATIVAS ASSOCIADAS AO
CONTROLE DE *Alternaria brassicicola* E *Colletotrichum higginsianum*
EM *Arabidopsis thaliana***

Tese submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do grau de Doutor em Ciências, área de concentração de Recursos Genéticos Vegetais. Orientador: Prof. Dr. Marciel J. Stadnik.

Florianópolis
2014

Ficha de identificação da obra elaborada pelo autor,
através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

de Freitas, Mateus Brusco

Caracterização química e atividade de ulvanas na indução de respostas oxidativas associadas ao controle de *Alternaria brassicicola* e *Colletotrichum higginsianum* em *Arabidopsis thaliana* / Mateus Brusco de Freitas ; orientador, Marciel João Stadnik - Florianópolis, SC, 2014. 109 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Agrárias. Programa de Pós-Graduação em Recursos Genéticos Vegetais.

Inclui referências

1. Recursos Genéticos Vegetais. 2. Indução de resistência de plantas. 3. Modificações químicas em polissacarídeos algais. 4. Estresse oxidativo. I. Stadnik, Marciel João. II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Recursos Genéticos Vegetais. III. Título.

MATEUS BRUSCO DE FREITAS

CARACTERIZAÇÃO QUÍMICA E ATIVIDADE DE ULVANAS NA
INDUÇÃO DE RESPOSTAS OXIDATIVAS ASSOCIADAS AO
CONTROLE DE *Alternaria brassicicola* E *Colletotrichum*
higginsianum EM *Arabidopsis thaliana*

Florianópolis, 28 de novembro de 2014.

Prof. Rubens Onofre Nodari, Dr.

Coordenador do Programa de Pós-Graduação em Recursos Genéticos
Vegetais

Banca Examinadora:

Prof., Dr. Marciel J. Stadnik,

Orientador,

Universidade Federal de Santa Catarina

Profa., Dra. Roberta Paulert,

Universidade Federal do Paraná

Prof., Dr. Carlos L. Ballaré,

Universidade de Buenos Aires

Profa., Dra. Ana Carolina M. Arisi,

Universidade Federal de Santa Catarina

Prof., Dr. Robson M. Di Piero,

Universidade Federal de Santa Catarina

Dra. Érika S. M. Koshikumo,

Universidade Federal de Santa Catarina

*À mulher da minha vida Adrielle pelo apoio incondicional e por trazer
para esse mundo o João Pedro, nosso maior tesouro.
Aos meus pais, Pedro e Maria pelo carinho, educação, incentivo e
apoio.*

AGRADECIMENTOS

Ao Programa de Pós-Graduação em Recursos Genéticos Vegetais (RGV) da Universidade Federal de Santa Catarina pela oportunidade;

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e a Programa de Reestruturação e Expansão das Universidades Federais (REUNI) pela concessão da bolsa de estudos;

Ao Professor Dr. Marciel J. Stadnik, pelos ensinamentos, orientação e incentivo em todas as etapas deste trabalho;

Aos Professores do Programa de Pós-Graduação, pelos ensinamentos;

Ao Dr. Miguel D. Nosedá e as Doutoradas Luciana G. Ferreira e Maria Eugênia R. Duarte da Universidade Federal do Paraná, pela realização das análises de HPSEC-MALLS e RMN do material sulfatado e dos oligossacarídeos utilizados neste trabalho;

Ao Professor Dr. Marcelo Maraschin, pelo auxílio técnico na obtenção das oligoulvanas;

Ao Professor Dr. Pradeep Kachroo da Universidade do Kentucky, pelo fornecimento das sementes do mutante *AtrbohF* de *Arabidopsis*;

Aos responsáveis pelo Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal – LFDG, Profs. Miguel P. Guerra e Rubens O. Nodari, a Bióloga Camila Martins e ao Técnico em Química André F. Knop por permitirem a utilização dos equipamentos do laboratório;

Ao Laboratório de Química Analítica da UFSC pelo fornecimento do cianeto de potássio;

A todos os colegas e amigos do Laboratório de Fitopatologia – LABFITOP pela convivência e amizade, Alessandro, Aline, Daniele, Érika, Felipe, Marlon e Mathias;

Aos colegas e amigos do grupo da *Arabidopsis*, Caroline Hawerth e Matheus Bússolo pelo apoio e parceria durante a realização deste trabalho;

A toda a minha família pelos bons momentos que vieram e que ainda estão por vir;

A todos que direta ou indiretamente contribuíram para a realização deste trabalho.

*“Por vezes sentimos que aquilo que fazemos não é senão uma gota de
água no mar. Mas o mar seria menor se lhe faltasse uma gota”
Madre Teresa de Calcutá.*

Caracterização química e atividade de ulvanas na indução de respostas oxidativas associadas ao controle de *Alternaria brassicicola* e *Colletotrichum higginsianum* em *Arabidopsis thaliana*

RESUMO

Atualmente, a busca por métodos alternativos no controle de doenças de plantas vem aumentando. Neste contexto, a indução da resistência de plantas surge como uma alternativa amigável ao meio ambiente. Dentre os indutores de resistência, pode-se destacar a ulvana, um heteropolissacarídeo sulfatado extraído das paredes celulares de algas verdes do gênero *Ulva* spp. Este polissacarídeo tem potencial no controle de vários patógenos em culturas economicamente importantes, tais como macieira e feijão. Assim, o presente trabalho teve por objetivo avaliar a eficiência de ulvanas sulfatadas quimicamente e parcialmente depolimerizadas e estudar os mecanismos bioquímicos de defesa relacionados ao estresse oxidativo no controle de *Alternaria brassicicola* e *Colletotrichum higginsianum* em plantas de *Arabidopsis thaliana*. Para a avaliação do efeito da sulfatação química e da depolimerização parcial da ulvana, plantas de *A. thaliana* genótipo WT foram pulverizadas com ulvanas com teor de sulfato variando de 18,9 a 36,6%, com 8 frações com diferentes pesos moleculares ou ramnose e inoculadas com *A. brassicicola* ou *C. higginsianum*. A severidade das doenças foi quantificada 5 dias após a inoculação. Para a avaliação do estresse oxidativo, plantas dos genótipos WT, *AtrbohF* e *AtrbohD* foram tratadas com água (testemunha) ou ulvana (1 mg mL^{-1}) e três dias depois inoculadas com *A. brassicicola*. A severidade da doença foi quantificada cinco dias após a inoculação. As atividades de peroxidases, NADPH oxidases, catalases, ascorbato peroxidases, glutationa redutases e superóxido dismutases foram determinadas às 6, 12, 24 e 48 horas após a inoculação (h.a.i). A perda de eletrólitos devido a danos na membrana celular foi determinada às 12 h.a.i, enquanto que o crescimento do fungo *in planta* e o acúmulo de peróxido de hidrogênio foram visualizados 72 h.a.i. A pulverização tanto de ulvana quanto dos seus derivados com diferentes teores de sulfato reduziu a severidade de *A. brassicicola* e *C. higginsianum* de forma semelhante. As frações DU1, DU3, DU5 e DU7 reduziram de forma mais eficiente a severidade de *A. brassicicola* quando comparadas com a ulvana. Por outro lado, as frações com baixo peso molecular DU6 e DU8 falharam em controlar o patógeno. Estes resultados sugerem que a eficiência em induzir respostas de defesa em *A. thaliana* pode estar mais relacionada com características estruturais do que o grau de polimerização da ulvana. A ramnose, o principal açúcar

componente da ulvana, reduziu tanto a severidade de *A. brassicicola* quanto a de *C. higginsianum* de forma semelhante ao polissacarídeo. Foi possível observar que o tratamento com ulvana reduziu a colonização dos tecidos e, conseqüentemente, a severidade de *A. brassicicola* nos genótipos WT e *AtrbohF* e aumentou a atividade de NADPH oxidase e os níveis de peróxido de hidrogênio. Além disso, o polissacarídeo tendeu a incrementar a atividade de enzimas envolvidas com a remoção de espécies ativas de oxigênio, sugerindo um controle rígido do sistema antioxidante. A ulvana não protegeu o mutante *AtrbohD* e plantas WT previamente infiltradas com difenileno iodonio, ambas afetadas na atividade de NADPH oxidase e no acúmulo de peróxido de hidrogênio.

Palavras-chave: *Arabidopsis thaliana*, *Alternaria brassicicola*, *Colletotrichum higginsianum*, ulvana, resistência induzida, espécies reativas de oxigênio, *AtrbohF*, *AtrbohD*, teor de sulfato e grau de polimerização.

Chemical characterization and activity of ulvans in inducing oxidative responses associated to the control of *Alternaria brassicicola* and *Colletotrichum higginsianum* in *Arabidopsis thaliana*

ABSTRACT

Nowadays, there is an increasing demand for alternative disease control methods in plants. In this context, the induction of plant resistance arises as an eco-friendly alternative. Among resistance inducers, ulvan, a sulfated heteropolysaccharide extracted from green seaweed belonging to *Ulva* spp., has potential to control several pathogens in economically important crops such as apple and beans. Thus, the present work aimed to evaluate the efficiency of chemically sulfated and partially depolymerized ulvans and to study the biochemical defense mechanisms related to oxidative stress in the control of *Alternaria brassicicola* and *Colletotrichum higginsianum* in *Arabidopsis thaliana* plants. In order to evaluate the effect of chemical sulfation and partial depolymerization of ulvan, WT *A. thaliana* plants were sprayed with ulvans (sulfate content varying from 18.9 to 36.6%), eight fractions with different molecular weight or rhamnose and inoculated with *A. brassicicola* or *C. higginsianum*. The severity of both diseases was quantified five days after inoculation. In order to evaluate the oxidative stress responses, *A. thaliana* plants ecotypes WT, *AtrbohF* and *AtrbohD* were sprayed with water (control) or ulvan (1 mg mL⁻¹) and inoculated three days later with *A. brassicicola*. The disease severity was quantified five days after inoculation. The activities of peroxidases, NADPH oxidases, catalases, ascorbate peroxidases, glutathione reductases and superoxide dismutases were determined at 6, 12, 24 and 48 hours after inoculation (h.a.i). Electrolyte loss due to damage in cell membrane was quantified at 12 h.a.i, whereas the colonization of host tissues by the fungus and hydrogen peroxide accumulation were visualized at 72 h.a.i. The spraying of ulvan and its derivatives with different sulfate content reduced the severity of *A. brassicicola* and *C. higginsianum* at a similar extent. Fractions DU1, DU3, DU5 and DU7 reduced the disease more efficiently when compared to ulvan. On the other hand, low molecular weight fractions DU6 and DU8 failed to control the pathogen suggesting that the efficiency in inducing resistance in *A. thaliana* may be related to structural features than chain size itself. On the other hand, the main sugar component of ulvan namely rhamnose reduced the severity of both diseases at a similar extent as the polysaccharide did. It was possible to observe that ulvan reduced the colonization of host tissues and, consequently, the severity of *A. brassicicola* and increased the activity of NADPH oxidases as well as

hydrogen peroxide levels in WT and *AtrbohF* plants. Ulvan did not protect the *AtrbohD* mutant and WT plants previously sprayed with diphenyleneiodonium both impaired in NADPH oxidases activity and hydrogen peroxide accumulation.

Keywords: *Arabidopsis thaliana*, *Alternaria brassicicola*, *Colletotrichum higginsianum*, ulvan, induced resistance, reactive oxygen species, *AtrbohF*, *AtrbohD*, sulfate content and polymerization degree.

LISTA DE FIGURAS

Capítulo 1

Figura 1.1. Estratégia de infecção utilizada por *Colletotrichum higginsianum*. AP: apressório, CM: célula morta; CO: conídio, HP: hifa primária, HS: hifa secundária, MP: membrana plasmática do hospedeiro. Adaptado de O'Connell et al. (2012). 33

Figura 1.2. Estratégia de infecção utilizada por fungos do gênero *Alternaria*. HI: hifa infectiva, SP: esporo. Adaptado de Agrios (2005).35

Figura 1.3. Interação de MAMPs e MIMPs com o hospedeiro. A. A molécula proveniente do patógeno (estrela) contém um MAMP (parte escura) que é reconhecido diretamente por um receptor no hospedeiro. B. a atividade intrínseca da molécula proveniente do patógeno no hospedeiro produz um MIMP que é reconhecido por um receptor. Adaptado de Mackey; McFall (2006)..... 38

Figura 1.4. Estrutura dos dois principais dissacarídeos constituintes da ulvana. Adaptado de Robic et al. (2009) e Stadnik e de Freitas (2014).45

Capítulo 2

Figure 2.1. HPSEC-RI analysis of the native ulvan (NU) and its chemically sulfated products SU3 and SU5 (A) and partially depolymerized ulvans DU2, DU3, DU4 and DU5 (B). *Peak corresponding to salt. 57

Figure 2.2. ¹³C NMR spectra of the native ulvan NU (A) and chemically sulfated ulvans SU3 (B) and SU5 (C). 60

Figure 2.3. HSQC NMR spectra (anomeric region) of the native ulvan NU (A) and chemically sulfated ulvans SU3 (B) and SU5 (C)..... 61

Figure 2.4. Percentage of disease reduction of *Alternaria brassicicola* (A) and *Colletotrichum higginsianum* (B) five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with different sulfate content ulvans (from 18.9 to 36.6%). Disease reduction values were calculated in relation to control plants. Columns with the same letter are

not significantly different (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean. 63

Figure 2.5. Percentage of disease reduction of *Alternaria brassicicola* five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with water (control), ulvan or different partially depolymerized ulvans (DU1 to DU8). Letters indicate significant differences (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean..... 64

Capítulo 3

Figure 3.1. Mycelium weight of *Alternaria brassicicola* 7 days after growing in PDB supplemented with different ulvan concentrations at 24°C under continuous shaking in dark. *Letters indicate significant differences (Tukey's Test, $p \leq 0.05$). Bars indicate the standard deviation of mean (n=3)..... 83

Figure 3.2. Percentage of disease reduction (A) and symptoms (B) caused by *Alternaria brassicicola* five days after inoculation of wild type (WT), *AtrbohF* and *AtrbohD* *Arabidopsis thaliana* plants previously sprayed with water or ulvan (1 mg mL⁻¹). *Indicate significant disease reduction (Tukey's test, $p \leq 0.05$, n=3). ns: not significant. 84

Figure 3.3. Changes in ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GSR), guaiacol peroxidase (GPX), NADPH oxidase (NOX) and superoxide dismutase (SOD) activities at 6, 12, 24 and 48 hours after inoculation represented as percentage of control plants (water – mock). *Differs from control plants (t Test, $p \leq 0.05$ or ** $p \leq 0.01$, n=3). n.d.: not determined. Baseline at 100% represents the enzymatic activity of control plants. W-Ab: water-*Alternaria brassicicola*. U-Ab: Ulvan-A. *brassicicola*. U-Mock: Ulvan-Mock. 86

Figure 3.4. Percentage of diaminobenzidine-stained leaf area at 72 hours after inoculation of WT, *AtrbohF* and *AtrbohD* *Arabidopsis thaliana* leaves. Plants were sprayed with water (control) or ulvan (1 mg mL⁻¹) and inoculated three days later with *Alternaria brassicicola* (Ab) or water (mock). *Letters indicate significant differences (Tukey's Test, $p \leq 0.05$). Bars indicate the standard deviation of mean (n=3)..... 87

Figure 3.5. Rate of electrolyte loss (A) and trypan blue staining for fungal hyphae (B) evaluated respectively at 24 and 72 hours after inoculation of WT, *AtrbohF* and *AtrbohD* leaves. Plants were sprayed with water (control) or ulvan (1 mg mL⁻¹) and inoculated three days later with *Alternaria brassicicola* (*Ab*) or water (mock). *Letters indicate significant differences (Scott-Knott's test, $p \leq 0.05$, $n=3$). 88

Figure 3.6. Percentage of *Alternaria brassicicola*-affected leaf area five days after inoculation of wild type (WT) plants previously sprayed with water or ulvan (1 mg mL⁻¹). *Leaves were infiltrated with water (control) or diphenyleneiodonium (DPI, 5 μ M) one hour before treatment application. Letters indicate significant differences (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean ($n=4$). 89

Figure 3.7. General model showing the defense responses induced by ulvan in plant tissues. Ulvan is recognized by an unknown receptor (?) triggering a calcium influx, which is required for the activation of membrane-bound NADPH oxidase (NADPHox) [12,16]. Superoxide produced in the apoplast by NADPH oxidase dismutates spontaneously or by the action of superoxide dismutase (SOD) into hydrogen peroxide. H₂O₂ enters the cytoplasm and activates a series of defense responses as well as initiate a reactive oxygen species (ROS) wave that propagates throughout the whole plant [2,6,12,16]. Ulvan does not induce disease resistance in *AtrbohD* plants because RBOHD, the main source of ROS, is not functional in this mutant [18]. On the other hand, the polysaccharide is able to induce resistance in *AtrbohF* plants since, although the RBOHF protein plays a major role in triggering of the hypersensitive response (i.e. localized cell death), it has only a minor role in ROS production [18]. DPI: diphenyleneiodonium. 92

LISTA DE TABELAS

Capítulo 1

Tabela 1.1. Efeitos da aplicação foliar de ulvana ou do extrato de algas do gênero *Ulva* contendo o polissacarídeo sobre doenças em diferentes patossistemas testados. 46

Capítulo 2

Table 2.1. Yield and sulfation degree of the native ulvan (NU) and its chemically sulfated and depolymerized derivatives. 56

Table 2.2. Chemical shift (ppm) assignments of ¹³C and ¹H NMR spectra of NU fraction. 62

Table 2.3. Lesion diameter of *Alternaria brassicicola* and *Colletotrichum higginsianum* five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with water (control), ulvan (NU) or L-rhamnose... 65

LISTA DE ABREVIATURAS

| | |
|-----------|---|
| <i>Ab</i> | <i>Alternaria brassicicola</i> |
| ANOVA | Análise de variância |
| AP | Apressório |
| APX | Ascorbato peroxidase |
| BSA | Albumina de soro bovino |
| CAT | Catalase |
| CE | Célula da epiderme |
| <i>Ch</i> | <i>Colletotrichum higginsianum</i> |
| CM | Célula do mesofil |
| CO | Conídio |
| DAB | Diaminobenzidina |
| DPI | Difenileno iodonio |
| DU | Ulvana depolimerizada |
| EDTA | Ácido etilenodiamino tetra-acético |
| GPX | Guaicol peroxidase |
| GSR | Glutationa redutase |
| GSSG | Glutationa oxidada |
| h.a.i | Horas após a inoculação |
| HF | Hifa primária |
| HR | Reação de hipersensibilidade (<i>Hypersesitive reaction</i>) |
| HS | Hifa secundária |
| ISR | Resistência sistêmica induzida (<i>Induced systemic resistance</i>) |
| n.d. | Não determinado |
| NOX | NADPH oxidase |
| NU | Ulvana nativa |
| PAL | Fenilalanina amônia liase |
| PAMP | Padrão molecular associado a micróbios (<i>Pathogen-associated molecular pattern</i>) |
| PIMP | Padrão molecular induzido por micróbios (<i>Pathogen-induced molecular pattern</i>) |
| PR | Proteína relacionada à patogênese |
| RBOH | Oxidases homóloga da explosão respiratória (<i>Respiratory burst oxidase homologue</i>) |
| ROS | Espécies reativas de oxigênio (<i>Reactive oxygen species</i>) |
| SAR | Resistência sistêmica adquirida (<i>Systemic acquired resistance</i>) |
| SOD | Superóxido dismutase |
| SU | Ulvana sulfatada |

| | |
|-----|------------------------------------|
| TCA | Ácido tricloroacético |
| TMV | Vírus do mosaico do tabaco |
| WT | Tipo selvagem (<i>Wild type</i>) |

SUMÁRIO

| | | |
|--------|--|----|
| 1. | INTRODUÇÃO E JUSTIFICATIVA | 27 |
| 2. | OBJETIVOS | 29 |
| 2.1. | GERAL | 29 |
| 2.2. | ESPECÍFICOS | 29 |
| 3. | CAPÍTULO 1 - REVISÃO DE LITERATURA | 31 |
| 3.1. | <i>Arabidopsis thaliana</i> | 31 |
| 3.2. | O GÊNERO <i>Colletotrichum</i> | 32 |
| 3.3. | O GÊNERO <i>Alternaria</i> | 35 |
| 3.4. | INDUÇÃO DE RESISTÊNCIA | 36 |
| 3.5. | A EXPLOÇÃO OXIDATIVA NA RESISTÊNCIA DE PLANTAS A PATÓGENOS | 40 |
| 3.6. | CARBOIDRATOS NA PROTEÇÃO DE PLANTAS CONTR PATÓGENOS | 42 |
| 3.7. | ULVANA | 44 |
| 3.8. | RELAÇÃO ENTRE ESTRUTURA, COMPOSIÇÃO E ATIVIDADE BIOLÓGICA DE POLISSACARÍDEOS ALGAIS | 48 |
| 4. | CAPÍTULO 2 – ULVANS INDUCE RESISTANCE AGAINST PLANT PATHOGENIC FUNGI INDEPENDENTLY OF ITS SULFATION AND POLYMERIZATION DEGREE | 49 |
| 4.1. | INTRODUCTION | 50 |
| 4.2. | MATERIAL AND METHODS | 52 |
| 4.2.1. | Obtaining of ulvan and rhamnose | 52 |
| 4.2.2. | Sulfation of ulvan | 52 |
| 4.2.3. | Preparation and fractionation of partially depolymerized ulvans | 52 |
| 4.2.4. | Analytical methods | 53 |
| 4.2.5. | High-pressure size-exclusion chromatography (HPSEC) analysis | 53 |
| 4.2.6. | Nuclear magnetic resonance (NMR) | 53 |
| 4.2.7. | Plant growth conditions and treatment | 54 |
| 4.2.8. | Inoculation and disease evaluation | 54 |
| 4.2.9. | Experimental design and statistical analysis | 54 |
| 4.3. | RESULTS | 55 |
| 4.3.1. | Extraction of ulvans, sulfation and partial depolymerization | 55 |
| 4.3.2. | Effect of sulfation degree of ulvans on the infection severity of <i>Alternaria brassicicola</i> and <i>Colletotrichum higginsianum</i> | 62 |
| 4.3.3. | Effect of ulvans molecular weight on the infection severity of <i>Alternaria brassicicola</i> | 64 |
| 4.3.4. | Effect of L-rhamnose on the infection severity of <i>Alternaria brassicicola</i> and <i>Colletotrichum higginsianum</i> | 64 |
| 4.4. | DISCUSSION | 65 |
| 4.5. | REFERENCES | 68 |

| | | |
|--------|---|----|
| 5. | CAPÍTULO 3 - ULVAN-INDUCED RESISTANCE IN <i>Arabidopsis thaliana</i> AGAINST <i>Alternaria brassicicola</i> REQUIRES REACTIVE OXYGEN SPECIES DERIVED FROM NADPH OXIDASE | 75 |
| 5.1. | INTRODUCTION..... | 75 |
| 5.2. | MATERIALS AND METHODS | 78 |
| 5.2.1. | Biological material..... | 78 |
| 5.2.2. | Effect of ulvan on fungus growth..... | 78 |
| 5.2.3. | Plant growth conditions and treatment..... | 79 |
| 5.2.4. | Inoculation and disease evaluation..... | 79 |
| 5.2.5. | Sampling | 79 |
| 5.2.6. | Enzyme activity assays | 80 |
| 5.2.7. | Histochemical analysis..... | 81 |
| 5.2.8. | Cell death measurement..... | 82 |
| 5.2.9. | Experimental design and statistical analysis | 82 |
| 5.3. | RESULTS | 83 |
| 5.3.1. | Effect of ulvan on fungus growth..... | 83 |
| 5.3.2. | Disease severity | 83 |
| 5.3.3. | Enzymatic activity | 84 |
| 5.3.4. | H ₂ O ₂ | 87 |
| 5.3.5. | Cell death measurement..... | 87 |
| 5.3.6. | Ulvan-induced resistance in DPI-treated plants | 88 |
| 5.4. | DISCUSSION | 89 |
| 5.5. | REFERENCES..... | 93 |
| 6. | CONSIDERAÇÕES FINAIS | 97 |
| 7. | REFERÊNCIAS BIBLIOGRÁFICAS | 99 |

1. INTRODUÇÃO E JUSTIFICATIVA

As doenças de plantas têm sido um problema contínuo para a sociedade desde o início da agricultura, persistindo mesmo com o surgimento de pesticidas e variedades resistentes (OERKE, 2006; WALTERS, 2009). Estima-se que as perdas ocasionadas por patógenos totalizaram 60% no período de 2001 a 2003 nas principais culturas a nível mundial (trigo, arroz, milho, batata, soja e algodão) (OERKE, 2006).

Devido aos problemas ambientais causados pela utilização de agrotóxicos e a grande variabilidade genética existente em alguns fitopatógenos, medidas alternativas têm sido requeridas para o manejo de doenças. A indução de resistência tem se mostrado uma alternativa promissora e complementar aos controles químico e genético convencionais. Entre os indutores, os polissacarídeos extraídos de macroalgas marinhas têm apresentado bons resultados quanto à capacidade de elicitar mecanismos de defesa em plantas contra patógenos (CLUZET et al., 2004; KLARZYNSKI et al., 2000; MERCIER et al., 2001; PAULERT et al., 2009).

Raramente a resistência induzida controla completamente a doença porém, reduz o tamanho e/ou número de lesões causadas pelo patógeno, levando assim, a um atraso em seu desenvolvimento (WALTERS, 2010 e 2013). Seus princípios podem ser aplicados na proteção de plantas contra um amplo espectro de doenças causadas por fungos, bactérias e vírus (HAMMERSCHMIDT, 1999; STADNIK; MARASCHIN, 2004).

Durante a interação planta-patógeno, uma das respostas de defesa iniciais é a explosão oxidativa, uma produção massiva de espécies ativas de oxigênio (ROS). As ROS são produzidas principalmente por NADPH oxidases (NOX). As NOX também conhecidas como oxidases homólogas da explosão respiratória (“*respiratory burst oxidase homologues*”, RBOH) constituem uma família multigênica com 10 genes diferentes em *A. thaliana* (*AtrbohA* – *AtrbohJ*). Esta explosão oxidativa é acompanhada por alterações no pH celular, influxo de íons, fosforilação de proteínas, expressão de genes de defesa, reação de hipersensibilidade, produção de fitoalexinas, deposição de calose e resistência sistêmica induzida (LAMB; DIXON, 1997; APEL; HIRT, 2004; O’BRIEN et al., 2012).

A ulvana é um heteropolissacarídeo sulfatado solúvel em água, extraído das paredes celulares de algas do gênero *Ulva* spp., representando de 8 a 29% do peso da alga (LAHAYE; ROBIC, 2007). Estudos recentes demonstram que a ulvana tem potencial para controlar a

ferrugem (*Uromyces appendiculatus*) (BORSATO; DI PIERO; STADNIK, 2010), antracnose (*Colletotrichum lindemuthianum*) (PAULERT et al., 2009) e o oídio (*Erysiphe polygoni*) em feijão e cereais (PAULERT et al., 2010; JAULNEAU et al., 2011) e *Colletotrichum gloeosporioides* em maçã (ARAUJO et al., 2008).

A atividade biológica de polissacarídeos algais pode ser afetada por vários fatores incluindo peso molecular, composição química, conformação da cadeia e quantidade e posição de grupos sulfato (ALVES et al., 2013). Assim, a modificação química de polissacarídeos pode resultar em moléculas com atividades biológicas novas ou melhoradas (QI et al., 2012). Por exemplo, ulvanas sulfatadas artificialmente (30,8 e 32,8% de sulfato) exibiram um incremento na atividade antioxidante *in vitro* quando comparadas à ulvana (19,5% de sulfato). Além disso, a ulvana sulfatada (32,8% de sulfato) apresentou uma maior atividade anti-hiperlipidêmica em camundongos quando compara à ulvana (QI et al., 2012). O grau de sulfatação da ulvana parece afetar a sua atividade elicitora também em plantas, porque a sua completa dessulfatação suprime a sua habilidade de aumentar a atividade de fenilalanina amônia liase (PAL) em plântulas de tomate infectadas com *Fusarium oxysporum* f.sp. *lycopersici* (EL MODAFAR et al., 2012).

Evidências sugerem que a ulvana induz diretamente a produção de peróxido de hidrogênio, via peroxidases (DE FREITAS; STADNIK, 2012) e que sua atividade biológica *in vitro* e em animais é fortemente influenciada por modificações em seu tamanho e teor de sulfato (QI et al., 2012). Desta forma, o presente trabalho foi desenvolvido para estudar e comparar os mecanismos de defesa relacionados ao estresse oxidativo induzidos por ulvana nos ecotipos WT, *AtrbohF* e *AtrbohD* contra *A. brassicicola* e avaliar a eficiência do polissacarídeo sulfatado quimicamente e oligulvanas no controle de *A. brassicicola* e *C. higginsianum* em plantas de *A. thaliana*.

2. OBJETIVOS

2.1. GERAL

Avaliar a eficiência de ulvanas sulfatadas quimicamente e parcialmente depolimerizadas e estudar os mecanismos de defesa bioquímicos relacionados ao estresse oxidativo no controle de *Alternaria brassicicola* e *Colletotrichum higginsianum* em plantas de *Arabidopsis thaliana*.

2.2. ESPECÍFICOS

- Comparar a eficiência da ulvana nativa, sulfatada e seus oligômeros no controle de *A. brassicicola* e *C. higginsianum* em plantas de *A. thaliana*.
- Avaliar o efeito da aplicação foliar ulvana na severidade de *A. brassicicola* nos genótipos WT, *AtrbohF* e *AtrbohD* de *A. thaliana*;
- Monitorar a atividade das enzimas peroxidases, catalases, ascorbato peroxidases, NADPH oxidases, glutathione redutases e superóxido dismutases nos genótipos WT, *AtrbohF* e *AtrbohD* de *A. thaliana* tratados ou não com ulvana e inoculados ou não com *A. brassicicola*;
- Avaliar o crescimento do fungo *in planta*, acúmulo e peróxido de hidrogênio nos genótipos WT, *AtrbohF* e *AtrbohD* de *A. thaliana* tratados ou não com ulvana e inoculados ou não com *A. brassicicola*.

3. CAPÍTULO 1 - REVISÃO DE LITERATURA

3.1. *Arabidopsis thaliana*

Arabidopsis thaliana (L.) Heyhn. é uma planta pertencente à família da couve e da mostarda (Brassicaceae) (MEYEROWITZ, 1987; KOORNNEEF; SCHERES, 2001). Esta espécie encontra-se amplamente distribuída no hemisfério norte (MEYEROWITZ, 1987; KOORNNEEF; SCHERES, 2001).

Uma planta adulta de *A. thaliana* é constituída de uma roseta de pequenas folhas e uma haste principal com uma inflorescência terminal podendo atingir 30 a 40 cm de altura. O fruto de *A. thaliana* é do tipo síliqua e pode conter de 30 a 60 pequenas sementes (MEYEROWITZ, 1987; KOORNNEEF; SCHERES, 2001).

Apesar de estar na mesma família de plantas relevantes para o mercado mundial, *A. thaliana* não apresenta importância econômica (MEYEROWITZ, 1987; KOORNNEEF; SCHERES, 2001; LEONELLI, 2007). No entanto, algumas de suas características despertaram o interesse de cientistas na década de 40 para o seu potencial como organismo modelo (LEONELLI, 2007). Dentre estas características pode-se destacar o pequeno porte, facilidade de cultivo, autofecundação, ciclo de vida curto, grande produção de sementes, genoma relativamente pequeno e facilidade de transformação e obtenção de mutantes (MEYEROWITZ, 1987; LEONELLI, 2007).

Atualmente, *A. thaliana* vem sendo utilizada como organismo modelo nas mais diversas áreas da ciência, incluindo anatomia, fisiologia, genética, bioquímica e interação patógeno-hospedeiro (MEINKE et al., 1998; GLAZEBROOK; ROGERS; AUSUBEL, 1997). *A. thaliana* tem mostrado ser um bom organismo modelo por apresentar uma grande variação genética natural representada por mais de 150 genótipos, susceptibilidade a patógenos biotróficos, hemibiotróficos e necrotróficos e respostas de defesa semelhantes às descritas para outros hospedeiros. Estas respostas envolvem as resistências do tipo gene-a-gene e a sistêmica adquirida (SAR) (GLAZEBROOK; ROGERS; AUSUBEL, 1997).

Genes de resistência vem sendo identificados em *A. thaliana* contra fungos (*Peronospora parasítica*, *albugo cândida*, *Erysiphe cichoracearum* e *Plasmodiophora brassicae*), vírus (cauliflower mosaic virus e turnip crinkle virus) e bactérias (*Xanthomonas campestris* pv. *Campestris*) (GLAZEBROOK; ROGERS; AUSUBEL, 1997). Além

disso, a elucidação de vias de sinalização envolvidas na indução de resistência também vem sendo realizada em *A. thaliana*. Assim, por exemplo, os mutantes *etr1* e *ein2*, ambos insensíveis ao etileno, expressam a resistência sistêmica adquirida (SAR) normalmente quando tratados com os ácidos salicílico e dicloro isonicotínico. Estes resultados sugerem que o etileno não é necessário para a ativação da SAR (BLEECKER et al., 1988; GUZMAN; ECKER, 1990). Apesar de o mutante *etr1* ativar a SAR em resposta a infecção por *Pseudomonas syringae* pv. *Tomato* (DC3000/*avrRpt2*), a expressão do gene *PR1* foi menor do que a observada no tipo selvagem, sugerindo que o etileno pode ter algum papel regulador na SAR (LAWTON et al., 1995).

Genes que conferem a resistência de não-hospedeiro também vem sendo identificados em *A. thaliana*. Investigações iniciais mostram que 3 genes (*PENetration1*, *PEN2* e *PEN3*) são necessários para a resistência pré-invasão do fungo biotrófico *Blumeria graminis* f. sp. *hordei*. Por outro lado, a inibição do crescimento das hifas depende principalmente dos genes *EDS1* (*Enhanced Disease Susceptibility 1*), *PAD4* (*Phytoalexin Deficient 4*) e *SAG101* (*Senescence Associated Gene 101*) (LIPKA et al., 2005; STEIN et al., 2006). Os genes *PEN2*, *AGB1* (*Arabidopsis G-protein β -subunit*), *PMR5* (*Powdery Mildew Resistant 5*) e *MLO2* (*Mildew Resistance Locus O 2*) atuam tanto na pré quanto na pós infecção de *Magnaporthe oryzae* (MAEDA et al., 2009; NAKAO et al., 2011).

Espécies do gênero *Colletotrichum* podem utilizar dois modos de penetração de tecidos vegetais diferentes: por meio de apressório melanizado ou penetração em ferimentos somente com a hifa. A penetração por ferimentos ocorre de forma predominante em plantas não-hospedeiras. A resistência de *A. thaliana* à invasão por ferimentos de *Colletotrichum gloeosporioides* depende do gene *PEN2*. Por outro lado, o mecanismo de resistência à penetração via apressório ainda é desconhecido (HIRUMA et al., 2010).

3.2. O GÊNERO *Colletotrichum*

Colletotrichum é um dos gêneros de fungos fitopatogênicos mais importante, especialmente em regiões tropicais e subtropicais. Este gênero é responsável por doenças economicamente importantes em gramíneas, legumes, vegetais e culturas perenes, incluindo árvores frutíferas (BAILEY et al., 1992; PERFECT et al., 1999; O'CONNELL et al., 2000; O'CONNELL et al., 2012). Os sintomas da antracnose incluem

lesões necróticas deprimidas circulares ou angulares que podem ser visualizadas em toda a parte aérea da planta (BAILEY et al., 1992).

A espécie *Colletotrichum higginsianum* ataca vários membros da família Brassicaceae (previamente denominadas Cruciferae), incluindo o nabo, rabanete e repolho chinês. Este fungo também infecta plantas de *A. thaliana*, formando assim, um importante patossistema para a dissecação dos mecanismos de patogenicidade de fungos hemibiotróficos e da resistência do hospedeiro (NARUSAKA et al., 2004; O'CONNELL et al., 2004; BIRKER et al., 2009; O'CONNELL et al., 2012).

C. higginsianum exibe uma estratégia de infecção e colonização do tipo hemibiotrófica (Figura 1). Inicialmente, os conídios aderem à superfície foliar, germinam e, em seguida, formam o apressório que penetra a cutícula diretamente (BAILEY et al., 1992; PERFECT et al., 1999; O'CONNELL et al., 2000, 2004 e 2012). Após a penetração, inicia-se uma breve fase biotrófica confinada às primeiras células invadidas onde o fungo coloniza os tecidos formando hifas primárias intracelulares e vesículas de infecção. A fase necrotrófica inicia-se com a formação de hifas secundárias que, se ramificam intra e extracelularmente e matam rapidamente as células do hospedeiro (BAILEY et al., 1992; PERFECT et al., 1999; O'CONNELL et al., 2000, 2004 e 2012).

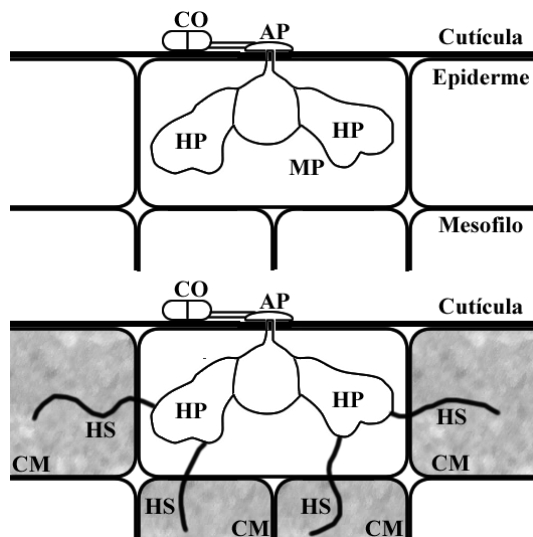


Figura 1.1. Estratégia de infecção utilizada por *Colletotrichum higginsianum*. AP: apressório, CM: célula morta; CO: conídio, HP: hifa primária, HS: hifa secundária, MP: membrana plasmática do hospedeiro. Adaptado de O'Connell et al. (2012).

Análises de genoma e transcriptoma revelaram que *C. higginsianum* possui vários genes relacionados à patogênese (O'CONNELL et al., 2012). Este patógeno dispõe de genes que codificam para efetores, transportadores e enzimas para a degradação de carboidratos e do metabolismo secundário. Esses genes são transcritos de forma coordenada em ondas sucessivas ligadas à transição patogênica onde, efetores e enzimas do metabolismo secundário são ativados antes da penetração e durante a fase biotrófica enquanto que a maioria das hidrolases e transportadores atuam mais tarde, na transição para a fase necrotrófica (O'CONNELL et al., 2012).

A transformação mediada por *Agrobacterium tumefaciens* vem sendo utilizada para a identificação de genes de patogenicidade em *C. higginsianum*. Desta forma, foram obtidos transformantes incapazes de melanizar seus apressórios, com habilidade de penetração reduzida, que induzem respostas de defesa ou não fazem a transição da fase biotrófica para a hemibiotrófica (HUSER et al., 2009). Os genes identificados são homólogos a um facilitador principal de uma superfamília de transportador de fosfato, importina β -2, ornitina descarboxilase, β -1,3(4)-glucanase, endoribonuclease ligadora de ATP, carbomiofosfato sintetase, e a poliproteína precursora de *N*-acetil glutamato quinase e de *N*-acetil glutamil fosfato redutase. Curiosamente, nenhum dos genes listados acima teve sua função relacionada com a patogênese em qualquer espécie do gênero *Colletotrichum* (HUSER et al., 2009).

A resistência genética de *A. thaliana* a *C. higginsianum* parece estar associada a um ou dois genes que podem ser dominantes ou recessivos e atuam no momento da penetração e estabelecimento da fase biotrófica. Assim, por exemplo, a resistência do acesso Eil-0 ao patógeno é conferida por um único gene dominante (*RCH1*) localizado no cromossomo 4 da planta (NARUSAKA et al., 2004). As respostas de defesa contra o patógeno envolvem a produção da fitoalexina camalexina (NARUSAKA et al., 2004) e a ativação das vias de sinalização do ácido salicílico e do etileno (O'CONNELL et al., 2004). Por outro lado, perfis de expressão de genes marcadores de defesa mostram que as vias de sinalização do jasmonato e etileno são mais importantes que a do ácido salicílico (NARUSAKA et al., 2004). Estes resultados sugerem que a resistência a patógenos hemibiotróficos tal como *Colletotrichum* pode requerer uma combinação das três vias (BIRKER et al., 2009).

3.3. O GÊNERO *Alternaria*

A grande maioria de espécies do gênero *Alternaria* é saprófita sendo comumente encontradas no solo ou em material vegetal em decomposição. Por outro lado, algumas espécies deste gênero são patógenos vegetais, causando danos econômicos em culturas economicamente importantes, tais como: cereais, ornamentais, olerícolas, vegetais como cenoura e batata e frutas como tomate, citros e maçã (ROTEM, 1998; THOMMA, 2003). Os sintomas típicos da mancha foliar de *Alternaria* são manchas necróticas circulares e concêntricas. Além das folhas, o fungo pode atacar as hastes e siliquis reduzindo a área fotossintética e acelerando a senescência e reduzindo a germinação de sementes e causando o tombamento das plântulas (ROTEM, 1998; THOMMA, 2003; NOWICKI et al., 2012).

A espécie *Alternaria brassicicola* infecta vários indivíduos da família Brassicaceae incluindo espécies economicamente importantes como repolho, couve-flor e brócolis. Além de espécies com valor econômico, *A. brassicicola* é capaz de colonizar plantas de *A. thaliana*, patossistema este que vem sendo amplamente utilizada como modelo no estudo de doenças causadas por fungos necrotróficos.

O processo infeccioso de fungos do gênero *Alternaria* inicia-se com a germinação dos esporos, produzindo um ou mais tubos germinativos. Em seguida, o fungo penetra através de estômatos, cutícula ou ferimentos formando ou não apressórios (ROTEM, 1998; THOMMA, 2003). Após a penetração, o patógeno utiliza um arsenal de enzimas e toxinas para matar as células do hospedeiro (ROTEM, 1998; THOMMA, 2003).

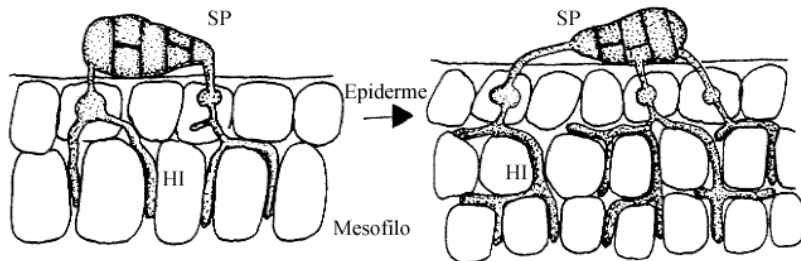


Figura 1.2. Estratégia de infecção utilizada por fungos do gênero *Alternaria*. HI: hifa infectiva, SP: esporo. Adaptado de Agrios (2005).

A maioria das toxinas produzidas por espécies do gênero *Alternaria* durante a colonização do hospedeiro são metabólitos secundários quimicamente diversos. Com base na seletividade, estas

toxinas podem ser divididas em hospedeiro inespecíficas e hospedeiro específicas (THOMMA et al., 2003; NOWICKI et al., 2012). De uma maneira geral, as toxinas hospedeiro inespecífica causam fitotoxicidade intermediária intensificando a severidade da doença, afetam um amplo espectro de espécies de plantas e não são absolutamente necessárias para o estabelecimento do patógeno. Por exemplo, a toxina brefeldina A desestrutura o complexo de Golgi e inibe a secreção de substâncias, enquanto que a curvularina afeta a montagem dos microtúbulos e, consequente, a divisão celular (THOMMA et al., 2003; NOWICKI et al., 2012).

As toxinas hospedeiro específicas são indispensáveis para o desenvolvimento da doença e, geralmente, causam efeitos severos em poucas espécies hospedeiras. Apesar de atuarem em diferentes sítios, todas estas toxinas causam a morte das células do hospedeiro. Assim, por exemplo, as toxinas AF, ACT e ACTG causam a permeabilização da membrana celular da célula vegetal (THOMMA et al., 2003; NOWICKI et al., 2012).

A resistência genética a *A. brassicicola* parece ser controlada por um ou vários genes parcialmente dominantes ou com efeito aditivo (NOWICKI et al., 2012). A resistência a patógenos deste gênero está associada com a via do ácido jasmônico, com incremento na atividade de polifenoloxidasas, peroxidases e catalases e no acúmulo de camalexina (THOMMA et al., 2003; NOWICKI et al., 2012).

3.4. INDUÇÃO DE RESISTÊNCIA

De forma geral, a evolução da imunidade inata pode ser explicada da seguinte forma: as defesas iniciais ou basais de plantas são ativadas através do reconhecimento de padrões moleculares associados a patógenos/ micróbios (PAMPs/ MAMPs) por receptores de reconhecimento de padrões (PRRs). Os PRRs são encontrados tanto em animais quanto em plantas e são quinases do tipo receptores transmembrana (RLKs) ou proteínas do tipo receptora (RLPs). O genoma de *A. thaliana* codifica para um grande número de RLKs (~615) com funções variando desde imunidade a autoincompatibilidade durante a polinização (SPOEL; DONG, 2012). Para superar esta linha de defesa, os patógenos passaram a secretar efetores. Como contra-ataque, as plantas desenvolveram proteínas “R” responsáveis pelo reconhecimento de efetores específicos originando a imunidade disparada por efetores (ETI). Este tipo de resistência era previamente conhecida como imunidade vertical ou baseada em genes “R”. Os resultados do reconhecimento são

semelhantes aos exibidos na resistência gene-a-gene e envolvem a reação de hipersensibilidade e o desenvolvimento da SAR (GOZZO; FAORO, 2013).

As plantas são frequentemente expostas a uma grande quantidade de patógenos, o que levou ao desenvolvimento de complexos mecanismos de defesa. De uma maneira geral, as plantas possuem mecanismos físicos e químicos pré-existentes, tais como: camadas de cera, cutícula, adaptações em estômatos e compostos fungitóxicos (AGRIOS, 2005). Além dos mecanismos pré-existentes, plantas possuem mecanismos que são ativados após o reconhecimento do patógeno, os quais envolvem a formação de tiloses, papilas, camadas de cortiça e de abscisão, produção de compostos como as fitoalexinas, proteínas relacionadas à patogênese (PR-proteínas) e espécies reativas de oxigênio (Reactive Oxygen Species – ROS) (AGRIOS, 2005; GOZZO; FAORO, 2013).

A habilidade das plantas se defenderem contra invasores está relacionada com a capacidade de detectá-los (CLUZET et al., 2004). A percepção de moléculas sinalizadoras (chamadas de elicitores), que podem ser de natureza química diversa, liberadas pelos patógenos, leva a um desencadeamento de mecanismos de sinalização. Estes mecanismos geralmente iniciam com um influxo de cálcio e uma explosão oxidativa, seguido da síntese de moléculas sinalizadoras tais como: ácido salicílico, ácido jasmônico e etileno (CLUZET et al., 2004).

Uma ampla variedade de moléculas pode atuar como elicitores, incluindo oligo- e polissacarídeos, peptídeos, lipídeos e proteínas (WALTERS, 2010). Estas moléculas podem ser divididas em dois grandes grupos: padrão molecular associado a micróbios ou PAMP (“*microbe-associated molecular pattern*” – MAMP) e padrão molecular induzido por micróbios ou PIMP (“*microbe-induced molecular pattern*” – MIMP) (MACKEY; MCFALL, 2006; PAULERT et al., 2010). Um MAMP representa qualquer molécula ou parte dela derivada de um patógeno que interage diretamente com um receptor de defesa no hospedeiro (Figura 3). Por exemplo, a proteína flagelina responsável pela estrutura do flagelo bacteriano contém um peptídeo com 22 aminoácidos (flg22) que interage diretamente com o receptor FLS2, codificado por plantas (CHINCHILLA et al., 2006). Efeitores fúngicos também contém MAMPs. Por exemplo, *Magnaporthe grisea* secreta o efector AvrPita que contém um MAMP q interage diretamente com a proteína Pi-ta do arroz (JIA et al., 2000).

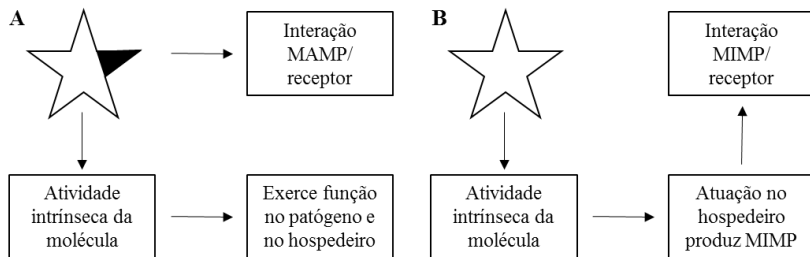


Figura 1.3. Interação de MAMPs e MIMPs com o hospedeiro. A. A molécula proveniente do patógeno (estrela) contém um MAMP (parte escura) que é reconhecido diretamente por um receptor no hospedeiro. B. a atividade intrínseca da molécula proveniente do patógeno no hospedeiro produz um MIMP que é reconhecido por um receptor. Adaptado de Mackey; McFall (2006).

Um MIMP representa uma molécula do hospedeiro que sofreu alguma modificação pela ação de efetores do patógeno e que é reconhecida por um receptor de defesa do hospedeiro (Figura 3) (MACKEY; MCFALL, 2006; PAULERT et al., 2010). Alguns efetores podem ser reconhecidos por produzirem um MIMP. Por exemplo, a bactéria *Pseudomonas syringae* secreta o efector AvrRpt2 que é reconhecido indiretamente pela proteína R chamada de RPS2 de *A. thaliana* (BENT et al., 1994). O efector AvrRpt2 é uma protease que cliva a proteína RIN4 de *A. thaliana* que, por sua vez, regula negativamente RPS3. A ativação constitutiva de RPS2 via mutação no gene de RIN4 resulta em morte do embrião (MACKEY et al., 2003). De forma resumida, a clivagem da proteína RIN4 por AvrRpt2 produz o MIMP reconhecido por RPS2. *P. syringae* secreta outro efector (AvrPphB) que cliva a proteína PBS1 de *A. thaliana* (SHAO et al., 2002; 2003). A PBS1 é necessária para o funcionamento de RPS5, uma proteína R citoplasmática que reconhece AvrPphB.

Raramente a resistência induzida controla completamente a doença porém, reduz o tamanho e/ou número de lesões causadas pelo patógeno, levando assim, a um atraso em seu desenvolvimento (WALTERS, 2010 e 2013). Seus princípios podem ser aplicados na proteção de plantas contra um amplo espectro de doenças causadas por fungos, bactérias e vírus (HAMMERSCHMIDT, 1999; STADNIK; MARASCHIN, 2004).

A resistência induzida pode ocorrer local ou sistemicamente, quando é expressa em locais não expostos diretamente ao agente indutor e pode ser dividida em duas categorias. A primeira delas, a resistência sistêmica adquirida (“*Systemic Acquired Resistance*” – SAR), pode se desenvolver local e sistemicamente em resposta a um patógeno causador de necrose. A SAR caracteriza-se pelo acúmulo de PR-proteínas e é mediada por uma

via dependente do ácido salicílico. As propriedades químicas permitem que o ácido salicílico realize várias atividades em reações e interações com enzimas e moléculas exercendo papéis importantes durante a SAR (GOZZO; FAORO, 2013). No entanto, a sua principal função descrita até o momento é induzir a expressão do gene PR-1, envolvendo a mediação da proteína NPR1. Estudos recentes mostram que a forma oligomérica da NPR1, presente no citoplasma, é desmontada e translocada para o núcleo onde seus monômeros promovem a expressão eficiente de genes de defesa (PR-1, por exemplo). O transporte para o núcleo ocorre somente após o ataque de um patógeno ou tratamento com ácido salicílico (MUKHTAR et al., 2009).

O segundo tipo de resistência induzida, conhecida como resistência sistêmica induzida (“*Induced Systemic Resistance*” – ISR), ocorre sistemicamente em resposta a ferimentos e danos causados por herbívoros. Além disso, a ISR pode ser induzida por microrganismos benéficos como bactérias colonizadoras de raízes, fungos formadores de micorrizas e *Trichoderma* spp. (GOZZO; FAORO, 2013). A ISR é mediada por uma via envolvendo jasmonato/etileno e não envolve o acúmulo de PR-proteínas (DURRANT; DONG, 2004, WALTERS, 2010 e 2013). Apesar de ser independente do ácido salicílico a ISR depende da proteína NPR1. Estudos mostram que a NPR1 assume uma função totalmente diferente daquela descrita na SAR durante a ISR. Tem-se sugerido que a NPR1 atua no citosol regulando a interação (“*cross-talk*”) entre as vias do ácido jasmônico e etileno (SPOEL et al., 2007; KOORNNEEF; PIETERSE, 2008). Caso a interação entre as duas vias ocorra sistemicamente durante a infecção por um patógeno biotrófico, pode-se esperar que a colonização subsequente por um necrotrófico seja facilitada. No entanto, a interação entre os ácidos salicílico e jasmônico é inibida em tecidos distantes daqueles tratados com efetores que disparam a ETI (SPOEL, et al., 2007).

Os indutores de resistência podem ser classificados, quanto à origem, em: bióticos e abióticos. Dentro dos indutores bióticos, pode-se destacar a pré-inoculação com patógenos virulentos e avirulentos e a pulverização com quitosana (Elexa[®], SafeScience), proteína harpina (Messenger[®], Plant Health Care) e polissacarídeos algais como carragenana, laminarana e ulvana. Dentro dos indutores abióticos, pode-se destacar a utilização de choque térmico, injúrias mecânicas, compostos químicos tais como: derivados do ácido salicílico, ácidos 2,6 dicloroisonicotínico e β -aminobutírico, probenazole e o acibenzolar-S-metil (Bion[®], Syngenta) (STADNIK; MARASCHIN, 2004, WALTERS, 2010 e 2013; STADNIK; DE FREITAS, 2014).

3.5. A EXPLOÇÃO OXIDATIVA NA RESISTÊNCIA DE PLANTAS A PATÓGENOS

A evolução de processos metabólicos tais como a respiração e a fotossíntese levaram à produção de espécies reativas de oxigênio (ROS). As ROS incluem ânion superóxido (O_2^-), radical hidroxila ($\cdot OH$), peróxido de hidrogênio (H_2O_2) e o oxigênio simples (1O_2). Em plantas, as ROS são formadas pela perda de elétrons pelas suas cadeias de transporte em cloroplastos, mitocôndrias e membrana plasmática ou como um subproduto de várias vias metabólicas localizadas em diferentes compartimentos celulares (APEL; HIRT, 2004; O'BRIEN et al., 2012; SHARMA et al., 2012).

A produção massiva de ROS é conhecida como explosão oxidativa, uma das respostas de defesa iniciais durante a interação patógeno-hospedeiro. Esta explosão oxidativa é acompanhada por alterações no pH celular, influxo de íons, fosforilação de proteínas, expressão de genes de defesa, reação de hipersensibilidade, produção de fitoalexinas deposição de calose e resistência sistêmica induzida (LAMB; DIXON, 1997; APEL; HIRT, 2004; O'BRIEN et al., 2012).

As ROS têm um alto poder de oxidação, danificando várias moléculas biológicas. Apesar do seu poder destrutivo, as ROS são mensageiros importantes em vários processos celulares vitais tais como crescimento, ciclo celular, morte celular programada, sinalização hormonal e respostas a estresses bióticos e abióticos (LAMB; DIXON, 1997; APEL; HIRT, 2004; MITTLER et al., 2004; O'BRIEN et al., 2012).

As ROS são produzidas por diferentes enzimas incluindo peroxidases, diamina oxidases, oxalato oxidases, amina oxidases, NADPH oxidases e xantina oxidases. Dentre estas, as NADPH oxidases (NOX) são as enzimas mais estudadas com um papel importante na produção de O_2^- durante a explosão oxidativa. As NOX também conhecidas como oxidases homólogas da explosão respiratória (“*respiratory burst oxidase homologues*”, RBOH) constituem uma família multigênica com 10 genes diferentes em *A. thaliana* (*AtrbohA* – *AtrbohJ*). NADPH oxidases são proteínas transmembrana que produzem O_2^- no apoplasto que dismuta para H_2O_2 espontaneamente ou pela ação da superóxido dismutase (SOD) (SUZUKI et al., 2011; O'BRIEN et al., 2012; BAXTER et al., 2014).

Estudos vem demonstrando a importância dos genes *AtrbohD* e *AtrbohF* na resistência de plantas a patógenos. Assim, por exemplo, a aplicação de oligogalacturonídeos, elicitores de defesa endógenos

produzidos pela degradação parcial da pectina presente na parede celular vegetal, induzem um forte acúmulo de ROS dependente do gene *AtrbohD* em *A. thaliana* (GALLETTI et al., 2009). A produção de ROS e a ativação da expressão de genes de defesa pela aplicação de metil jasmonato é dependente de genes *AtrbohD* e *AtrbohF* funcionais (MARUTA et al., 2011). Por outro lado, o fechamento estomático induzido pela aplicação de ácido abscísico é parcialmente e completamente afetado nos mutantes *AtrbohF* e *AtrbohD/F*, respectivamente. Além disso, o mutante duplo (*AtrbohD/F*) não acumula cálcio citosólico e ROS (KWAK et al., 2003). Mutantes de *A. thaliana* *AtrbohD* e *AtrbohF* infectados por *Pseudomonas syringae* e *Peronospora parasitica* mostram que o primeiro gene é responsável principalmente pela produção de ROS em resposta a patógenos virulentos, enquanto que, o segundo atua na regulação da reação de hipersensibilidade (TORRES et al., 2002).

Mutações nos genes *Rboh* aumentam a susceptibilidade de plantas a infecção por *Sclerotinia sclerotiorum* (PERCHEPIED et al., 2010). Além disso, estudos relatam que a tiamina ativa a via de sinalização de ROS dependente de NADPH oxidases afetando o desenvolvimento do patógeno. Trabalhos adicionais sugerem que o cálcio citosólico e proteínas quinases sensíveis a estaurosporina estão envolvidas na ativação de NADPH oxidases pela tiamina. Além destas respostas, a tiamina induz a deposição de lignina e fechamento estomático (ZHOU et al., 2013).

Para manter a concentração de ROS abaixo de níveis tóxicos, as plantas evoluíram mecanismos enzimáticos e não enzimáticos complexos de detoxificação. Os mecanismos antioxidantes não enzimáticos incluem ascorbato, glutatona, tocoferol, flavonóides, alcalóides e carotenóides. Os mecanismos enzimáticos de remoção de ROS incluem a ascorbato peroxidase (APX), SOD, guaiacol peroxidase (GPX), glutatona redutase (GSR) e catalase (CAT). A SOD atua na primeira linha de defesa contra ROS dismutando o O_2^- em H_2O_2 , que, em seguida, é convertido em H_2O pela ação da CAT. A GPX oxida doadores de elétrons aromáticos tais como guaiacol e pirogalol às custas de H_2O_2 . A GSR catalisa a redução da glutatona dissulfeto previamente oxidada pelas ROS, de volta a glutatona (APEL; HIRT, 2004; MITTLER et al., 2004).

O papel específico de cada enzima tem sido explorado por meio de transgenia. Assim, por exemplo, a superexpressão de SOD em cloroplastos não aumenta a tolerância de plantas de fumo ao estresse oxidativo (ALLEN, 1995). Por outro lado, a expressão de uma SOD extraída de ervilhas aumenta a resistência aos danos causados na

membrana celular pela aplicação de metil viologênio em fumo (ALLEN, 1995).

A catalase é indispensável para a tolerância ao balanço oxidativo pois, plantas transgênicas de fumo sem esta enzima acumulam mais ROS em resposta a estresses bióticos e abióticos (WILLEKENS et al., 1997). Estudos demonstram que plantas deficientes na atividade de CAT são mais suscetíveis ao estresse induzido por paraquat, sal e ozônio (WILLEKENS et al., 1997). Por outro lado, a superexpressão de um gene que codifica para CAT em *Brassica juncea* aumenta a tolerância ao estresse oxidativo induzido pelo cádmio em fumo (GUAN et al., 2009).

A guaiacol peroxidase é amplamente aceita como a enzima do estresse já que várias condições ambientais adversas induzem um incremento em sua atividade (SHARMA et al., 2012). Por exemplo, Radotic et al. (2000) encontraram correlação entre a atividade de GPX e reações oxidativas em condições de toxicidade por metais. Os autores sugerem que a GPX pode ser utilizada como biomarcador para doses subletais de metais em plantas. A tolerância de plantas de cártamo a altas concentrações de sal no solo tem sido atribuída a atividade elevada de GPX (TAYEFI-NASRABADI et al., 2011).

As enzimas do ciclo do ascorbato/glutationa também exercem papéis importantes na proteção de plantas contra os danos causados pelo estresse oxidativo. Vários trabalhos relatam incremento na atividade de APX em resposta a estresses abióticos tais como seca, salinidade, baixas temperaturas, toxicidade por metais e radiação UV (SHARMA et al., 2012). Por exemplo, a superexpressão de um gene que codifica para APX de ervilha ameniza os danos oxidativos causados em plantas de tomate por baixas temperaturas e altas concentrações de sal (WANG et al., 2005). Da mesma forma, a superexpressão do gene tApX aumenta a tolerância de plantas fumo e *A. thaliana* ao estresse oxidativo (YABUTA et al., 2002). A correlação entre o incremento na atividade de GSR e resistência ao estresse oxidativo também tem sido relatada na literatura. Assim, por exemplo, a superexpressão do gene que codifica para a GSR aumenta o conteúdo foliar de ascorbato e a tolerância ao estresse oxidativo em plantas de fumo e álamo (AONO et al., 1993; FOYER et al., 1995).

3.6. CARBOIDRATOS NA PROTEÇÃO DE PLANTAS CONTR PATÓGENOS

Os carboidratos produzidos pela fotossíntese são amplamente conhecidos pelo seu papel como fontes vitais de energia e estruturas de

carbono para compostos orgânicos e componentes de reserva. Além disso, estudos recentes mostram que os carboidratos exercem uma função essencial como moléculas sinalizadoras, atuando de forma semelhante aos hormônios (MOGHADDAM; VAN DEN ENDE, 2012. TROUVELOT et al., 2014).

O envolvimento de carboidratos como moléculas sinalizadoras na defesa de plantas levou ao desenvolvimento dos conceitos de “imunidade doce” (“*sweet-immunity*”) e “defesa incrementada por açúcares” (“*sugar-enhanced defense*”). De acordo com esses conceitos, pequenos açúcares (mono, di e pequenos oligossacarídeos) tais como sacarose, trealose e rafinose podem ativar respostas de defesa protegendo plantas contra patógenos (TROUVELOT et al., 2014). Assim, por exemplo, a adição de sacarose ao meio de cultura aumenta o teor de isoflavonóides e a atividade de PAL protegendo plantas de tremçoço (*Lupinus luteus*) contra *Fusarium oxysporum* f. sp. *lupini* (MORKUNAS et al., 2005). A presença de sacarose ou glicose no meio de cultivo, induz a expressão das proteínas relacionadas à patogênese PR-1, PR-2 e PR-5 em uma via dependente do ácido salicílico em culturas de células de *A. thaliana* (THIBAUD et al., 2004). Por outro lado, esses açúcares induzem a expressão de PR-Q e PAR-1 em folhas de tabaco de forma independente do ácido salicílico (HERBERS et al., 1996). O pré-tratamento com sacarose incrementa sistemicamente a expressão dos genes PR-1a, PR-1b, PVZ e PR-5 e protege plantas de arroz contra *Magnaporthe oryzae* (GÓMEZ-ARIZA et al., 2007). A trealose, um açúcar não redutor compostos por duas unidades de glicose, é capaz de proteger plantas de trigo contra o oídio ativando respostas de defesa como a deposição de papilas e incremento na atividade de PAL, peroxidases (REIGNAULT et al., 2001) e lipoxigenase (TAYEH et al., 2014).

O sistema de defesa de plantas também pode ser ativado pela aplicação de polissacarídeos. Assim, por exemplo, Mercier et al. (2001) observaram que, a aplicação de carragenana aciona diferentes sistemas de defesa em plantas de fumo, envolvendo as vias metabólicas de etileno, ácido jasmônico e ácido salicílico. Por outro lado, Sangha et al. (2010) demonstraram que a resistência de *Arabidopsis thaliana* induzida por λ -carragenana à *Sclerotinia sclerotiorum* correlaciona com a expressão de alguns genes da via de sinalização do ácido jasmônico, isto é, AOS, PDF1.2 e PR3, bem como aumenta a atividade de oxalato oxidase. Além disso, λ -carragenana é capaz de induzir resistência em um mutante de *A. thaliana* deficiente em ácido salicílico (*ics1*), sugerindo que indução de resistência é independente deste.

Laminaranas são capazes de estimular reações de defesa em suspensões de células de diversas espécies vegetais, tais como fumo (*Nicotiana tabacum*) (KLARZYNSKI et al., 2000), videira (*Vitis vinifera*) (AZIZ et al., 2003) e arroz (*Oryza sativa*) (INUI et al., 1997). Estas respostas típicas incluem a ativação de proteínas quinases, influxo de Ca^{2+} , explosão oxidativa e alcalinização do meio extracelular, expressão de genes relacionados à defesa da planta, aumento da atividade de quitinase e β -1,3-glucanase e, a produção de fitoalexinas. Quando aplicada em fumo ou videira, a laminarana induz, sem desencadear reação de hipersensibilidade, a acumulação de fitoalexinas e a expressão de um conjunto de proteínas PR (KLARZYNSKI et al., 2000; AZIZ et al., 2003). Isso acontece porque oligossacarídeos obtidos da laminarana mimetizam o ataque de um patógeno, pois estão naturalmente envolvidos nos mecanismos de reconhecimento celular, nas interações de planta-patógeno exógenas (resultantes da degradação das paredes celulares de fungos) ou endógenas (calose fragmentada no hospedeiro) (KLARZYNSKI et al., 2000).

O reconhecimento de açúcares pelas plantas é um processo altamente complexo e necessita de investigações adicionais. Até o presente momento, sabe-se que hexoses são reconhecidas por hexoquinases tal como o sensor intracelular de glicose HXK 1 em *A. thaliana* (SHEEN et al., 1999; SMEEKENS, 2000; ROLLAND et al., 2006). Por outro lado, vias de reconhecimento independentes de hexoquinases têm sido relatadas (LALONDE et al., 1999; SHEEN et al., 1999). Aparentemente, a sacarose e outros dissacarídeos são reconhecidos a nível de membrana plasmática (ROLLAND et al., 2006). Porém, a sacarose também pode ser hidrolisada por invertases apoplásticas resultando em hexoses que serão reconhecidas por receptores de membrana ou citosólicos (SHEEN et al., 1999).

3.7. ULVANA

A ulvana é um heteropolissacarídeo sulfatado solúvel em água, extraído das paredes celulares de algas marinhas do gênero *Ulva* representando de 8 a 29% do peso seco da alga. Espécies deste gênero, popularmente conhecidas como “alface do mar”, encontram-se amplamente distribuídas ao redor do mundo e vêm sendo utilizadas, ainda em pequena escala, na alimentação humana, medicina e agricultura. Por outro lado, indivíduos desta espécie estão frequentemente envolvidos em problemas ecológicos ao longo de áreas costeiras, devido a sua grande proliferação em ambientes eutrofizados, principalmente em regiões

quentes do globo (CLUZE et al., 2004; LAHAYE; ROBIC, 2007; PAULERT et al., 2007 e 2009).

A ulvana é composta por ramnose, xilose, glicose manose, galactose e ácidos urônicos. Estes açúcares estão estruturalmente agrupados em dois principais dissacarídeos repetitivos, os ácidos ulvanobiorônicos tipo A [(→4)-β-D-GlcA-(1→4)-α-L-Rha 3S-(1→)] e tipo B [(→4)-α-L-IdoA-(1→4)-α-L-Rha 3S-(1→)] (Figura 2). No entanto, a composição do polissacarídeo pode ser mais complexa e sofrer variações de ordem taxonômica e/ou ecofisiológica (LAHAYE; ROBIC, 2007; PAULERT et al., 2007; ROBIC; SASSI; LAHAYE, 2008; STADNIK; DE FREITAS, 2014).

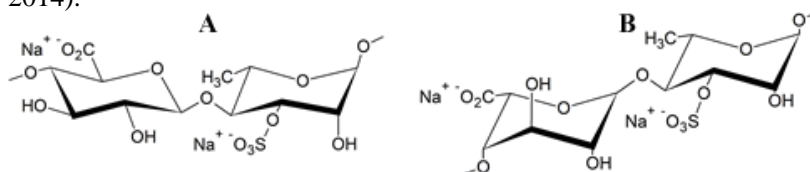


Figura 1.4. Estrutura dos dois principais dissacarídeos constituintes da ulvana. Adaptado de Robic et al. (2009) e Stadnik e de Freitas (2014).

Diversos trabalhos publicados até o presente momento demonstram que a ulvana tem potencial para controlar a ferrugem (*Uromyces appendiculatus*) (BORSATO et al., 2010), antracnose (*C. lindemuthianum*) (PAULERT et al., 2009; DE FREITAS; STADNIK, 2012) e o oídio (*E. polygoni*) (JAULEAU et al., 2011) do feijoeiro. Além disso, o polissacarídeo tem mostrado resultados promissores com outras espécies de plantas, tais como em macieira no controle da mancha foliar de *Glomerella* (*C. gloeosporioides*) (ARAÚJO et al., 2008) e em trigo (*Triticum aestivum*) e cevada (*Hordeum vulgare*) contra o oídio (PAULERT et al., 2010). Na Tabela 1 encontram-se sumarizados efeitos da ulvana em diferentes patossistemas testados até o momento.

Tabela 1.1. Efeitos da aplicação foliar de ulvana ou do extrato de algas do gênero *Ulva* contendo o polissacarídeo sobre doenças em diferentes patossistemas testados.

| Hospedeiro | Patógeno | Efeito | Referência |
|--------------|---|-----------------|--|
| Alfafa | <i>Colletotrichm trifolii</i> | Reduz | Cluzet et al. (2004) |
| Arabidopsis | <i>Alternaria brassicicola</i> | Reduz | Stadnik et al. (2009) |
| | <i>Burkholderia cepacia</i> | NA ¹ | Wordell Filho et al. (2007) |
| Cebola | <i>Peronospora destructor</i> | NA | Wordell Filho et al. (2007) |
| Cebolinha | <i>Alternaria porri</i> | Reduz | Araújo et al. (2012) |
| Cereais | <i>Blumeria graminis</i> | Reduz | Paulert et al. (2010) |
| | <i>Colletotrichum lindemuthianum</i> | Reduz | Paulert et al. (2009) |
| Feijoeiro | <i>Erysiphe polygoni</i> | Reduz | Jaulneau et al. (2011) |
| | <i>Uromyces appendiculatus</i> | Reduz | Borsato et al. (2010) |
| Macieira | <i>Colletotrichum gloeosporioides</i> | Reduz | Araújo et al. (2008) |
| | <i>Penicillium expansum</i> | Reduz | Abouraïcha et al. (2015) |
| Maçã (fruto) | <i>Botrytis cinerea</i> | Reduz | Abouraïcha et al. (2015) |
| Pepino | <i>Spherotheca fuliginea</i> | Reduz | Jaulneau et al. (2011) |
| Tomateiro | <i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> | Reduz | El Modafar et al. (2012) |
| | <i>Erysiphe necator</i> | Reduz | Jaulneau et al. (2011) |
| Videira | <i>Plasmopara viticola</i> | Reduz/ NA | Galvão et al. (2006) Peruch et al. (2007) |
| | <i>Sphaceloma ampelinum</i> | Reduz | Galvão et al. (2006) |

¹ Não afeta.

Em experimentos conduzidos em casa-de-vegetação, a aplicação foliar preventiva de ulvana ($10 \text{ mg}\cdot\text{mL}^{-1}$) reduziu a severidade da antracnose (*C. lindemuthianum*) em cerca de 50% e protegeu as plantas por até 9 dias após o tratamento (SCHONS et al., 2011). Considerando que a ulvana possui efeito sistêmico, mas não inibe o crescimento micelial e a germinação dos conídios do fungo *in vitro* e *in vivo*, o controle tem sido atribuído à indução da resistência da planta (PAULERT et al., 2009; DE FREITAS; STADNIK, 2012). De acordo com CLUZET et al. (2004), a aplicação preventiva de ulvana em plantas de alfafa elicitamente múltiplas respostas de defesa em resposta à infecção por *Colletotrichum trifolii*. Estas respostas incluíram a biossíntese de

fitoalexinas e de proteínas relacionadas à patogênese (PRPs) e, apresentaram sua máxima elicitação dois dias após a pulverização de 500 mg·mL⁻¹.

Não se constatou fitotoxicidade do extrato, nem alteração do metabolismo primário da planta devido a sua aplicação. A elicitação de respostas de defesa pela aplicação de ulvana tem sido relatada em outros patossistemas como, por exemplo, em feijoeiro contra *U. appendiculatus* e *C. lindemuthianum*, onde o polissacarídeo incrementa a atividade pós-infeccional de glucanases e peroxidases, respectivamente (BORSATO et al., 2010; DE FREITAS; STADNIK, 2012).

A elicitação de respostas de defesa por ulvana nas plantas parece ser dependente do genótipo e patógeno envolvidos. Assim por exemplo, BORSATO et al. (2010) observaram que a pulverização de ulvana aumentou a atividade de glucanases somente nas plantas de feijoeiro da cv. Pérola (moderadamente suscetível), mas não em Juriti, uma cultivar com maior nível de suscetibilidade a ferrugem. Ainda em feijoeiro, a ulvana elevou a atividade de peroxidases em plantas resistentes a antracnose, mas não em suscetíveis (DE FREITAS; STADNIK, 2012).

A indução de resistência está frequentemente associada com o *priming* onde a planta tratada com o agente indutor torna-se sensibilizada (*primed*) podendo ativar respostas de defesa mais rápidas e intensas se submetidas ao ataque de patógenos (BECKERS; CONRATH, 2007). A capacidade da ulvana de induzir o *priming* foi recentemente relatada (PAULERT et al. 2010), onde a pré-incubação de culturas de células de trigo e arroz com o polissacarídeo amplifica drasticamente a explosão oxidativa elicitada pela aplicação posterior de quitina. Por outro lado, em plantas de feijoeiro, o tratamento com ulvana promoveu, por si só, um incremento de cerca de 50% na atividade de peroxidases e glucanases (DE FREITAS; STADNIK, 2012).

Estudos recentes demonstram que a via de sinalização do ácido jasmônico está envolvida na indução de resistência pela aplicação de ulvana (JAULNEAU et al., 2010). Assim, este polissacarídeo pode ser eficiente contra patógenos necrotróficos, pois esta via de sinalização está envolvida em respostas de defesa contra indivíduos deste grupo (GLAZEBROOK, 2005). No entanto, tem-se demonstrado que a ulvana pode controlar, além de patógenos necrotróficos (ARAÚJO et al., 2012), biotróficos (BORSATO et al., 2010; PAULERT et al., 2010) e hemibiotróficos (PAULERT et al., 2009; DE FREITAS; STADNIK, 2012). Desta forma, pode-se sugerir que este polissacarídeo possa atuar em mais de uma via de sinalização já que a expressão da proteína relacionada

à patogênese PR-1 (marcador da via do ácido salicílico) tem sido observada após o tratamento com ulvana (CLUZET al., 2004).

3.8. RELAÇÃO ENTRE ESTRUTURA, COMPOSIÇÃO E ATIVIDADE BIOLÓGICA DE POLISSACARÍDEOS ALGAIS

A atividade biológica de polissacarídeos sulfatados extraídos de algas marinhas depende de vários fatores incluindo peso molecular, composição química, conformação de cadeia e conteúdo e posição de grupamentos sulfato. Conseqüentemente, conhecer os efeitos destes fatores na atividade de polissacarídeos vem trazendo oportunidade para a obtenção de moléculas com atividades elicitoras novas e melhoradas. Por exemplo, o pré-tratamento de plantas de *A. thaliana* com a altamente sulfatada (35%) λ -carragenana induz resistência a *Sclerotinia sclerotiorum* resultando em menos dano foliar, enquanto que, a ι -carragenana, com um teor de sulfato menor (30%), aumenta a severidade da doença (SANGHA et al., 2010). A laminarana, um polissacarídeo linear não sulfatado composto por β -1,3 glucanas, induz a expressão de proteínas dependentes da via de sinalização do etileno, enquanto que, o seu derivado sulfatado ativa proteínas tanto da via do etileno quanto do ácido salicílico (MÉNARD et al., 2004). De acordo, a laminarana sulfatada mostrou maior eficiência na redução dos sintomas do TMV em plantas de tabaco do que o polissacarídeo natural não sulfatado (MÉNARD et al., 2005).

Mudanças na atividade biológica devido a modificações químicas também têm sido relatadas para a ulvana. Por exemplo, oligoulvanas (28,2 kDa) tem melhor atividade antioxidante *in vitro* que a ulvana (151,7 kDa). De acordo com El Modafar et al. (2012), a infiltração de oligoulvanas em plântulas de tomate aumenta de forma mais eficiente a atividade de fenilalanina amônia-liase (FAL) e o acúmulo de compostos fenólicos e ácido salicílico em tecidos não tratados.

Ulvanas sulfatadas quimicamente (30,8 e 32,8% de sulfato) exibem um incremento na atividade antioxidante, poder redutor e habilidade quelante de íons de ferro *in vitro* quando comparadas com a ulvana natural (QI et al., 2005). O grau de sulfatação da ulvana parece afetar a sua atividade elicitora também em plantas, porque a sua completa dessulfatação suprime a sua habilidade de aumentar a atividade de PAL em plântulas de tomate infectadas com *Fusarium oxysporum* f.sp. *lycopersici* (EL MODAFAR et al., 2012).

4. CAPÍTULO 2 – ULVANS INDUCE RESISTANCE AGAINST PLANT PATHOGENIC FUNGI INDEPENDENTLY OF ITS SULFATION AND POLYMERIZATION DEGREE

Mateus B. de Freitas¹, Caroline Hawerth¹, Matheus Bússolo¹, Luciana G. Ferreira², Maria Eugênia R. Duarte², Miguel D. Nosedá² and Marciel J. Stadnik^{1*}

¹ Laboratory of phytopathology, Federal University of Santa Catarina, 88034-001, Florianópolis, SC, Brazil

² Department of biochemistry and molecular biology, Federal University of Paraná, 81531-990, Curitiba, PR, Brazil.

*Corresponding author. Tel: +55 48 3721 5338; fax: +55 48 3721 5335. E-mail addresses: mateus.brusco@ufsc.br (M.B. de Freitas), mdn@ufpr.br (M.D.Nosedá) marciel.stadnik@ufsc.br (M.J.Stadnik).

ABSTRACT

The present work aimed to evaluate the defense responses induced by chemically sulfated and partially depolymerized ulvans in *Arabidopsis thaliana* plants against phytopathogenic fungi *Alternaria brassicicola* and *Colletotrichum higginsianum*. The sulfated polysaccharide (18.9% of sulfate) and its chemically sulfated derivatives (20.9-36.6%) similarly reduced the severity of both pathogenic fungi infection. Partially depolymerized ulvans DU1, DU3, DU5 and DU7 were more efficient than native ulvan to reduce the fungal disease. However, DU6 and DU8 oligoulvans failed to control the pathogen suggesting that the efficiency in inducing resistance in *A. thaliana* may be related to other structural features rather than chain size. Also, L-rhamnose, reduced the severity of both fungi at a similar extent as the polysaccharide did. Collectively, our results suggest that ulvan induces resistance against both fungal pathogens independently of its sulfation and polymerization degree and that rhamnose seems to play an important role in the polysaccharide-induced resistance.

Keywords: *Arabidopsis thaliana*, *Alternaria brassicicola*, *Colletotrichum higginsianum*, ulvan, induced resistance, sulfate content, polymerization degree.

4.1. INTRODUCTION

Seaweeds represent a rich but still underexploited source of bioactive compounds. Among them, carbohydrates are probably the most abundant organic compounds in the oceans with a great molecular diversity (Alves, Sousa & Reis, 2013; Khan et al., 2009; Sharma, Fleming, Selby, Rao & Martin, 2014; Stadnik & de Freitas, 2014). Ulvan is a unique water-soluble sulfated heteropolysaccharide extracted from the cell walls of the green macroalgae *Ulva* spp. It has been one of the most studied algal carbohydrates with potential application in several areas, including agriculture (Alves et al., 2013; Stadnik & de Freitas, 2014).

Ulvan is composed by rhamnose (16-45%), xylose (2-12%), glucose (0.5-6.5%), uronic acid (6.5-19%) and sulfate (16-23%). This sulfated polysaccharide is structurally constituted by two main repeating disaccharides, the ulvabiuronic acid type A [(\rightarrow 4)- β -D-GlcA-(1 \rightarrow 4)- α -L-Rha 3S-(1 \rightarrow)] and type B [(\rightarrow 4)- α -L-IdoA-(1 \rightarrow 4)- α -L-Rha 3S-(1 \rightarrow)] (Alves et al., 2013; Lahaye & Robic, 2007; Stadnik & de Freitas, 2014). Ulvan is known to exhibit different biological activities in the pharmaceutical and biomedical context (Alves et al., 2013). In crop sciences, studies have revealed that it can be applied in to stimulate growth and defense of plants (Alves et al., 2013; Stadnik & de Freitas, 2014).

The current worldwide concern on toxic residues in the environment and public health has fostered the development of clean technologies. In this scenario, the induction of resistance using algal polysaccharides arises as an eco-friendly strategy to control plant diseases. Studies have shown that ulvan is able to protect bean plants against a broad range of fungal diseases such as rust (*Uromyces appendiculatus*) (Delgado, de Freitas & Stadnik, 2013), anthracnose (*Colletotrichum lindemuthianum*) (de Freitas & Stadnik, 2012) and powdery mildew (*Erysiphe poligoni*) (Jaulneau et al., 2011). Moreover, it has shown promising results against powdery mildew (*Blumeria graminis*) on wheat and barley (Paulert, Ebbinghaus, Urluss & Moerschbacher, 2010) and Glomerella leaf spot (*C. gloeosporioides*) on apple (Araújo & Stadnik, 2013).

The biological activity of sulfated polysaccharides from marine algae has been frequently associated with several structural factors including molecular weight, chemical composition, chain conformation, and content and positioning of sulfate groups (Alves et al., 2013; Cassolato et al., 2008; Duarte et al., 2004; Faria-Tischer et al., 2006). As

consequence, knowing how such factors can affect the activity of polysaccharides has provided opportunities for obtaining molecules with new, or even, enhanced elicitor activity (Qi et al., 2005a, 2012). For instance, the pre-treatment with a highly sulfated red seaweed polysaccharide λ -carrageenan (35%) induces resistance to *Sclerotinia sclerotiorum* resulting in less foliar damage while the ι -carrageenan, with a lower degree of sulfation (30%), increases disease severity (Sangha, Ravichandran, Prithiviraj, Critchley & Prithiviraj, 2010). Laminarin, an unsulfated β -1,3 linear glucan induces the expression of ethylene-dependent proteins while its sulfated derivative can activate the expression of both ethylene- and salicylic acid-dependent proteins (Ménard et al., 2004). Accordingly, sulfated laminarin is more efficient to reduce tobacco mosaic virus-symptoms in tobacco plants than the unsulfated natural polysaccharide (Ménard et al., 2005).

Changes in biological activity due to chemical modifications have also been reported for the algal polysaccharide ulvan. Oligoulvans (28.2 kDa) presented higher *in vitro* antioxidant activity than the native ulvan (151.7 kDa) (Qi et al., 2005a). According to El Modafar et al. (2012), when compared to ulvan, the infiltration of oligoulvans in tomato seedlings increases more efficiently the activity of phenylalanine ammonia-lyase (PAL) in systemic tissues, as well as the accumulation of phenolic compounds and salicylic acid. Oligoulvans were also more efficient to reduce the lesion caused by both *Penicillium expansum* and *Botrytis cinerea* in apple fruit when compared to ulvan (Abouraïcha et al., 2015). In comparison with native ulvan (19.5% of sulfate), chemically sulfated ulvans (30.8 and 32.8% of sulfate) exhibit enhanced scavenging activity on hydroxyl radical, reducing power and chelating ability on ferrous ion (Qi et al., 2005b). The degree of sulfation of ulvans seems to affect its elicitor activity on plants as the complete desulfation suppresses its ability to enhance the activity of PAL in tomato seedlings infected by *Fusarium oxysporum* f.sp. *lycopersici* (El Modafar et al., 2012).

Although some information relating the effect of chemical modification of ulvans on biological activity is already available for animals (Qi et al., 2005a, 2005b, 2012), there is a lack of studies regarding plant-pathogen models. Therefore, this work aimed to evaluate the efficiency of chemically sulfated ulvans and oligoulvans to control *Alternaria brassicicola* and *Colletotrichum higginsianum* in *Arabidopsis thaliana*.

4.2. MATERIAL AND METHODS

4.2.1. Obtaining of ulvan and rhamnose

Ulva fasciata Delile samples were harvested in December 2011 at Armação beach (27.4454°S; 48.2956°W) in Florianópolis-SC, Brazil. Ulvan was obtained as previously described by Paulert et al. (2009). Briefly, the ground dried alga (100 g) was autoclaved for 2 h at 110 °C in distilled water (1 L). The resulting aqueous solution was filtered and the polysaccharide precipitated with ethanol (3 v) for 48 h at -20 °C. The precipitate was filtered, washed three times with ethanol and dissolved in distilled water. The solution was dialyzed against tap water for 48 h and against distilled water for another 48 h using 3600 Da Mw cutoff dialysis membrane. The resulting products were concentrated under vacuum, lyophilized, kept at -20 °C until use and named Native Ulvans (NU). L-Rhamnose (99% pure) was purchased from Sigma-Aldrich.

4.2.2. Sulfation of ulvan

Ulvan derivatives with five different degrees of sulfation were produced as described by Ménard et al. (2004), with some modifications. Dry ulvan (2 g; 18.9% of sulfate) was added to 80 mL of formamide and the mixture was stirred at 60 °C for 30 min in order to disperse it into solvent. Sulfation was performed by continuous addition of SO₃-pyridine complex (5.9 g) for 2 h at 60 °C. Stirring was continued for another 2 h. Derivatives with growing sulfate content were obtained by increasing the amount of SO₃-pyridine complex added during the reaction. After cooling to room temperature, the mixture was neutralized with a 2 M NaOH solution and precipitated with ethanol (3 v) for 24 h at -20 °C. The precipitated compound was filtered, washed three times with ethanol and dissolved in distilled water. The solution was dialyzed against tap water for 48 h and against distilled water for another 48 h using 3600 Da Mw cutoff dialysis membrane. The resultant retained material was concentrated under vacuum, lyophilized and kept at -20 °C until use.

4.2.3. Preparation and fractionation of partially depolymerized ulvans

Partially depolymerized ulvans were obtained as described by Lahaye, Ray, Baumberger, Quemener & Axelos (1996). For that, ulvan NU (10 mg mL⁻¹; 18.9% of sulfate) was hydrolyzed in trifluoroacetic acid

(0.1 M) at 100 °C for 75 min. After hydrolysis, the solution was concentrated under vacuum, lyophilized and kept at -20 °C until use. The lyophilized material was dissolved in distilled water (10 mg mL⁻¹) and chromatographed through a Sepharose CL6B column (44 x 1.6 cm, Sigma Aldrich) eluted by distilled water (30 mL h⁻¹). Samples were collected every 2 mL rendering the partially depolymerized fractions DU-1 to DU-8.

4.2.4. Analytical methods

Total carbohydrates were estimated by the phenol-sulfuric acid method according to Dubois, Gilles, Hamilton, Rebers & Smith (1956), using rhamnose as standard.

Sulfate was estimated according to the turbidimetric method of Dodgson & Price (1962), using potassium sulfate as standard.

4.2.5. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC was carried out with a 1 mg mL⁻¹ solution of the polysaccharide, using a multidetection equipment with a Waters 2410 differential refractometer (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector adapted on-line. Four Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled to the multi-detection equipment. The eluent was a solution of 0.1 M NaNO₂ containing NaN₃ (0.2 g L⁻¹). All experiments were carried out at 25 °C.

4.2.6. Nuclear magnetic resonance (NMR)

NMR analyses were performed on a Bruker Advance DRX400 NMR spectrometer equipped with a 5-mm multinuclear inverse detection probe. The base frequency was 100.63 and 400.13 MHz for ¹³C and ¹H nuclei, respectively. Analyses were recorded at 70 °C. For ¹³C NMR spectra, the samples were dissolved in D₂O (40 mg mL⁻¹). For ¹H and 2D NMR experiments, samples were deuterium exchanged with D₂O 99.9% (3×) and then dissolved in D₂O (20-25 mg mL⁻¹). ¹³C and ¹H NMR acquisition parameters were previously reported (Ascêncio, Orsato, França, Duarte & Nosedá, 2006). Chemical shifts are expressed relative to an internal acetone standard at 31.45 and 2.225 ppm for ¹³C and ¹H NMR spectra, respectively.

4.2.7. Plant growth conditions and treatment

Arabidopsis thaliana wild-type ecotype Col-0 seeds were sown on pots (6 x 6 x 6 cm) containing a mixture of fertilized black soil and vermiculite (1:1, v/v). Seeds were vernalized for two days at 4 °C after sowing. About 20 days after incubation in a growth chamber (23 ± 3 °C, 12 h of light and a photon flux density of 160 µE m⁻² sec⁻¹) plants were transplanted to new pots and grown under the same conditions.

Shortly before use, rhamnose, partially depolymerized ulvans, native ulvan and its derivatives with different sulfate content were completely dissolved in distilled water under continuous stirring at room temperature. Six-weeks-old *A. thaliana* plants were sprayed once (i.e. three days before inoculation) with water (control), L-rhamnose (0.5 mg mL⁻¹), native ulvan, its chemically sulfated derivatives or partially depolymerized products (1 mg mL⁻¹). A volume of 0.9 mL was delivered per plant.

4.2.8. Inoculation and disease evaluation

The strains CBS 125088 and IMI 349063 of *Alternaria brassicicola* and *Colletotrichum higginsianum*, respectively were used in the experiments. *A. brassicicola* and *C. higginsianum* were grown to sporulate at 25 °C and 12 h photoperiod for 15 days on Petri dishes containing V8 and oat meal culture media, respectively. Thereafter, Petri dishes were flooded with 10 mL of distilled water and the conidial suspension was collected and filtered twice to remove mycelial fragments. The number of conidia was estimated using a Neubauer counting chamber and inoculum concentration was adjusted to 1 x 10⁵ conidia mL⁻¹ with distilled water.

Plants were inoculated by placing on each leaf a 10 µL drop of a homogeneous suspension of *A. brassicicola* or *C. higginsianum*. Plants were kept under a highly humid condition (humidity > 90%) for 48 h and the severity of both diseases was assessed five days after inoculation by measuring lesion diameter using a caliper rule.

4.2.9. Experimental design and statistical analysis

The experiment testing the efficiency of chemically sulfated ulvans and oligoulvans was carried out with three replications (pots). On the other hand, the effect of L-rhamnose on the severity of *A. brassicicola*

and *C. higginsianum* was evaluated with ten replications. The experiments were repeated at least twice with similar results.

After verification of homogeneity of the variances of the datasets, data were subjected to a one-way analysis of variance. Tukey's test at 5% of significance level was used for separation of means. When necessary, data transformation was used before analysis in order to meet ANOVA assumptions. The statistical analysis was performed using the software Statistica (v. 10).

4.3. RESULTS

4.3.1. Extraction of ulvans, sulfation and partial depolymerization

Native ulvan fraction (NU) was obtained from *U. fasciata* by hot-water extraction followed by precipitation with the addition of ethanol. In these conditions, NU fraction presented yield of 30% based on dried and milled seaweed (Table 1). Chemical analysis revealed that the sulfation degree of this polysaccharide was 18.9%.

After NU chemical sulfation, five derivatives with sulfate content varying from 20.9 to 36.6% were obtained (Table 1). About 20-25% of the polysaccharide was recovered after the sulfation process.

Partial depolymerization of NU followed by chromatographic fractionation gave rise to eight partially depolymerized fractions (Table 1). These fractions were named DU1 to DU8 (1.4 – 8.0% yield). Due to the low yield, sulfate content was not determined for these samples.

Table 2.1. Yield and sulfation degree of the native ulvan (NU) and its chemically sulfated and depolymerized derivatives.

| Fraction ^a | Yield (%) | Sulfate (%) |
|-----------------------|-------------------|-------------------|
| NU | 32.2 ^b | 18.9 |
| SU1 | 22.5 ^c | 20.9 |
| SU2 | 20.0 ^c | 28.0 |
| SU3 | 23.4 ^c | 28.6 |
| SU4 | 22.1 ^c | 33.5 |
| SU5 | 24.7 ^c | 36.6 |
| DU1 | 1.4 ^c | n.d. ^d |
| DU2 | 1.8 ^c | n.d. |
| DU3 | 2.0 ^c | n.d. |
| DU4 | 2.2 ^c | n.d. |
| DU5 | 8.0 ^c | n.d. |
| DU6 | 3.4 ^c | n.d. |
| DU7 | 5.4 ^c | n.d. |
| DU8 | 2.8 ^c | n.d. |

^a NU: Native ulvan, SU: Chemically sulfated ulvan, DU: Partially depolymerized ulvan.

^b Yield (%) based on a dried and milled seaweed.

^c Yield (%) based on NU fraction.

^d n.d.: not determined.

The HPSEC-RI analyses of the native ulvan NU and of its chemically sulfated derivatives SU3 and SU5 showed similar chromatograms with a broad bimodal peak indicating heterogeneous elution profiles (Figure 1A). The HPSEC-RI analysis of the partially depolymerized ulvans DU2, DU3, DU4 and DU5 showed that they have low molecular weights, since they were eluted (55-65 mL) close to the total volume of the chromatographic system (70 mL). Since the partially depolymerized ulvans showed heterogeneous elution profiles (Figure 1B), we were unable to determine their molecular weights.

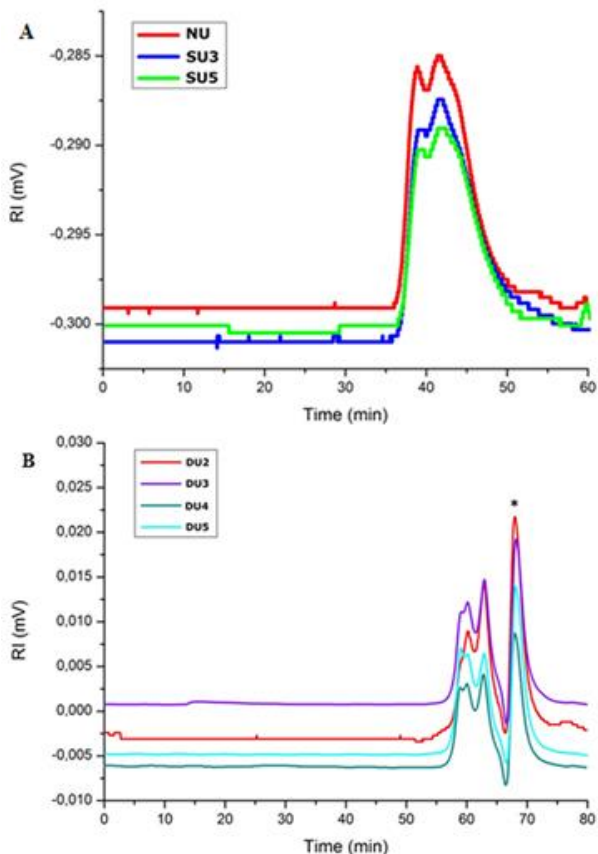


Figure 2.1. HPSEC-RI analysis of the native ulvan (NU) and its chemically sulfated products SU3 and SU5 (A) and partially depolymerized ulvans DU2, DU3, DU4 and DU5 (B). *Peak corresponding to salt.

The ^{13}C NMR spectra of the native ulvan NU (18.9% of NaSO_3) and the chemically sulfated ulvans SU3 (28.6%) and SU5 (36.6%) are shown in Figure 2. SU3 and SU5 ^{13}C NMR spectra are complex, showing broad signals consistent with the α - and β -effects promoted by chemical sulfation of free hydroxyl groups of native ulvan. The increment of sulfate groups on SU3 and SU5 can be corroborated by the comparison between NMR spectra of the native and chemically sulfated polysaccharides (Figures 2 and 3).

NU ^{13}C - and ^1H -NMR assignments were determined using spectral information together with literature data showing that the native

ulvan NU is constituted by three main diads, as follows: 4-linked β -D-glucuronic acid-(1 \rightarrow 4)-linked to α -L-rhamnose 3-sulfate (C-1/H-1 at 104.4/4.65 and 101.0/4.85 ppm, respectively) (Lahaye, 1998; Lahaye, Brunel & Bonnin, 1997; Lahaye, Inizan & Vigoureux, 1998); 4-linked β -D-xylose-(1 \rightarrow 4)-linked to α -L-rhamnose 3-sulfate (C-1/H-1 at 104.7/4.65 and 98.9/4.91 ppm, respectively) and 4-linked β -D-xylose 2-sulfate-(1 \rightarrow 4)-linked to α -L-rhamnose 3-sulfate (C-1/H-1 at 100.5/4.97 and 99.3/4.95 ppm, respectively) (Lahaye, 1998). The complete assignments of these diads are shown in Table 2.

In SU3 and SU5 ^{13}C NMR spectra, the introduction of sulfate groups at C-2 of the 4-linked α -L-rhamnosyl 3-sulfate units present in the native polysaccharide can be observed by the increase of the signals at 77.1-77.6 ppm (C-2 of 4-linked α -L-rhamnosyl 2,3-disulfate) and the concomitant decrease of the signal at 70.4 ppm (C-2 of 4-linked α -L-rhamnose 3-sulfate). Additionally, SU3 and SU5 spectra presented C-1 (groups of signals centered at 98.6, 98.3 and 97.3 ppm) and C-3 (~77.6 ppm) of the 4-linked 2,3-disulfate rhamnosyl units upfield shifted (β -effect) when compared to C-1 (101.0, 99.3 and 98.9 ppm) and C-3 (79.4, 79.6 ppm) of the 4-linked 3-sulfate rhamnosyl units. This C-2 downfield shift (α -shift) of +6.5-7.2 ppm after C-2 sulfation, as well as C-1 and C-3 upfield shift are in good agreement with the effect promoted by sulfate groups at C-2 of the 3-linked rhamnosyl units of sulfated heterorhamnans obtained from *Gayralia oxysperma* (Ropellato et al., 2015).

Sulfation of the 4-linked xylosyl units was observed on SU3 and SU5 ^{13}C NMR spectra by the increase and/or appearance of the signals at ~78.9 and ~78.4 ppm, corresponding to C-2 and C-3, respectively, of the 4-linked xylosyl 2,3-disulfate units and the concomitant decrease of the signals at 74.8 and 75.3 ppm (C-2 and C-3, respectively, of the 4-linked xylosyl units). These assignments were made considering the simultaneous α - and β -shift effects suffered by C-2 and C-3 after sulfation. In addition, C-1 and C-4 of these disulfated xylose residues (~100.5 and ~72.1 ppm, respectively) are ~4.2 and ~3.4 ppm upfield shifted when compared to C-1 and C-4 of the 4-linked non-sulfated xylosyl units (104.7 and 75.5 ppm, respectively). These data are in accordance with those described by Kovac et al. (1980) and Lahaye (1998) for xylooligosaccharides and oligosaccharides obtained from *Ulva rigida* ulvan, respectively.

The anomeric region of SU3 and SU5 ^{13}C NMR spectra also showed the presence of signals at 104.1-104.4 ppm corresponding to 4-linked glucuronic acid not sulfated at C-2 and groups of signals at ~103.7 and ~103.3 ppm attributed to glucuronic acid 2,3-disulfate and 2-sulfate,

respectively. The sulfation of C-2 and/or C-3 of these acidic units is also observed in the ring region of SU3 and SU5 spectra due to the decrease of the signals at 75.1 and 75.3 ppm (C-2 and C-3 of 4-linked glucuronic acid, respectively) together with the appearance of two groups of signals at ~81.5 and ~82.5 ppm, corresponding to C-2 and/or C-3 of 4-linked glucuronic acid 2-, 3- and/or 2,3-sulfated. Additionally, SU3 and SU5 spectra showed the appearance of the signal at ~79.1 ppm, which was attributed to C-4 of 4-linked glucuronic acid 2- and 2,3-sulfated, concomitant with the decrease of the signal at 80.2 ppm (C-4 of 4-linked glucuronic acid). These data are in agreement with Roger et al. (2004).

The chemical sulfation of SU3 and SU5 was also verified in the HSQC spectra of these fractions. In the anomeric regions (Figure 3), the presence of the new correlations at ~97.3/5.16 and 98.3-98.6/5.19-5.40 ppm were attributed to C-2 sulfation of the 4-linked rhamnosyl 3-sulfate units constituent of the different diads present in the polysaccharide. Moreover, the new correlation at 103.2/4.75-4.87 ppm corresponded to 4-linked glucuronic acid units sulfated at C-2, C-3 and/or C-2 and C-3. The correlation corresponding to sulfated xylosyl units was overlapped and could not be assigned.

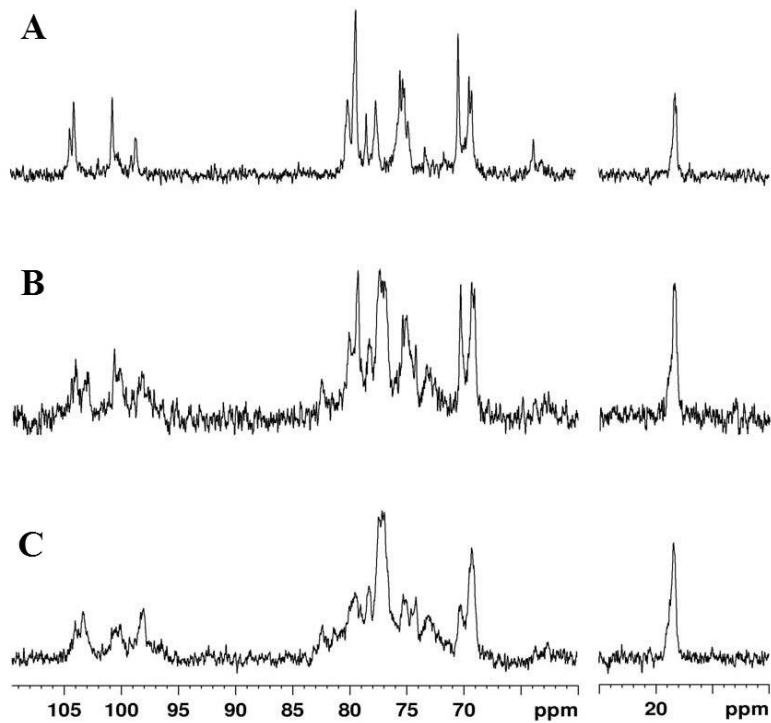


Figure 2.2. ^{13}C NMR spectra of the native ulvan NU (A) and chemically sulfated ulvans SU3 (B) and SU5 (C).

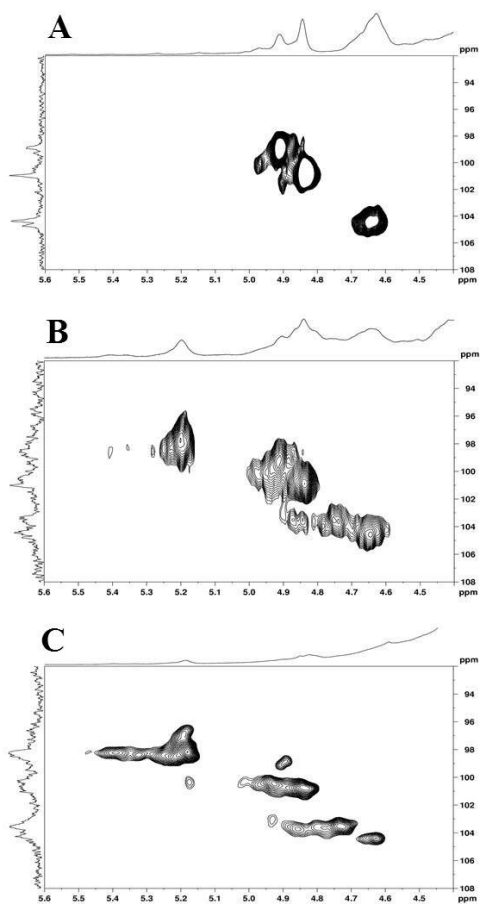


Figure 2.3. HSQC NMR spectra (anomeric region) of the native ulvan NU (A) and chemically sulfated ulvans SU3 (B) and SU5 (C).

Table 2.2. Chemical shift (ppm) assignments of ¹³C and ¹H NMR spectra of NU fraction.

| Diads | Chemical shift (ppm) | | | | | |
|----------------------|----------------------|---------------|--------------|----------------|--------------------|-------------------|
| | C-1 H-1 | C-2 H-2 | C-3 H-3 | C-4 H-4 | C-5 H-5 | C-6 H-6 |
| [→4)-β-D-GlcA-(1→ | 104.4 4.65 | 75.1 3.36 | 75.3 3.66 | 80.2 3.66 | 77.7 3.76 | 176.2 - |
| 4)-α-L-Rha 3-S-(1→] | 101.0 4.85 | 70.4 4.25 | 79.4 4.63 | 79.4 3.80 | 69.4 4.17 | 18.2 1.30-1.34 |
| [→4)-β-D-Xyl-(1→ | 104.7 4.65 | 74.8 3.36 | 75.1 3.66 | 75.5 3.62 | 63.7 3.41, 4.10 | - - |
| 4)-α-L-Rha 3-S-(1→] | 98.9 4.91 | 70.4 4.25 | 79.6 4.63 | 78.5/ ~3.81 | 69.2 4.07 | 18.2 1.30-1.34 |
| [→4)-β-D-Xyl 2-S-(1→ | 100.5 4.97 | ~79.5 4.15 | 72.7 3.86 | 75.5 ~3.70 | 63.0 3.50, 4.15 | - - |
| 4)-α-L-Rha 3-S-(1→] | 99.3 4.95 | 70.4 4.25 | 79.6 4.63 | 78.5 ~3.81 | 69.2 ~4.10 | 18.2 1.30-1.34 |

4.3.2. Effect of sulfation degree of ulvans on the infection severity of *Alternaria brassicicola* and *Colletotrichum higginsianum*

The average lesion diameter reached 5.3 and 5.7 mm in *A. brassicicola*- and *C. higginsianum*-inoculated control plants, respectively. In this situation, native ulvan (NU, 18.9% of sulfate) reduced significantly the lesion diameter by 60 and 35% in *A. brassicicola*- and *C. higginsianum*-inoculated plants, respectively (Figure 4). In both inoculated plants, the increase in sulfate content did not change the disease control efficiency of chemically sulfated ulvans when compared to NU (Figure 4).

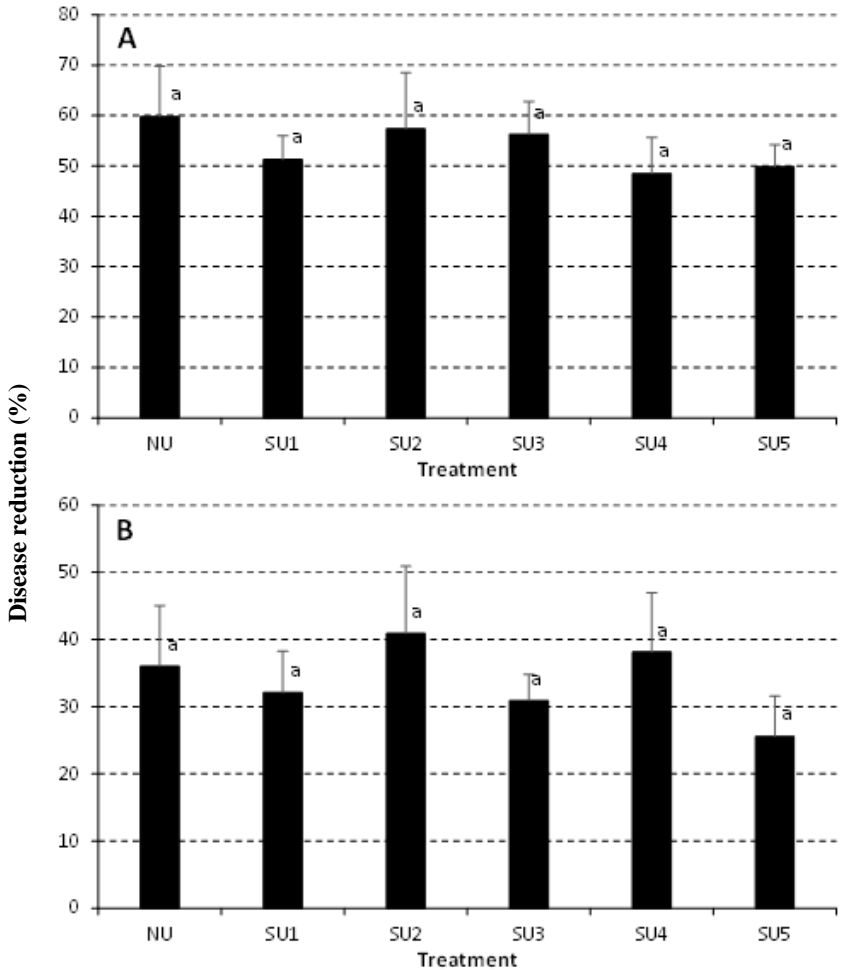


Figure 2.4. Percentage of disease reduction of *Alternaria brassicicola* (A) and *Colletotrichum higginsianum* (B) five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with different sulfate content ulvans (from 18.9 to 36.6%). Disease reduction values were calculated in relation to control plants. Columns with the same letter are not significantly different (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean.

4.3.3. Effect of ulvans molecular weight on the infection severity of *Alternaria brassicicola*

The average lesion diameter reached 2.2 mm in control plants. Ulvan (NU) and the partially depolymerized ulvans DU2 and DU4 similarly reduced the disease severity by 60% (Figure 5). On the other hand, DU1, DU3, DU5 and DU7 reduced the severity of *A. brassicicola* by 90%, differing from NU. Fractions DU6 and DU8 were not able to reduce the severity of *A. brassicicola*.

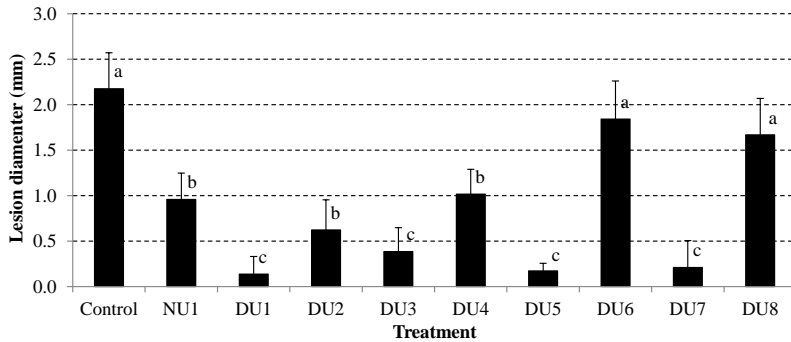


Figure 2.5. Percentage of disease reduction of *Alternaria brassicicola* five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with water (control), ulvan or different partially depolymerized ulvans (DU1 to DU8). Letters indicate significant differences (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean.

4.3.4. Effect of L-rhamnose on the infection severity of *Alternaria brassicicola* and *Colletotrichum higginsianum*

A. thaliana plants previously sprayed with water, NU or L-rhamnose (major monosaccharide component of ulvan), were inoculated with *A. brassicicola* or *C. higginsianum*. In this situation, NU and rhamnose spraying similarly reduced the lesion diameter of both *A. brassicicola* and *C. higginsianum* by 25% (Table 3).

Table 2.3. Lesion diameter of *Alternaria brassicicola* and *Colletotrichum higginsianum* five days after inoculation of *Arabidopsis thaliana* plants previously sprayed with water (control), ulvan (NU) or L-rhamnose.

| Pathogen | Treatment | Lesion diameter (mm) |
|------------------------|------------|----------------------|
| <i>A. brassicicola</i> | Control | 2.3 ^{a*} |
| | NU | 1.9 ^b |
| | L-Rhamnose | 1.8 ^b |
| <i>C. higginsianum</i> | Control | 2.5 ^a |
| | NU | 1.9 ^b |
| | L-Rhamnose | 1.7 ^b |

*Letters indicate significant differences (Tukey's test, $p \leq 0.05$).

4.4. DISCUSSION

In this work, we obtained ulvan derivatives with higher sulfate content than the native polysaccharide NU (18.9% of NaSO_3). The chemically sulfated ulvans SU1-SU5 showed a sulfate degree varying between 20.9 to 36.6% (Table 1). The ^{13}C and HSQC NMR spectra analysis showed that the chemical sulfation process was effective to introduce sulfate on free hydroxyl groups of the native ulvan, and confirmed the different sulfation degrees of NU, SU3 and SU5. In comparison with NU and SU5, SU3 fraction showed the most complex anomeric region in the HSQC spectra. This result is consistent with a higher heterogeneous sulfation pattern of SU3 when compared with those of the native ulvan (NU) and the most sulfated ulvan SU5.

Increasing sulfate content of ulvan did not lead to better disease reduction (Figure 4). Thus, it can be argued that ulvan-induced resistance in *A. thaliana* does not depend on sulfation degree at least against *A. brassicicola* (necrotroph) and *C. higginsianum* (hemibiotroph fungus). However, it seems that some sulfate groups are required for ulvan to induce resistance since El Modafar et al. (2012) reported that unsulfated ulvans are not able to elicit defense reactions in tomato plants.

Although not fully understood, ulvan recognition in plants is proposed to be specifically associated to rhamnose and uronic acid residues (Jaulneau et al., 2010). L-Rhamnose is an uncommon sugar found primarily in plants, algae and some bacteria (Alves et al., 2013). In plants, it is a component of cell wall pectic polysaccharides

rhamnogalactouronans I and II and it is also present in several secondary metabolites including flavonoids, anthocyanins and triterpenoids (Watt, Leof, Harper & Bar-Peled, 2004). As a simple sugar, rhamnose is present in some plants from the *Moringaceae* family (Isitua & Ibeh, 2013). In Gram-negative bacteria such as *Pseudomonas aeruginosa*, rhamnose is present in lipopolysaccharides, the major components (about 75%) of the external membrane (Varnier et al., 2009).

In the present work, eight fractions with reduced molecular weight were obtained after partial hydrolysis of ulvan. Fractions DU1, DU3, DU5 and DU7 reduced the disease more efficiently than the native ulvan (Figure 5). On the other hand, low molecular weight fractions DU6 and DU8 failed to control the pathogen. Thus, it seems that the efficiency in inducing resistance in *A. thaliana* against *A. brassicicola* does not depend on the polymerization degree of ulvans. In contrast, El Modafar et al. (2012) reported that when compared with ulvan, small oligoulvans induced a stronger (*ca.* 2 times) increase in PAL activity in leaves of tomato seedlings inoculated with *Fusarium oxysporum* f.sp. *lycopersici*, a vascular pathogen. Moreover, oligoulvans increased more efficiently the activity of catalase, superoxide dismutase, peroxidase, phenylalanine ammonia-lyase and polyphenoloxidase and the accumulation of H₂O₂, protecting apple fruits against *P. expansum* and *B. cinerea* (Abouraïcha et al., 2015). The increased efficiency in reducing disease severity observed for fractions DU1, DU3, DU5 and DU7 may be related to other structural features and not only to chain size itself. These fractions could be composed by different sugar proportions and their chains could assume different conformations. These features in turn, could affect their biological activity. In general, rhamnose-rich polysaccharides possess increased biological activity, at least in animal models (Alves et al., 2013; Ropellato et al., 2015). Indeed, rhamnans with secondary structures formed by 1 or 2 coils induce defense responses more efficiently in *A. thaliana* plants when compared to the uncoiled oligosaccharide (Bedini et al., 2005). Nevertheless, further studies are necessary in order to better understand the structure and composition of the depolymerized ulvans presented here.

Considering that sulfate content and degree of polymerization seems to not directly affect protection efficiency of ulvans, follow-up experiments were carried out to determine whether the main sugar component of ulvan (rhamnose) alone could affect both diseases. Indeed, our results demonstrate that rhamnose is able to reduce the disease severity of both *A. brassicicola* and *C. higginsianum* similarly to ulvan. Thus, it seems reasonable to assume that rhamnose plays an important

role in ulvan recognition by *A. thaliana*. In this way, rhamnose could directly induce defense responses in *A. thaliana* plants. It is well known that sugars are involved in several metabolic and signaling pathways in plants, including disease resistance (Morkunas & Ratajczak, 2014; Tayeh, Randoux, Vincent, Bourdon & Reignault, 2014; Reignault et al., 2001).

The involvement of sugars as signaling molecules in plant defense has led to the concepts of “sweet-immunity” and “sugar-enhanced defense”. According to these concepts, small sugars (i.e. mono-, di- or small oligosaccharides) such as sucrose and trehalose are able to activate plant defense responses and increase their resistance to pathogens (Trouvelot et al., 2014). For instance, pre-treatment with sucrose, the major product of photosynthesis and transport carbohydrate in plants (Trouvelot et al., 2014), systemically upregulates the expression of defense-related genes such as PR1a, PR1b, PBZ and PR5 and protects rice plants against the pathogenic fungus *Magnaporthe oryzae* (Gómez-Arisa et al., 2007). Trehalose, a nonreducing sugar composed of two glucose units α,α -(1 \rightarrow 1)-linked can protect wheat against powdery mildew by activating plant defense responses such as papilla deposition and increasing PAL, peroxidases (Reignault et al., 2001) and lipoxygenases activities (Tayeh et al., 2014).

L-Rhamnose also seems to be involved in inducing defense responses in plants. For instance, bacterial rhamnolipids induce a wide range of defense responses including Ca^{2+} influx, mitogen-activated protein kinases activation and reactive oxygen species production in grapevine cell suspension cultures and protect plants against *Botrytis cinerea* (Varnier et al., 2009). Despite the lipid moiety present in the rhamnolipids, the oligosaccharide component alone can elicit specific responses (Newman, Dow, Molinaro & Parrilli, 2007). For instance, rhamnans composed of 3, 6 or 9 L-rhamnose units induce the expression of PR-1 and PR-2 in *A. thaliana* plants (Bedini et al., 2005). However, hydrophilic sugars such as rhamnose encounter the cuticle, a highly hydrophobic barrier and, therefore, are only able to penetrate through specific channels (Morkunas & Ratajczak, 2014). Anyway, even at low concentrations, some sugars can be perceived by plants and provoke metabolic changes leading to increased resistance.

Alternatively, L-rhamnose could be affecting fungus by increasing the activity of its endopolygalacturonase, that in turn, could release elicitors of plant defense through the degradation of pectin, one of the main polymers of the primary plant cell wall and middle lamella (Vorwerk, Somerville & Somerville, 2004). Accordingly, Hugouvieux, Centis, Lafitte & Esquerre-Tugayé (1997) reported that the production of

endopolygalacturonase by *Colletotrichum lindemuthianum* was enhanced when the fungus was grown in liquid media containing L-rhamnose as the sole carbon source. However, it seems reasonable to accept that the monosaccharide indirectly affected disease severity, since studies (Moline & Gross, 1984) have demonstrated that rhamnose does not inhibit *in vitro* a large number of fungal pathogens. Hence, comparing how both ulvan and rhamnose can induce resistance will be an exciting challenge for future research.

In conclusion, the present results show that ulvan-induced resistance in *A. thaliana* against *A. brassicicola* and *C. higginsianum* seems to not be directly affected by its sulfation and polymerization degrees. Additionally, L-rhamnose, the major constituent of ulvan, seems to play an important role in the induction of resistance.

ACKNOWLEDGEMENTS

Authors thank the Coordination for the Improvement of Higher Education Personnel – CAPES and National Counsel of Technological and Scientific Development – CNPq for Ph.D. and postdoctoral scholarships for the first and fourth authors, respectively and financial support. MED, MDN and MJS are Research Members of CNPq. Authors also thank Prof. Marcelo Maraschin for technical support in obtaining the partially depolymerized ulvans.

4.5. REFERENCES

- Abouraïcha, E., El Alaoui-Talibi, Z., El Boutachfai, R., Petit, E., Courtois, B., Courtois, J., & El Modafar, C. (2015). Induction of natural defense and protection against *Penicillium expansum* and *Botrytis cinerea* in apple fruit in response to bioelicitors isolated from green algae. *Scientia Horticulturae*, *181*, 121-128.
- Alves, A., Sousa, R.A., & Reis, R. (2013). A practical perspective on ulvan extracted from green algae. *Journal of Applied Phycology*, *25*, 407-424.
- Araújo, L., & Stadnik, M.J. (2013). Cultivar-specific and ulvan-induced resistance of apple plants to *Glomerella* leaf spot are associated with enhanced activity of peroxidases. *Acta Scientiarum Agronomy*, *35*, 287-293.
- Ascêncio, S.D., Orsato, A., França, R.A., Duarte, M.E.R., & Nosedá, M.D. (2006). Complete ^1H and ^{13}C NMR assignment of digeneaside, a

low-molecular-mass carbohydrate produced by red seaweeds. *Carbohydrate Research*, 341, 677-682.

Bedini, E., de Castro, C., Erbs, G., Mangoni, L., Dow, J.M., Newman, M.A., Parrilli, M., & Unverzagt, C. (2005). Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. *Journal of the American Chemical Society*, 127, 2414-2416.

Cassolato, J.E.F., Nosedá, M.D., Pujol, C.A., Pellizzari, F.M., Damonte, E.B., & Duarte, M.E.R. (2008). Chemical structure and antiviral activity of the sulfated heterorhamnan isolated from the green seaweed *Gayralia oxysperma*. *Carbohydrate Research*, 343, 3085-3095.

de Freitas, M.B., & Stadnik, M.J. (2012). Race-specific and ulvan-induced defense responses in bean (*Phaseolus vulgaris*) against *Colletotrichum lindemuthianum*. *Physiological and Molecular Plant Pathology*, 78, 8-13.

Delgado, D.Z., de Freitas, M.B., & Stadnik, M.J. (2013). Effectiveness of saccharin and ulvan as resistance inducers against rust and angular leaf spot in bean plants (*Phaseolus vulgaris*). *Crop Protection*, 47, 67-73.

Dodgson, K.S., & Price, R.G. (1962). A note on the determination of the ester sulfate content of sulphated polysaccharides. *Biochemical Journal*, 84, 106-110.

Duarte, M.E.R., Cauduro, J.P., Nosedá, D.G., Nosedá, M.D., Gonçalves, A.G., Pujol, C.A., Damonte, E.B., & Cerezo, A.S. (2004). The structure of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) and its antiviral activity. Relation between structure and antiviral activity in agarans. *Carbohydrate Research*, 339, 335-347.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350-356.

El Modafar, C., Elgadda, M., El Boutachfai, R., Abouraicha, E., Zehhar, N., Petit, E., Alaoui-Talibi, Z., Courtois, B., & Courtois, J. (2012). Induction of natural defense accompanied by salicylic acid-dependant systemic acquired resistance in tomato seedlings in response to bioelicitors isolated from green algae. *Scientia Horticulturae*, 138, 55-63.

Faria-Tischer, P.C.S., Talarico, L.B., Nosedá, M.D., Guimarães, S.M.P.B., Damonte, E.B., & Duarte, M.E.R. (2006). Chemical structure and antiviral activity of carrageenans from *Meristiella gelidium* against herpes simplex and dengue virus. *Carbohydrate Polymers*, *63*, 459-465.

Gómez-Ariza, J., Campo, S., Rufat, M., Estopá, M., Messeguer, J., San Segundo, B., & Coca, M. (2007). Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. *Molecular Plant-Microbe Interactions*, *20*, 832-842.

Hugouvieux, V., Centis, S., Lafitte, C., & Esquerré-Tugayé, M.T. (1997). Induction by α -L-arabinose and α -L-rhamnose of endopolygalacturonase gene expression in *Colletotrichum lindemuthianum*. *Applied and Environmental Microbiology*, *63*, 2287-2292.

Isitua, C.C., & Ibeh, I.N. (2013). Toxicological assessment of aqueous extract of *Moringa oleifera* and *Caulis bambusae* leaves in rabbits. *Journal of Clinical Toxicology*, *S2*, 003.

Jaulneau, V., Laffite, C., Corio-Costet, M.F., Stadnik, M.J., Salamagne, S., Briand, X., Esquerré-Tugayé, M.T., & Dumas, B. (2011). An *Ulva armoricana* extract protects plants against three powdery mildew pathogens. *European Journal of Plant Pathology*, *131*, 393-401.

Jaulneau, V., Lafitte, C., Jacquet, C., Fournier, S., Salamagne, S., Briand, X., Esquerré-Tugayé, M.T., & Dumas, B. (2010). Ulvan, a sulfated polysaccharide from green algae, activates plant immunity through the jasmonic acid pathway. *Journal of Biomedicine Biotechnology*, *525291*.

Khan, W., Rayirath, U.P., Subramanian, S., Jithesh, M.N., Rayorath, P., Hodges, D.M., Critchley, A.T., Craige, J.S., Norrie, J., & Prithiviraj, B. (2009). Seaweed extracts as biostimulants of plant growth and development. *Journal of Plant Growth Regulation*, *28*, 386-399.

Kovac, P., Hirsch, J., Shashkov, A.S., Usov, A.I., Yarotsky, S.V. (1980). ^{13}C -n.m.r. spectra of xylo-oligosaccharides and their application to the elucidation of xylan structures. *Carbohydrate Research*, *85*, 177-185.

Lahaye, M. (1998). NMR spectroscopic characterization of oligosaccharides from two *Ulva rigida* ulvan samples (Ulvales, Chlorophyta) degraded by a lyase. *Carbohydrate Research*, *314*, 1-12.

- Lahaye, M., Brunel, M., & Bonnin, E. (1997). Fine chemical structure analysis of oligosaccharides produced by an ulvan-lyase degradation of the water-soluble cell-wall polysaccharides from *Ulva* sp. (Ulvales, Chlorophyta). *Carbohydrate Research*, *304*, 325-333.
- Lahaye, M., Inizan, F., & Vigoureux, J. (1998). NMR analysis of the chemical structure of ulvan and of ulvan-boron complex formation. *Carbohydrate Polymers*, *36*, 239-249.
- Lahaye, M., Ray, B., Baumberger, S., Quemener, B., & Axelos, M.A.V. (1996). Chemical characterization and gelling properties of cell wall polysaccharides from species of *Ulva* (Ulvales, Chlorophyta). *Hydrobiologia*, *326/327*, 473-480.
- Lahaye, M., & Robic, A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules*, *8*, 1765-1774.
- Ménard, R., Alban, S., Ruffray, P., Jamois, F., Franz, G., Fritig, B., Yvin, J.C., & Kauffmann, S. (2004). β -1,3 glucan sulfate, but not β -1,3 glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis. *The Plant Cell*, *16*, 3020-3032.
- Ménard, R., Ruffray, P., Fritig, B., Yvin, J.C., & Kauffmann, S. (2005). Defense and resistance-inducing activities in Tobacco of the sulfated β -1,3 glucan PS3 and its synergistic activities with the unsulfated molecule. *Plant and Cell Physiology*, *46*, 1964-1972.
- Moline, H.E., & Gross, K.C. (1984). Growth of five pathogenic fungi on cell wall-related monosaccharides. *Plant Science Letters*, *33*, 15-21.
- Morkunas, I., & Ratajczak, L. (2014). The role of sugar signaling in plant defense responses against fungal pathogens. *Acta Physiologiae Plantarum*, *36*, 1607-1619.
- Newman, M.A., Dow, J.M., Molinaro, A., & Parrilli, M. (2007). Priming, induction and modulation of plant defense responses by bacterial lipopolysaccharides. *Journal of Endotoxin Research*, *13*, 69-84.
- Paulert, R., Ebbinghaus, D., Urlass, C., & Moerschbacher, B.M. (2010). Priming of the oxidative burst in rice and wheat cell cultures by ulvan, a polysaccharide from green macroalgae, and enhanced resistance against powdery mildew in wheat and barley plants. *Plant Pathology*, *59*, 634-642.

Paulert, R., Talamini, V., Cassolato, J.E.F., Duarte, M.E.R., Nosedá, M.D., Smania Júnior, A., & Stadnik, M.J. (2009). Effects of sulfated polysaccharide and alcoholic extracts from Green seaweed *Ulva fasciata* on anthracnose severity and growth of common bean (*Phaseolus vulgaris* L.). *Journal of Plant Disease and Protection*, *116*, 263-270.

Qi, H., Huang, L., Liu, X., Liu, D., Zhang, Q., & Liu, S. (2012). Antihyperlipidemic activity of high sulfate content derivative of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Carbohydrate Polymers*, *87*, 1637-1640.

Qi, H., Zhang, Q., Zhao, T., Chen, R., Niu, X., & Li, Z. (2005b). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules*, *37*, 195-199.

Qi, H., Zhao, T., Zhang, Q., Li, Z., Zhao, Z., & Xing, R., (2005a). Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *Journal of Applied Phycology*, *17*, 527-534.

Reignault, P.H., Cogan, A., Muchembled, J., Sahraoui, A.L.H., Durand, R., & Sancholle, M. (2001). Trehalose induces resistance to powdery mildew in wheat. *New Phytologist*, *149*, 519-529.

Roger, O., Kervarec, N., Ratiskol, J., Collic-Jouault, S., Chevolut, L. (2004). Structural studies of the main exopolysaccharide produced by the deep-sea bacterium *Alteromonas infernus*. *Carbohydrate Research*, *339*, 2371-2380.

Ropellato, J., Carvalho, M.M., Ferreira, L.G., Nosedá, M.D., Zuconelli, C.R., Gonçalves, A.G., Ducatti, B.R., Kenski, J.C., Nasato, P.L., Winnischofer, S.M., & Duarte, M.E. (2015). Sulfated heterorhamnans from the green seaweed *Gayralia oxysperma*: partial depolymerization, chemical structure and antitumor activity. *Carbohydrate Polymers*, *117*, 475-485.

Sangha, J.S., Ravichandran, S., Prithiviraj, K., Critchley, A.T., & Prithiviraj, B. (2010). Sulfated macroalgal polysaccharides λ -carrageenan and ι -carrageenan differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*. *Physiological and Molecular Plant Pathology*, *75*, 38-45.

- Sharma, H.S.S., Fleming, C., Selby, C., Rao, J.R., & Martin, T. (2014). Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *Journal of Applied Phycology*, *26*, 465-490.
- Stadnik, M.J., & de Freitas, M.B. (2014). Algal polysaccharides as source of plant resistance inducers. *Tropical Plant Pathology*, *39*, 111-118.
- Tayeh, C., Randoux, B., Vincent, D., Bourdon, N., & Reignault, P. (2014). Exogenous trehalose induces defenses in wheat before and during a biotic stress caused by powdery mildew. *Phytopathology*, *104*, 293-305.
- Trouvelot, S., Héloir, M.C., Poinssot, B., Gauthier, A., Paris, F., Guillier, C., Combiér, M., Trdá, L., Daire, X., & Adrian, M. (2014). Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. *Frontiers in Plant Science*, *5*, 592.
- Varnier, A.L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brugger, A., Sorokin, A., Renault, J.H., Kauffmann, S., Pugin, A., Clement, C., Baillieul, F., & Dorey, S. (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant, Cell & Environment*, *32*, 178-193.
- Vorwerk, S., Somerville, S., & Somerville, C. (2004). The role of plant cell wall polysaccharides composition in disease resistance. *Trends in Plant Science*, *9*, 203-209.
- Watt, G., Loeff, C., Harper, A.D., & Bar-Peled, M. (2004). A bifunctional 3,5-epimerase/4-keto reductase for nucleotide-rhamnose synthesis in Arabidopsis. *Plant Physiology*, *134*, 1337-1346.

5. CAPÍTULO 3 - ULVAN-INDUCED RESISTANCE IN *Arabidopsis thaliana* AGAINST *Alternaria brassicicola* REQUIRES REACTIVE OXYGEN SPECIES DERIVED FROM NADPH OXIDASE¹

Mateus B. de Freitas and Marciel J. Stadnik*

Laboratory of phytopathology, Federal University of Santa Catarina, 88034-001, Florianópolis, SC, Brazil

*Corresponding author. Tel: +55 48 3721 5338; fax: +55 48 3721 5335.

E-mail addresses: mateusbrusco@gmail.com (M.B. de Freitas), marciel.stadnik@ufsc.br (M.J.Stadnik).

ABSTRACT

The present work aimed to study the role of reactive oxygen species derived from NADPH oxidase in the ulvan-induced resistance against *Alternaria brassicicola* in *Arabidopsis thaliana*. Foliar spraying of ulvan, a water-soluble algal polysaccharide, reduced the colonization of host tissues and, consequently, the severity of *A. brassicicola* by 90% in both wild type and *AtrbohF* plants, and it increased NADPH oxidase activity and hydrogen peroxide levels. Ulvan also tended to enhance the activity of enzymes related to the removal of reactive oxygen species (APX, GSR, CAT and SOD) suggesting a tight control of the antioxidant system. Ulvan did not protect the *AtrbohD* mutant as well as wild type plants previously infiltrated with diphenyleioidonium, both impaired in NADPH oxidase activity and hydrogen peroxide accumulation. Based on our results and those available in the literature, we propose a general model for ulvan-induced defense responses in plant tissues. Collectively, our results suggest that ulvan-induced resistance in *A. thaliana* against *A. brassicicola* requires reactive oxygen species derived from the respiratory burst oxidase homologue D (RBOHD) NADPH oxidase.

Keywords: *Arabidopsis thaliana*, *Alternaria brassicicola*, ulvan, induced resistance, reactive oxygen species, *AtrbohF*, *AtrbohD*.

5.1. INTRODUCTION

The current interest in the environment and human health together with an increase in the production costs and continuous breakdown of resistance in commercial cultivars have fostered the

¹ Artigo aceito para publicação no periódico Physiological and Molecular Plant Pathology.

development of alternative disease control methods. In this scenario, algal polysaccharides arise as a convenient and eco-friendly strategy [1].

Ulvan is a water-soluble heteropolysaccharide extracted from the cell walls of the marine green algae *Ulva* spp.. Ulvan spraying can induce defense responses in several crop plant species to a broad range of fungal pathogens. In alfalfa infected by *Colletotrichum trifolii*, responses included the biosynthesis of phytoalexins and pathogenesis-related (PR) proteins (PR-1 and 10) [2]. The elicited defense activated by ulvan has been reported for bean plants against rust (*Uromyces appendiculatus*) [3] and anthracnose (*Colletotrichum lindemuthianum*) [4], where the polysaccharide increased the post-infection activity of glucanase and peroxidase, respectively. Increase in peroxidase activity has been also found in apple plants at 72 h after infection with *C. gloeosporioides*, but not earlier and without affecting glucanase [5]. Furthermore, the ability of ulvan to induce priming, where a pretreated plant activates faster and stronger defense responses when exposed to pathogen attack, was recently demonstrated [6]. Thus, although ulvan itself did not change the production of hydrogen peroxide in suspension-cultured wheat or rice cells, its previous addition in the first one enhanced both the chitin- and chitosan-elicited oxidative burst about four fold. In rice-cultured cells, the production of hydrogen peroxide was strongly primed by pretreatment with ulvan, increasing the burst triggered by chitin and chitosan 150 and 80 times, respectively [6].

Although not fully understood, recent studies have indicated that the jasmonic acid signaling pathway is activated by ulvan, which could explain its efficiency in inducing resistance to necrotrophic pathogens [7]. Nevertheless, it seems possible that ulvan also activates salicylic acid-dependent responses, since it has been able to protect plants against biotrophic and hemibiotrophic pathogens [1,2,6]. Accordingly, the expression of PR-1, a known marker for the salicylic acid pathway, has been observed after the treatment of alfalfa with ulvan [2]. Indeed, Truman et al. [8] show that jasmonates may play a central role in systemic defense, acting as the initial signal for the classic Systemic Acquired Resistance (SAR).

During a plant-pathogen interaction, one of the first defense response occurring is the so-called oxidative burst, a massive production of reactive oxygen species (ROS) at the site of attempted invasion. This burst is accompanied by changes in cellular pH, ion influxes, protein phosphorylation and immobilization, defense gene expression, hypersensitive reaction (HR), cell wall protein cross-linking, phytoalexin production, callose deposition and SAR [9-14].

ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$) or hydroxyl radical ($\cdot\text{OH}$) possess a strong oxidizing potential that leads to damage to a variety of biological molecules. They are therefore unwelcome byproducts of normal metabolic processes such as photosynthesis or glycolysis in all aerobic organisms. Despite their destructive activity, ROS are well-described messengers in several important cellular processes [9-14].

Several enzymes have been implicated in the generation of ROS within cellular compartments including cell wall peroxidase, diamine oxidases, oxalate oxidases, amine oxidases, NADPH oxidases and xanthine oxidase. Among these, NADPH oxidases (NOX) are the most well-studied so far and plays a pivotal role in the production of $\text{O}_2^{\cdot-}$ during the oxidative burst. NOX also referred to as respiratory burst oxidase homologues (RBOH) constitutes a multigenic family with ten different genes in the model plant *Arabidopsis thaliana* (*AtrbohA* – *AtrbohJ*). NOX are transmembrane proteins that produce ($\text{O}_2^{\cdot-}$) at the apoplast, which dismutates to H_2O_2 spontaneously or catalytically by the action of superoxide dismutase (SOD). These proteins play key roles in several biological processes. For instance, *AtrbohB* is active during seed germination and after-ripening. On the other hand, *AtrbohC* regulates cell expansion during root hair formation and is responsible for cell wall integrity [11,12,15-17].

While most RBOH are tissue specific, *AtrbohD* and *AtrbohF* genes are expressed throughout the whole plant. Both of them are involved in pathogen defense responses and in abscisic acid- and ethylene-induced stomatal closure. Furthermore, RBOHD was shown to be required for the initiation of a cascade of cell-to-cell signals resulting in the formation of a ROS wave that propagates throughout different tissues and for long distances. Both *AtrbohD* and *AtrbohF* mutants presumably give rise to nonfunctional proteins impairing hydrogen peroxide accumulation and hypersensitive reaction triggering, respectively [16,18-20].

In order to keep ROS levels under a toxic threshold, plants have evolved complex arrays of nonenzymatic and enzymatic detoxification mechanisms. The nonenzymatic antioxidants include ascorbate, glutathione, tocopherol, flavonoids, alkaloids and carotenoids. The enzymatic ROS scavenging mechanisms in plants include ascorbate peroxidase (APX), SOD, guaiacol peroxidase (GPX), glutathione reductase (GSR) and catalase (CAT). SOD acts as the first line of defense against ROS, dismutating $\text{O}_2^{\cdot-}$ to H_2O_2 , which is converted to H_2O and O_2 by CAT and APX. GPX oxidizes aromatic electron donors such as

guaiacol and pyrogallol at the expense of H_2O_2 . GSR catalyzes the reduction of glutathione disulfide (GSSG), previously oxidized by ROS, back to glutathione (GSH) [10,14,21].

Although evidences suggest that ulvan directly induces the production of hydrogen peroxide, at least in bean plants [4], there is no information regarding the signalization involved in this process. Thus, the present work was carried out in order to compare ulvan-induced oxidative stress responses in wild type, *AtrbohD* and *AtrbohF* *A. thaliana* plants and to elucidate the role of NADPH oxidase-derived reactive oxygen species in the induced resistance against *Alternaria* infection.

5.2. MATERIALS AND METHODS

5.2.1. Biological material

Arabidopsis thaliana (L.) Heynhold wild type ecotype Col-0 (WT) and its mutants *AtrbohD* and *AtrbohF* were used in the experiments.

Ulva fasciata Delile samples were harvested in December 2011 at Armação beach in Florianópolis-SC, Brazil (27.4454°S; 48.2956°W). Ulvan was obtained as previously described by Paulert et al. [22]. Briefly, the ground dried alga (100 g) was autoclaved for 2 h at 110 °C in distilled water (1 L). The resulting aqueous solution was filtered and the polysaccharide precipitated with ethanol for 48 h at -20 °C. The precipitate was filtered, washed three times with ethanol and dissolved in distilled water. The solution was dialyzed against tap water for 48 h and against distilled water for another 48 h using 3600 Da Mw cutoff dialysis membrane. The resulting product named ulvan was concentrated under vacuum, lyophilized and kept at -20 °C until use.

The strain CBS 125088 of *Alternaria brassicicola* (*Ab*; Schweinitz, Wiltshire) was used in the experiments. *A. brassicicola* was grown to sporulate at 25 °C and 12 h photoperiod for 15 days in Petri dishes containing V8 culture media. Thereafter, Petri dishes were flooded with 10 mL of distilled water and the conidial suspension was collected and filtered twice to remove mycelial fragments. The number of conidia was determined using a Neubauer's counting chamber and inoculum concentration was adjusted to 1×10^5 conidia mL⁻¹ with distilled water.

5.2.2. Effect of ulvan on fungus growth

Mycelial growth of *A. brassicicola* was measured on potato dextrose broth (PDB: 200 g potato + 20 g dextrose + 1 L distilled water)

supplemented or not with ulvan at final concentrations of 1, 10 and 100 mg mL⁻¹. For that, one 4-mm-diameter fungal disc was taken from advancing zones of mycelia of *A. brassicicola* cultured in PDA plates and transferred to a 250 mL conical flask containing PDB (30 mL). The culture flasks were incubated at 24 ± 1°C under continuous shaking (84 rpm) in dark for 7 days. Then, mycelial mat was collected by vacuum suction on pre-weighed filter paper (Whatman n°40), washed, dried at 105 °C for 48 h, and dry weight was recorded.

5.2.3. Plant growth conditions and treatment

Arabidopsis thaliana seeds were sown in pots (6 x 6 x 6 cm) containing a mixture of organic compost and vermiculite (1:1, v/v). Seeds were vernalized for two days at 4 °C after sowing. About 20 days after growing in a chamber (23 ± 3 °C, 12 hours of light and a photon flux density of 160 µE m⁻² sec⁻¹) plants were transplanted to new pots and grown under the same conditions as above.

Shortly before use, ulvan was completely dissolved in distilled water under continuous stirring at room temperature. Six-week-old *A. thaliana* plants were sprayed once (i.e. three days before inoculation) with water (control) or ulvan (1 mg mL⁻¹) [6, 23]. A volume of 0.9 mL was delivered per plant.

Diphenyleneiodonium (DPI, 5 µM) and water (control) were directly infiltrated into WT *A. thaliana* leaves using a needless syringe one hour before treatment application [24].

5.2.4. Inoculation and disease evaluation

Three days after treatment, plants were inoculated by spraying a homogeneous suspension of *Ab* and placed under highly humid conditions (humidity > 90%) for 48 h. A mock-inoculation was performed by spraying plants with distilled water. The severity of *Ab* was assessed five days after inoculation by quantifying the percentage of necrotic leaf area using a software program (Quant v. 1.01, Viçosa – MG).

5.2.5. Sampling

For enzyme activity determination, approximately 200 mg of leaves from each replication were collected at 6, 12, 24 and 48 hours after inoculation (h.a.i). Leaves were weighted, snap-frozen in liquid nitrogen and stored at -80°C until analysis.

For visualization of hydrogen peroxide (H_2O_2) and fungal colonization in plant tissues, four fully expanded leaves were randomly sampled from each replication at 72 h.a.i and immediately placed into a staining solution.

For cell death measurement, 12 fully expanded leaves were randomly collected from each replication at 24 h.a.i. Leaves were washed extensively with water and submerged into a tube with 30 mL of deionized water.

5.2.6. Enzyme activity assays

5.2.6.1. Extraction

The plant extract for enzyme activity assays was obtained as described by Rao et al. [25], with modifications. For that, frozen *A. thaliana* leaves were homogenized with 50 mM potassium phosphate (pH 7.0) in a ratio of 5 mL buffer g^{-1} of leaf fresh mass. The homogenate was centrifuged at 20,000 $\times g$ for 30 min at 4°C and the supernatant was collected and placed on ice until analysis. Protein content was determined according to the method of Bradford [26] using BSA as a standard.

Enzymatic activity were determined as described by Rao et al. [25], with modifications and expressed in katal, where 1 katal represents the amount of enzyme converting 1 M of substrate s^{-1} .

5.2.6.2. Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was determined in a reaction mixture composed of 0.1 mL of enzyme extract and 2 mL 50 mM potassium phosphate buffer (pH 7) containing 0.5 mM hydrogen peroxide and 0.5 mM ascorbic acid (extinction coefficient 2.8 mM cm^{-1}). After addition of enzyme extract, absorbance at 290 nm was recorded for three minutes.

5.2.6.3. Catalase

Catalase (CAT) activity was determined in a reaction mixture composed of 0.050 mL of enzyme extract and 2 mL of 50 mM potassium phosphate buffer (pH 7) containing 13.3 mM hydrogen peroxide (extinction coefficient 39.4 mM cm^{-1}). After addition of enzyme extract, absorbance at 240 nm was recorded for three minutes.

5.2.6.4. Glutathione reductase

Glutathione reductase (GSR) activity was determined in a reaction mixture composed of 0.010 mL of enzyme extract, 0.890 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.75 mM 5,5-dithio-bis-(2-nitrobenzoic acid) and 0.1 mM NADPH (extinction coefficient 6.2 mM cm^{-1}) and 0.1 mL of GSSG. After addition of GSSG, absorbance at 412 nm was recorded for three minutes.

5.2.6.5. Guaiacol peroxidase

Guaiacol peroxidase (GPX) activity was determined in a reaction mixture composed of 0.1 mL of the enzyme extract and 2.9 mL of 10 mM sodium phosphate buffer (pH 6) containing 12.6 mM hydrogen peroxide and 0.31 M guaiacol (extinction coefficient 25.2 mM cm^{-1}). After addition of enzyme extract, absorbance at 470 nm was recorded for three minutes.

5.2.6.6. NADPH oxidase

NADPH oxidase activity (NOX) was determined in a reaction mixture composed of 0.100 mL of enzyme extract and 2 mL of 50 mM potassium phosphate buffer (pH 7) containing 300 μM NADPH (extinction coefficient 6.2 mM cm^{-1}) and 25 μM KCN. After addition of enzyme extract, absorbance at 340 nm was recorded for three minutes.

5.2.6.7. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined in a reaction mixture composed of 0.040 mL of enzyme extract, 2 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 10 mM methionine and 56 μM nitroblue tetrazolium and 150 μM of riboflavin. After the addition of riboflavin, samples were placed in a box lined with aluminum foil and illuminated for 15 minutes (15 watts white lamp at approximately 12 cm from samples). Duplicate samples kept in the dark for the same time were used as blanks. Absorbance was determined at 560 nm and enzymatic activity was calculated using purified SOD as standard.

5.2.7. Histochemical analysis

To visualize hydrogen peroxide *in situ*, 3,3'-diaminobenzidine (DAB) staining was performed on *A. thaliana* leaves as described by

Hückelhoven et al. [27], with modifications. Briefly, leaves were immediately placed on a DAB solution (1 mg mL^{-1}) for 12 h. Then, the DAB solution was switched to a trichloroacetic acid 0.15% in ethanol and chloroform (4:1; v/v) solution to bleach tissues. After 24 h, the leaves were placed in a conservation solution (lactic acid: glycerol: water; 1:1:1; v/v/v). Hydrogen peroxide was visualized as a reddish-brown coloration [27]. Leaves were scanned and the percentage of DAB-stained leaf area was calculated using the Threshold tool of ImageJ software (National Institutes of Health, USA).

The *in situ* staining of fungal hyphae was performed according to Pogány et al. [28], with modifications. For that, leaves were submerged into a trypan blue solution (2.5 mg mL^{-1} in lactophenol and ethanol; 1:2; v/v), heated in a boiling water bath for 2 min. and kept in the solution for 1 h at room temperature. Then, the trypan blue solution was replaced by chloral hydrate (2.5 g mL^{-1}) to bleach tissues. After 12 h, leaves were placed in a conservation solution (70% glycerol).

5.2.8. Cell death measurement

Electrolyte leakage due to damages in the plasmatic membrane was quantified according to Dellagi et al. [29], with modifications. For that, conductivity measurements were taken every hour for 12 h using a conductivity meter (Lutron, model CD-4301). The slope calculated for each replication was used in the statistical analysis.

5.2.9. Experimental design and statistical analysis

The experiment with ulvan on the fungus mycelial growth was conducted in a completely randomized design with three replications.

The assessment of oxidative stress responses induced by ulvan against *Ab* in WT, *AtrbohD* and *AtrbohF* plants was carried out in a factorial completely randomized design with three factors: ecotype (WT, *AtrbohD* or *AtrbohF*), treatment (water or ulvan) and inoculation (*Ab*- or mock inoculated plants). This experiment was carried out with 15 replications each one containing four plants. Samples for all analysis were collected from three different replications randomly selected at each time point.

The DPI-experiment was carried out in a factorial completely randomized design with two factors: treatment (water or ulvan) and DPI (with or without it) and four replications each one containing four plants.

After verification of homogeneity of the variances of the datasets, data were subjected to a two-way analysis of variance. Tukey's, Scott-Knott's or *t* tests at 5% of significance level were used for separation of means. When necessary, data transformation was used before analysis in order to meet ANOVA assumptions. Statistical analyzes were performed using the software Statistica (v. 10). All experiments were repeated independently once with similar results.

5.3. RESULTS

5.3.1. Effect of ulvan on fungus growth

Alternaria brassicicola produced about 0.185 g of mycelium in the control PDB (i.e. without ulvan). Increasing concentrations of ulvan stimulated the mycelial growth (Figure 1).

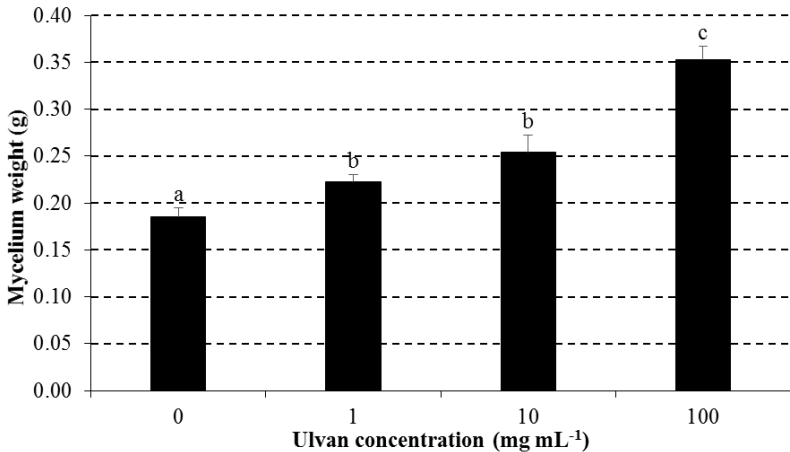


Figure 3.1. Mycelium weight of *Alternaria brassicicola* 7 days after growing in PDB supplemented with different ulvan concentrations at 24°C under continuous shaking in dark. *Letters indicate significant differences (Tukey's Test, $p \leq 0.05$). Bars indicate the standard deviation of mean ($n=3$).

5.3.2. Disease severity

The percentage of affected leaf area reached 40, 40 and 45% in WT, *AtrbohF* and *AtrbohD* inoculated plants, respectively. In this situation, ulvan spraying reduced the severity of *Ab* by 90% in both WT and *AtrbohF* ecotypes (Figures 2A and B), but failed to control it on

AtrbohD plants. Trypan blue staining revealed that WT and *AtrbohF* control plants had their tissues more strongly colonized by the fungus than ulvan-treated ones (Figure 5).

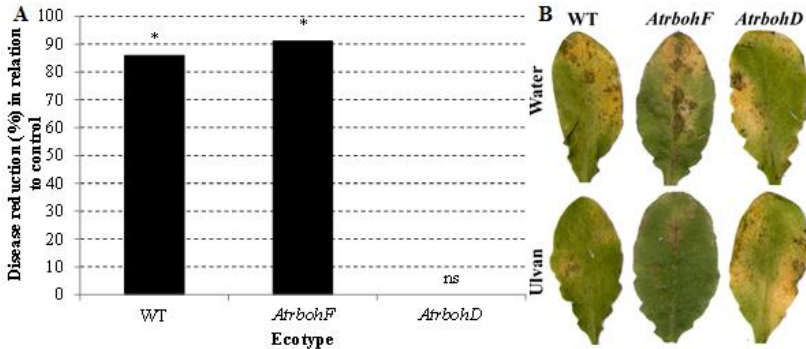


Figure 3.2. Percentage of disease reduction (A) and symptoms (B) caused by *Alternaria brassicicola* five days after inoculation of wild type (WT), *AtrbohF* and *AtrbohD* *Arabidopsis thaliana* plants previously sprayed with water or ulvan (1 mg mL^{-1}). *Indicate significant disease reduction (Tukey's test, $p \leq 0.05$, $n=3$). ns: not significant.

5.3.3. Enzymatic activity

The activity of enzymes related to production and removal of reactive oxygen species is represented in figure 3 as percent in relation to control (plants treated with water and non-inoculated). The enzymatic activity was not determined at 48 h.a.i in the *AtrbohD* mutant due to plant mortality where some of these plants stop growing and die as described by Torres et al. [18].

5.3.3.1. Ascorbate peroxidase (APX)

In WT, *AtrbohF* and *AtrbohD* control plants, APX activity ranged around 2,428, 8,379 and 505 $\mu\text{katal mg of protein}^{-1}$ over time, respectively. In WT and *AtrbohD* plants, both *Ab* and ulvan strongly increased APX activity (Figure 3). In *AtrbohF* inoculated plants, *Ab* decreased and ulvan transiently increased APX activity.

5.3.3.2. Catalase (CAT)

In WT, *AtrbohF* and *AtrbohD* control plants, CAT activity ranged around 4,693, 7,416 and 4,831 $\mu\text{katal mg of protein}^{-1}$ over time, respectively. In WT plants, both *Ab* and ulvan increased CAT activity (Figure 3). In *AtrbohF* plants, ulvan increased CAT activity in both *Ab*- and mock-inoculated plants. On the other hand, ulvan transiently decreased CAT activity in *AtrbohD* plants inoculated with *Ab*.

5.3.3.3. Glutathione reductase (GSR)

In WT, *AtrbohF* and *AtrbohD* control plants, GSR activity ranged around 15,755, 19,760 and 2,813 $\mu\text{katal mg of protein}^{-1}$ over time, respectively. In WT plants inoculated with *Ab*, ulvan increased GSR activity (Figure 3). On the other hand, the polysaccharide affected variably GSR activity over time in non-inoculated WT plants. Both *Ab* and ulvan increased more markedly GSR activity in *AtrbohF* plants than in other ecotypes. Neither ulvan spraying nor *Ab*-inoculation affected GSR activity in *AtrbohD* plants.

5.3.3.4. Guaiacol peroxidase (GPX)

In WT, *AtrbohF* and *AtrbohD* control plants, GPX activity ranged around 39,122, 252,462 and 33,247 $\mu\text{katal mg of protein}^{-1}$ over time, respectively. Both *Ab* and ulvan increased more markedly GPX activity in WT plants than in other ecotypes (Figure 3). The polysaccharide transiently increased GPX activity only in non-inoculated *AtrbohF* and *AtrbohD* plants.

5.3.3.5. NADPH oxidase (NOX)

The mean value of NOX activity for WT and *AtrbohF* control plants was 10 and 21 $\mu\text{katal mg of protein}^{-1}$ over time, respectively, whereas, no enzymatic activity was detected in the *AtrbohD* mutant. Ulvan strongly increased NOX activity in both WT and *AtrbohF* plants inoculated with *Ab* (Figure 3). Ulvan increased NOX activity in non-inoculated WT plants. On the other hand, the polysaccharide affected variably the enzymatic activity over time in non-inoculated *AtrbohF* plants.

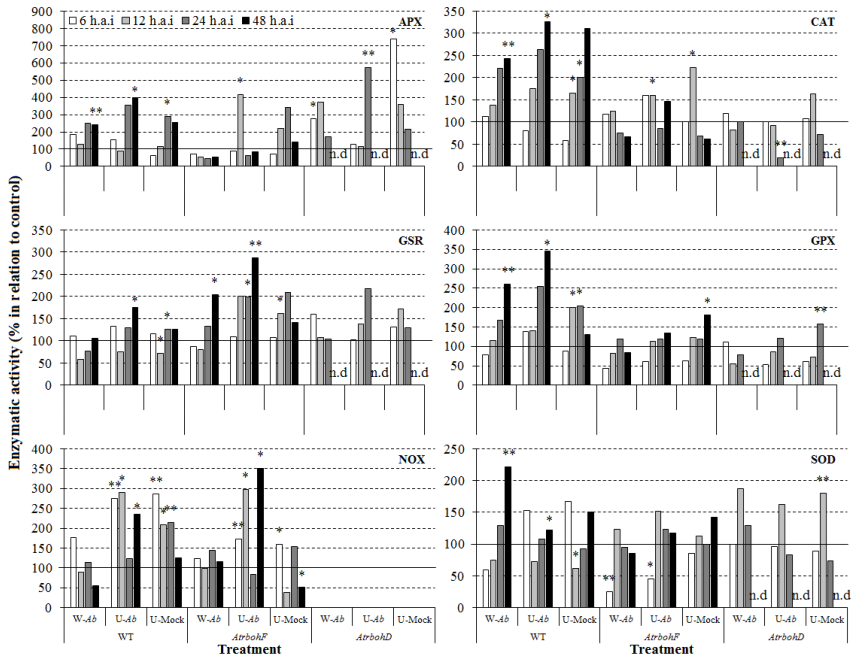


Figure 3.3. Changes in ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GSR), guaiacol peroxidase (GPX), NADPH oxidase (NOX) and superoxide dismutase (SOD) activities at 6, 12, 24 and 48 hours after inoculation represented as percentage of control plants (water – mock). *Differs from control plants (t Test, $p \leq 0.05$ or ** $p \leq 0.01$, $n=3$). n.d.: not determined. Baseline at 100% represents the enzymatic activity of control plants. W-*Ab*: water-*Alternaria brassicicola*. U-*Ab*: Ulvan-*A. brassicicola*. U-Mock: Ulvan-Mock.

5.3.3.6. Superoxide dismutase (SOD)

In WT, *AtrbohF* and *AtrbohD* control plants, SOD activity remained around 11, 24 and 2 $\mu\text{katal mg of protein}^{-1}$ over time, respectively. In WT plants, *Ab* strongly increased SOD activity (Figure 3). Ulvan transiently increased the enzymatic activity in the WT ecotype inoculated with *Ab*. On the other hand, the polysaccharide momentarily decreased SOD activity in non-inoculated WT plants. In *AtrbohF* inoculated plants, both *Ab* and ulvan transiently decreased SOD activity. On the other hand, ulvan temporarily increased SOD activity in mock-inoculated *AtrbohD* plants.

5.3.4. H₂O₂

In WT and *AtrbohF* plants inoculated with *Ab*, DAB-stained leaf area was 19 and 17% at 72 h.a.i, respectively (Figure 4). In these plants, ulvan increased H₂O₂ levels by 1.8 times. In mock-inoculated WT and *AtrbohF* plants, DAB-stained leaf area reached 4 and 5%, respectively. The polysaccharide increased H₂O₂ levels 4.4 times in those ecotypes. In *AtrbohD* plants, the DAB-stained leaf area remained around 5% in all treatments (Figure 4).

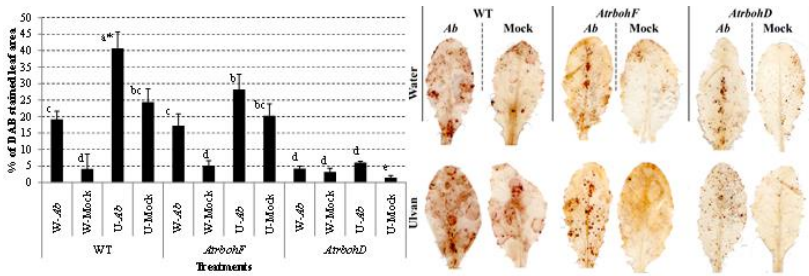


Figure 3.4. Percentage of diaminobenzidine-stained leaf area at 72 hours after inoculation of WT, *AtrbohF* and *AtrbohD* *Arabidopsis thaliana* leaves. Plants were sprayed with water (control) or ulvan (1 mg mL⁻¹) and inoculated three days later with *Alternaria brassicicola* (*Ab*) or water (mock). *Letters indicate significant differences (Tukey's Test, $p \leq 0.05$). Bars indicate the standard deviation of mean (n=3).

5.3.5. Cell death measurement

In WT, *AtrbohF* and *AtrbohD* plants inoculated with *Ab*, the rates of electrolyte leakage quantified at 24 h.a.i were 0.7, 0.5 and 0.4, respectively. Ulvan reduced the electrolyte loss by 130% in WT and *AtrbohF* plants, but failed to control it in the *AtrbohD* mutant (Figure 5). Overall, the rate of electrolyte loss was 2 times higher in *Ab*- than in non-inoculated plants at 24 h.a.i.

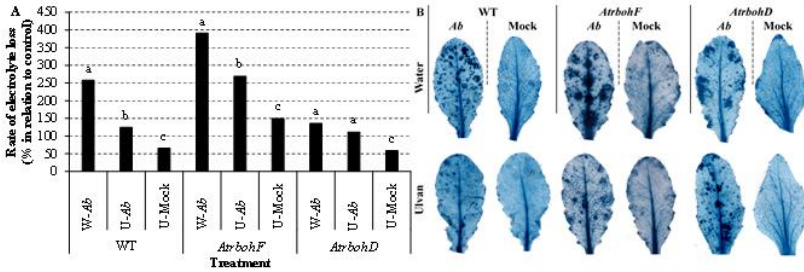


Figure 3.5. Rate of electrolyte loss (A) and trypan blue staining for fungal hyphae (B) evaluated respectively at 24 and 72 hours after inoculation of WT, *AtrbohF* and *AtrbohD* leaves. Plants were sprayed with water (control) or ulvan (1 mg mL⁻¹) and inoculated three days later with *Alternaria brassicicola* (Ab) or water (mock). *Letters indicate significant differences (Scott-Knott's test, $p \leq 0.05$, $n=3$).

5.3.6. Ulvan-induced resistance in DPI-treated plants

This experiment was carried out in order to confirm that ulvan-induced resistance requires functional NADPH oxidase. Thus, WT plants were infiltrated with water (control) or DPI (5 μ M), a specific inhibitor of NADPH oxidase, one hour before treatment application. The percentage of affected leaf area reached 20 and 25% in water-treated plants infiltrated with water or DPI, respectively (Figure 6). In ulvan-treated plants not previously infiltrated with DPI, the percentage of affected leaf area reached 7%. DPI suppressed the ulvan-induced resistance to *A. brassicicola*.

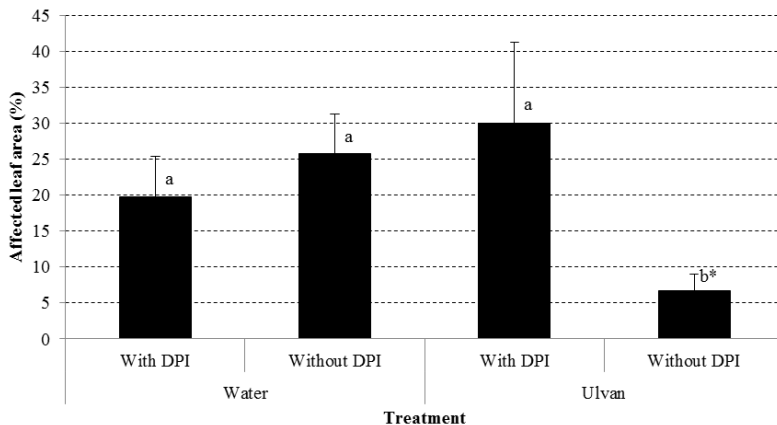


Figure 3.6. Percentage of *Alternaria brassicicola*-affected leaf area five days after inoculation of wild type (WT) plants previously sprayed with water or ulvan (1 mg mL⁻¹). *Leaves were infiltrated with water (control) or diphenyleneiodonium (DPI, 5 μ M) one hour before treatment application. Letters indicate significant differences (Tukey's test, $p \leq 0.05$). Bars indicate the standard deviation of mean (n=4).

5.4. DISCUSSION

No inhibitory effect of ulvan has been observed against different microorganisms including bacteria, fungi and yeast [22, 30]. Accordingly, in the present work, ulvan did not inhibit mycelial growth of *A. brassicicola* (Figure 1).

Ulvan reduced the severity of *A. brassicicola* infection by 90% in both WT and *AtrbohF* plants (Figure 2). Varying levels of protection by ulvan ranging from 40% to nearly complete disease control have been reported in the literature in several crop species against different kind of pathogens [2-7,22,23]. Thus, ulvan-induced resistance seems to be consistent and able to provide a high protection level, at least in *A. thaliana*.

The rate of electrolyte leakage due to damages in the plasmatic membrane was 2 times higher in *Ab*- than in mock-inoculated plants at 24 h.a.i (Figure 5). At this time point, the fungus already penetrated the cuticle and started colonizing host tissues [31]. Thus, an increase in ion leakage due to cell disruption would be expected in *Ab*-inoculated plants. On the other hand, ulvan reduced the rate of electrolyte loss in both inoculated WT and *AtrbohF* plants (Figure 5). This result could be explained by the defense mechanisms induced by the polysaccharide

causing a delay in the tissue colonization by the fungus. Indeed, trypan blue staining revealed that ulvan-treated WT and *AtrbohF* leaves were colonized at a lower extent than those sprayed with water (Figure 5).

Reactive oxygen species (ROS) are involved in several processes in plants. However, their levels in cells must be tightly regulated because of their toxicity. In order to keep H_2O_2 levels under control plants are equipped with antioxidant systems including CAT, SOD, GSR and APX [21]. In the present work, ulvan treatment trended to increase the activities of GPX, NOX, CAT, APX and GSR. These results seem to be contradictory because GPX and NOX are involved in producing whereas CAT, APX and GSR in removing H_2O_2 . However, this apparent contradiction could be explained by an antioxidant regulatory system. It is well known that a concurrent increase in ROS production and decrease in their removal is crucial for the onset of hypersensitive reaction [10]. On the other hand, ulvan induced resistance has been considered to be HR-independent [2,4,6]. Hence, an intervention of the antioxidant system would be expected to keep H_2O_2 level below the HR-inducing threshold.

Both *AtrbohD* and *AtrbohF* mutants showed different activity levels for the enzymes analyzed in the present work in comparison to WT plants (Figure 3). Considering that both mutants are derived from Col-0 plants [18], it would be expected some level of similarities in the enzymatic activity of these three ecotypes. However, both mutants show varying levels of H_2O_2 when compared to WT plants. RBOHF exhibits a minor effect while RBOHD gene is required for most of ROS produced during defense responses [18]. Thus, some variation in the enzymatic activity of the antioxidant system among the genotypes would be necessary since each one must deal with different levels of ROS.

Ulvan spraying increased transiently the activity of SOD in mock-inoculated *AtrbohD* plants and of GPX in both mutants (Figure 3). Similarly, the polysaccharide increased GPX activity in resistance mock-inoculated bean plants [4]. Previous works [2,7] demonstrated that ulvan treatment is able to induce profound cellular modifications in plants upregulating the expression of genes related to defense and primary and secondary metabolism.

Ulvan spraying did not reduce the disease severity in *AtrbohD* plants (Figure 2). The polysaccharide also failed to reduce the tissue colonization as well as electrolyte loss due to damages in the plasmatic membrane (Figure 5). Additionally, no NOX activity was detected in these plants (Figure 3) confirming the loss of function of the RBOHD gene reported in the literature [18,28]. Accordingly, these plants failed to accumulate H_2O_2 after ulvan treatment and inoculation with *A.*

brassicicola (Figure 4). Thus, we propose that ulvan induces resistance through a NOX-dependent pathway. It is well known that the two major sources of ROS in plants are NOX and class III peroxidase [12]. Therefore, even though *AtrbohD* plants have a functional class III peroxidase-dependent H₂O₂ generation system, they would not be able to respond to ulvan since NOX is impaired. In fact, ulvan did not reduce the disease severity in WT plants previously infiltrated with DPI, a widely used irreversible inhibitor of NADPH oxidases [24] (Figure 6). This result confirms our assumption that NADPH oxidase likely plays a key role in ulvan-induced resistance.

Further evidence supporting this proposition can be provided if we assume that rhamnose participates in the recognition process of ulvan [7]. Rhamnose linked to lipids, called rhamnolipids, extracted from *Pseudomonas aeruginosa* have been reported to induce a wide range of responses including Ca²⁺ influx, mitogen-activated protein kinase activation and ROS production in grapevine cell suspension cultures [24]. Understanding mechanisms and finding possible cell membrane-bound receptors involved in the plant recognition of rhamnolipids and ulvans will be an exciting challenge for future research.

The role of ROS in plant defense against necrotrophic pathogens is still unclear since its accumulation and the onset of HR are not predicted to limit their growth [32,33]. Rather than a direct antimicrobial effect, it has been suggested that ROS play a key role to trigger appropriate defense responses according to the nature of invading pathogen [32].

Considering our results and those available in the literature, we propose a general schematic representation of the defense responses induced by ulvan (Figure 7). In our model, the recognition of ulvan by an unknown receptor triggers a calcium influx, which is required for the activation of membrane-bound NADPH oxidase [12,16]. The superoxide produced in the apoplast by NADPH oxidase dismutates spontaneously or by the action of superoxide dismutase into hydrogen peroxide. Then, H₂O₂ enters the cytoplasm and activates a series of defense responses as well as initiates a ROS wave that propagates throughout the whole plant [2,6,12,16]. Ulvan does not induce disease resistance in *AtrbohD* plants because RBOHD, the main source of ROS, is not functional in this mutant [18]. On the other hand, the polysaccharide is able to induce resistance in *AtrbohF* plants since, although the RBOHF protein plays a major role in triggering of the hypersensitive response (i.e. localized cell death), it has only a minor role in ROS production [18].

In summary, our findings demonstrate that ulvan is able to protect *A. thaliana* plants against *A. brassicicola* through a pathway that is NADPH oxidase-dependent but HR-independent.

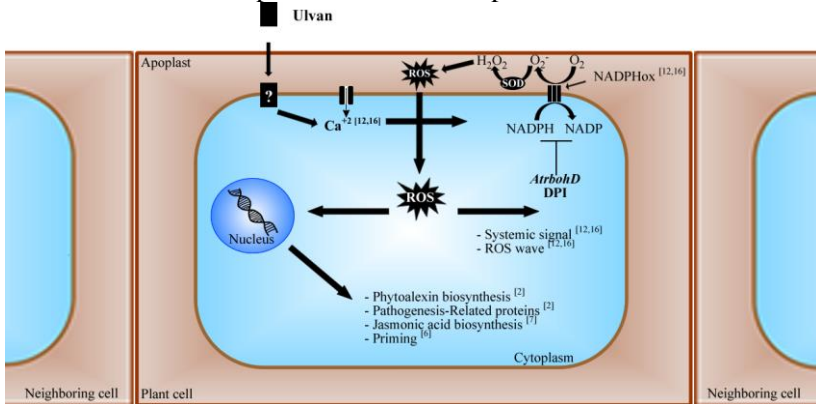


Figure 3.7. General model showing the defense responses induced by ulvan in plant tissues. Ulvan is recognized by an unknown receptor (?) triggering a calcium influx, which is required for the activation of membrane-bound NADPH oxidase (NADPHox) [12,16]. Superoxide produced in the apoplast by NADPH oxidase dismutates spontaneously or by the action of superoxide dismutase (SOD) into hydrogen peroxide. H₂O₂ enters the cytoplasm and activates a series of defense responses as well as initiate a reactive oxygen species (ROS) wave that propagates throughout the whole plant [2,6,12,16]. Ulvan does not induce disease resistance in *AtrbohD* plants because RBOHD, the main source of ROS, is not functional in this mutant [18]. On the other hand, the polysaccharide is able to induce resistance in *AtrbohF* plants since, although the RBOHF protein plays a major role in triggering of the hypersensitive response (i.e. localized cell death), it has only a minor role in ROS production [18]. DPI: diphenyleneiodonium.

ACKNOWLEDGEMENTS

The first and second authors thank the Coordination for the Improvement of Higher Education Personnel – CAPES and the National Counsel of Technological and Scientific Development – CNPq for the Ph.D. scholarship and the research productivity fellowship, respectively. We are also grateful to Dr. Pradeep Kachroo for kindly providing us seeds from the *AtrbohF* mutant.

5.5. REFERENCES

- [1] Stadnik MJ, de Freitas MB. Algal polysaccharides as source of plant resistance inducers. *Trop Plant Pathol* 2014; 39: 111-8.
- [2] Cluzet S, Torregrosa C, Jacquet C, Lafitte C, Fournier J, Mercier L, et al. Gene expression profiling and protection of *Medicago truncatula* against a fungal infection in response to an elicitor from green algae *Ulva* spp. *Plant, Cell Environ* 2004; 27: 917-28.
- [3] Borsato LC, Di Piero RM, Stadnik MJ. Mecanismos de defesa elicitados por ulvana contra *Uromyces appendiculatus* em três cultivares de feijoeiro. *Trop Plant Pathol* 2010; 35: 318-22.
- [4] de Freitas MB, Stadnik MJ. Race-specific and ulvan-induced defense responses in bean (*Phaseolus vulgaris*) against *Colletotrichum lindemuthianum*. *Physiol Mol Plant Pathol* 2012; 78: 8-13.
- [5] Araujo L, Stadnik MJ. Cultivar-specific and ulvan-induced resistance of apple plants to Glomerella leaf spot are associated with enhanced activity of peroxidases. *Acta Sci Agron* 2013; 35: 287-93.
- [6] Paulert R, Ebbinghaus D, Urlass C, Moerschbacher BM. Priming of the oxidative burst in rice and wheat cell cultures by ulvan, a polysaccharide from green macroalgae, and enhanced resistance against powdery mildew in wheat and barley plants. *Plant Pathol* 2010; 59: 634-42.
- [7] Jaulneau V, Lafitte C, Jacquet C, Fournier S, Salamagne S, Briand X, et al. Ulvan, a sulfated polysaccharide from green algae, activates plant immunity through the jasmonic acid pathway. *J Biomed Biotech* 2010, 525291.
- [8] Truman W, Bennett MH, Kubigsteltig I, Turnbull C, Grant M. *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc Natl Acad Sci* 2007; 104: 1075-80.
- [9] Lamb C, Dixon RA. The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 1997; 48: 251-75.

- [10] Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annu Rev Plant Biol* 2004; 55: 373-99.
- [11] Nanda AK, Andrio E, Marino D, Pauly N, Dunand C. Reactive oxygen species during plant-microorganism early interaction. *J Integr Plant Biol* 2010; 52: 195-204.
- [12] O'Brien JA, Daudi A, Butt VS, Bolwell GP. Reactive oxygen species and their role in plant defense and cell wall metabolism. *Planta* 2012; 236: 765-79.
- [13] Petrov VD, Breusegem FV. Hydrogen peroxide – a central hub for information flow in plant cells. *AoB Plants* 2012, pls014.
- [14] Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage and antioxidative defense mechanism in plants under stressful conditions. *J Bot* 2012, 217037.
- [15] Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R. Respiratory burst oxidases: the engines of ROS signaling. *Curr Opin Plant Biol* 2011; 14: 691-9.
- [16] Baxter A, Mittler R, Suzuki N. ROS as key players in plant stress signaling. *J Exp Bot* 2014; 65: 1229-40.
- [17] Kaur G, Sharma A, Guruprasad K, Pati PK. Versatile roles of plant NADPH oxidases and emerging concepts. *Biotech Adv* 2014; 32: 551-63.
- [18] Torres MA, Dangl JL, Jones JDG. *Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci* 2002; 99: 517-22.
- [19] Maruta T, Inoue T, Tamoi M, Yabuta Y, Yoshimura K, Ishikawa T, et al. *Arabidopsis* NADPH OXIDASES, *AtrbohD* and *AtrbohF* are essential for jasmonic acid-induced expression of genes regulated by MYC2 transcription factor. *Plant Sci* 2011; 180: 655-60.

- [20] Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vadepoele K, et al. ROS signaling: the new wave? *Trends Plant Sci* 2011; 16: 300-9.
- [21] Mittler R, Vanderauwera S, Gollery M, van Breusegem F. Reactive oxygen gene network of plants. *Trends Plant Sci* 2004; 9: 490-8.
- [22] Paulert R, Talamini V, Cassolato JEF, Duarte MER, Noseda MD, Smania Júnior A, et al. Effects of sulfated polysaccharide and alcoholic extracts from Green seaweed *Ulva fasciata* on anthracnose severity and growth of common bean (*Phaseolus vulgaris* L.). *J Plant Dis Prot* 2009; 116: 263-70.
- [23] Delgado DZ, de Freitas MB, Stadnik MJ. Effectiveness of saccharin and ulvan as resistance inducers against rust and angular leaf spot in bean plants (*Phaseolus vulgaris*). *Crop Prot* 2013; 47: 67-73.
- [24] Varnier AL, Sanchez L, Vatsa P, Boudesocque L, Garcia-Brugger A, Rabenoelina F, et al. Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant Cell Environ* 2009; 32: 178-93.
- [25] Rao MV, Paliyath G, Ormrod DP. Ultraviolet-B- and ozone-induced biochemical changes on antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol* 1996; 110: 125-36.
- [26] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
- [27] Hückelhoven R, Fodor J, Preis C, Kogel KH. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. *Plant Physiol* 1999; 119: 1251-60.
- [28] Pogány M, von Rad U, Grün S, Dongó A, Pintye A, Simoneau P, et al. Dual roles of reactive oxygen species and NADPH oxidase RBOHD in *Arabidopsis-Alternaria* pathosystem. *Plant Physiol* 2009; 151: 1459-75.

[29] Dellagi A, Brisset MN, Paulin JP, Expert D. Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. *Mol Plant-Microbe Interact* 1998; 11: 734-42.

[30] Paulert R, Smania Jr A, Stadnik MJ, Pizzolatti MG. Antifungal and antibacterial properties of the ulvan and crude extracts from the green seaweed *Ulva fasciata* Delile. *Algol Stud* 2007; 123: 123-9.

[31] Thomma BPHJ. *Alternaria* spp.: from general saprophyte to specific parasite. *Mol Plant Pathol* 2003, 4: 225-36.

[32] Tierens KFMJ, Thomma BPHJ, Bari RP, Garmier M, Eggermont K, Brouwer M, et al. *Esa1*, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds. *Plant J* 2002; 29: 131-40.

[33] Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 2005; 43: 205-227.

6. CONSIDERAÇÕES FINAIS

O presente trabalho demonstra que a ulvana é capaz de proteger plantas de *A. thaliana* frente a *A. brassicicola*, provavelmente por uma via de sinalização dependente de peróxido de hidrogênio produzido por NADPH oxidases, porém independente da reação de hipersensibilidade. A resistência induzida por ulvana é relacionada com um forte incremento na atividade de NADPH oxidases a partir de 6 horas após a inoculação. Além disso, o polissacarídeo tende a incrementar a atividade de enzimas envolvidas na remoção de espécies reativas de oxigênio sugerindo um controle rígido dos seus níveis.

A eficiência em induzir respostas de defesa de seis derivados de ulvana com teor de sulfato crescente (de 20,9% a 56,5%), bem como de oito frações obtidas a partir da hidrólise do polissacarídeo foi testada em plantas de *A. thaliana* contra *A. brassicicola* e *C. higginsianum*. A pulverização de ulvana e dos seus derivados com diferentes teores de sulfato reduziu de forma semelhante a severidade de ambos os patógenos. As frações DU1, DU3, DU5 e DU7 reduziram de forma mais eficiente a severidade de *A. brassicicola* quando comparadas com a ulvana. Por outro lado, as frações com baixo peso molecular DU6 e DU8 falharam em controlar o patógeno. Estes resultados sugerem que a eficiência em induzir respostas de defesa em *A. thaliana* pode estar mais relacionada com características estruturais do que o grau de polimerização da ulvana. Considerando que o grau de sulfatação e polimerização parecem não afetar a eficiência da ulvana, novos experimentos foram conduzidos para verificar se a ramnose, o principal açúcar componente da ulvana, poderia afetar a severidade de ambos os patógenos. De fato, nossos resultados demonstram que a ramnose é capaz de reduzir o diâmetro das lesões causadas por *A. brassicicola* e *C. higginsianum* de forma semelhante a ulvana, sugerindo que esse açúcar desempenha um papel importante na indução de resistência.

7. REFERÊNCIAS BIBLIOGRÁFICAS

AGRIOS, G. N. (Ed.). **Plant Pathology**. San Diego: Academic Press, 2005. 635p.

ALVES, A.; et al. A practical perspective on ulvan extracted from green algae. **Journal of Applied Phycology**, v. 25, p. 407-424, 2013.

ALLEN, R.D. Dissection of oxidative stress tolerance using transgenic plants. **Plant Physiology**, v. 107, p. 1049-1054, 1995.

AONO, M.; KUBO, A.; SAJI, H.; TANAKA, K.; KONDO, N. Enhanced tolerance to photooxidative stress of transgenic *Nicotiana tabacum* with high chloroplastic glutathione reductase activity. **Plant and Cell Physiology**, v. 34, p. 129-135, 1993.

APEL, K.; HIRT, H. Reactive oxygen species: metabolism, oxidative stress and signal transduction. **Annual Reviews of Plant Biology**, v. 55, p. 373-399, 2004.

ARAUJO, I. B.; PERUCH, L. A. M.; STADNIK, M. J. Efeito do extrato de alga e da argila silicatada na severidade da alternariose e na produtividade da cebolinha comum (*Allium fistulosum* L.). **Tropical Plant Pathology**, v. 37, p. 363-367, 2012.

ARAUJO, L.; STADNIK, M. J.; VALDEBENITO-SANHUEZA, R. M. Fosfito de potássio e ulvana no controle da mancha foliar a gala em macieira. **Tropical Plant Pathology**, v. 33, p. 148-152, 2008.

AZIZ, A.; POINSSOT, B.; DAIRE, X.; ADRIAN, M.; BÉZIER, A.; LAMBERT, B.; JOUBERT, J.M.; PUGIN, A. Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopora viticola*. **Molecular Plant-Microbe Interactions**, v.16, p. 1118-1128, 2003.

BAILEY, J.A.; O'CONNELL, R.J.; PRING, R.J.; NASH, C. Infection strategies of *Colletotrichum* species. In: BAILEY, A. J.; JEGER, J. M. **Colletotrichum: biology, pathology and control**. Oxford: British Society for Plant Pathology, 1992. p.88-120.

BAXTER, A.; MITTLER, R.; SUZUKI, N. ROS as key players in plant stress signaling. **Journal of Experimental Botany**, v. 65, p. 1229-1240, 2014.

BECKERS, G. J. M.; CONRATH, U. Priming for stress resistance: from the lab to the field. **Current Opinion in Plant Biology**, v. 10, p. 425-431, 2007.

BENT, A.F.; KUNKEL, B.N.; DAHLBECK, D.; BROWN, K.L.; SCHMIDT, R.; GIRAUDAT, J.; LEUNG, J.; STASKAWICS, B.J. RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. **Science**, v. 265, p. 1850-1860, 1994.

BIRKER, D.; HEIDRICH, K.; TAKAHARA, H.; NARUSAKA, M.; DESLANDES, L.; NARUSAKA, Y.; et al. A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct *Arabidopsis* accessions. **The Plant Journal**, v. 60, p. 602-613, 2009.

BLEECKER, A.B.; ESTELLE, M.A.; SOMMERVILLE, C.; KENDE, H. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. **Science**, v. 241, p. 1086-1089. 1988.

BORSATO, L. C.; DI PIERO, R. M.; STADNIK, M. J. Mecanismos de defesa elicitados por ulvana contra *Uromyces appendiculatus* em três cultivares de feijoeiro. **Tropical Plant Pathology**, v. 35, p. 318-322, 2010.

CHINCHILLA, D.; BAUER, Z.; REGENASS, M.; BOLLER, T.; FELIX, G. The *Arabidopsis* receptor Kinase FLS2 binds flg22 and determines the specificity of Flagellin perception. **Plant Cell**, v. 18, p. 465-476, 2006.

CLUZET, S.; TORREGROSA, C.; JACQUET, C.; LAFITTE, C.; FOURNIER, J.; MERCIER, L.; SALAMAGNE, S.; BRIAND, X.; ESQUERRÉ-TUGAYÉ, M. T.; DUMAS, B. Gene expression profiling and protection of *Medicago truncatula* against a fungal infection in response to an elicitor from green algae *Ulva* spp. **Plant, Cell and Environment**, Great Britain, v. 27, n. 7, p. 917-928, 2004.

DE FREITAS, M. B.; STADNIK, M. J. Race-specific and ulvan-induced defense responses in bean (*Phaseolus vulgaris*) against *Colletotrichum*

lindemuthianum. **Physiological and Molecular Plant Pathology**, v.78, p. 8-13, 2012.

DURRANT, W. E.; DONG, X. Systemic acquired resistance. **Annual Reviews of Phytopathology**, v. 42, p. 185-209, 2004.

EL MODAFAR, C. K.; ELGADDA, M.; EL BOUTACHFAITI, R.; ABOURAICHA, E.; ZEHHRAR, N.; PETIT, E.; EL ALAOUI-TALIBI, Z.; COURTOIS, B.; COURTOIS, J. Induction of natural defence accompanied by salicylic acid-dependant systemic acquired resistance in tomato seedlings in response to bioelicitors isolated from green algae. **Scientia Horticulturae**, v. 138, p. 55-63, 2012.

FOYER, C.H.; SOURIAU, N.; PERRET, S.; LELANDAIS, M.; KUNERT, K.J.; PRUVOST, C.; JOUANIN, L. Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. **Plant Physiology**, v. 109, p. 1047-1057, 1995.

GALLETTI, R.; DE LORENZO, G.; FERRARI, S. Host-derived signals activate plant innate immunity. **Plant Signaling and Behavior**, v. 3, p. 33-34, 2009

GALVÃO, S.; STADNIK, M.J.; PERUCH, L.A.M.; BRUNA, E.D. Avaliação da eficiência de produtos alternativos para o controle do míldio e da antracnose em videira, cultivar Niágara Branca. **Agropecuária Catarinense**, v.19, p.91-93, 2006.

GLAZEBROOK, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. **Annual Review of Phytopathology**, v. 43, p. 205-227, 2005.

GLAZEBROOK, J.; ROGERS, E. E.; AUSUBEL, F. M. Use of *Arabidopsis* for genetic dissection of plant defense responses. **Annual Review of Genetics**, v. 31, 547-569, 1997.

GOMÉZ-ARISA, J.; CAMPOS, S.; RUFAT, M.; ESTOPÀ, M.; MESSEGUER, J.; SAN SEGUNDO, B.; COCA, M. Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. **Molecular Plant-Microbe Interactions**, v. 20, p. 832-842, 2007.

GOZZO, F.; FAORO, F. Systemic acquired resistance (50 years after discovery): moving from the lab to the field. **Journal of Agricultural and Food Chemistry**, v. 61, p. 12473-12491, 2013.

GUAN, Z.; CHAI, T.; ZHANG, Y.; XU, J.; WEI, W. Enhancement of Cd tolerance in transgenic tobacco plants overexpressing a Cd-induced catalase cDNA. **Chemosphere**, v. 76, p. 623-630, 2009.

GUZMANN, P.; ECKER, J. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. **Plant Cell**, v. 2, p. 513-523, 1990.

HAMMERSCHIMIDT, R. Induced Disease Resistance: How to Induce Plants Stop Pathogens? **Physiological and Molecular Plant Pathology**, v. 55, p. 77-84, 1999.

HERBERS, K.; MEUWLY, P.; MÉTRAUX, J. P.; SONNEWALD, U. Salicylic acid independent induction of pathogenesis-related protein transcripts by sugars is independent on leaf developmental stage. **FEBS Letters**, v. 397, p. 239-244, 1996.

HIRUMA, K.; ONOZAWA-KOMORI, M.; TAKAHASHI, F.; ASAKURA, M.; BEDNAREK, P.; OKUNO, T.; SCHULZE-LEFERT, P.; TAKANO, Y. Entry mode-dependent function of an indole glucosinolate pathway in *Arabidopsis* for nonhost resistance against anthracnose pathogens. **Plant Cell**, v. 22, p. 2429-2443, 2010.

HUSER, A.; TAKAHARA, H.; SCHMALENBACH, W.; O'CONNELL, R. Discovery of pathogenicity genes in the crucifer anthracnose fungus, *Colletotrichum higginsianum*, using random insertional mutagenesis. **Molecular Plant-Microbe Interactions**, v. 22, p. 143-156, 2009.

INUI, H.; YAMAGUCHI, Y.; HIRANO, S. Elicitor actions of N-acetylchitoooligosaccharides and laminarioligosaccharides for chitinase and L-phenylalanine ammonia-lyase induction in rice suspension culture. **Bioscience, Biotechnology and Biochemistry**, v.61, p. 975-978, 1997.

JAULENEAU, V.; LAFITTE, C.; CORIO-COSTET, M. F.; STADNIK, M. J.; SALAMAGNE, S.; BRIAND, X. et al. An *Ulva armoricana* extract protects plants against three powdery mildew pathogens. **European Journal of Plant Pathology**, v. 131, p. 393-401, 2011.

JIA, Y., MCADAMS, S.A.; BRYAN, G.T.; HERSHEY, H.P.; VALENT, B. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. **EMBO Journal**, v. 19, p. 4004-4014, 2000.

KLARZYNSKI, O.; PLESSE, B.; JOUBERT, J. M.; YVIN, J. C.; KOPP, M.; KLOAREG, B.; FRITIG, B. Linear beta-1,3 glucans are elicitors of defense responses in tobacco. **Plant Physiology**, v. 124, n. 3, p. 1027-1038, 2000.

KOORNNEEF, A.; PIETERSE, C.M.J. Cross talk in defense signaling. **Plant Physiology**, v. 146, p. 839-844, 2008.

KOORNNEEF, M.; SCHERES, B. *Arabidopsis thaliana* as an experimental organism. **Encyclopedia of Life Sciences**, 2001.

KWAK, J.M.; MORI, I.C.; PEI, Z.; LEONHARDT, N.; TORRES, M.A.; DANGL, J.L.; BLOOM, R.E.; BODDE, S.; JONES, J.D.G.; SCHROEDER, J.I. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling *Arabidopsis*. **The EMBO journal**, v.22, p. 2623-2633, 2003.

LAMB, C.; DIXON, R. A. The oxidative burst in plant disease resistance. **Annual Reviews of Plant Physiology and Plant Molecular Biology**, v. 48, p. 251-275, 1997.

LAHAYE, M.; ROBIC, A. Structure and functional properties of ulvan, a polysaccharide from green seaweeds. **Biomacromolecules**, v. 8, n. 6, p. 1765-1774, 2007.

LALONDE, S.; BOLES, E.; HELLMANN, H.; BARKER, L.; PATRICK, J. W.; FROMMER, W. B.; WARD, J. M. The dual function of sugar carriers. Transport and sugar sensing. **Plant Cell**, v. 11, p. 707-726, 1999.

LAWTON, K.; WEYMANN, R.; FRIEDRICH, L.; VERNOOIJ, B.; UKNES, S.; RYALS, J. Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. **Molecular Plant-Microbe Interactions**, v. 8, p. 863-870, 1995.

LEONELLI, S. *Arabidopsis*, the botanical *Drosophila*: from mouse cress to model organism. **Endeavour**, v. 31, p. 34-38, 2007.

LIMA-FILHO, J. V. M.; CARVALHO, A. F. F. U.; FREITAS, S. M.; MELO, V. M. M. Antibacterial activity of extracts of six macroalgae from the northeastern Brazilian coast. **Brazilian Journal of Microbiology**, v.33, n. 4, p. 311-314, 2002.

LIPKA, V.; DITTGEN, J.; BEDNAREK, P.; BHAT, R.; WIERMER, M.; STEIN, M.; LANDTAG, J.; BRANDT, W.; ROSAHL, S.; SCHEEL, D.; LLORENT, F.; MOLINA, A.; PARKER, J.; SOMMERVILLE, S.; SCHULZE-LEFERT, P. Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. **Science**, v. 310, p. 1180-1183, 2005.

LOFFAGUEN, J. C.; HARTMANN, O. E. L.; TALAMINI, V.; STADNIK, M. J. Extratos naturais no controle da antracnose e na produtividade do feijoeiro. In: Congresso Brasileiro de Fitopatologia, 2004, **Fitopatologia Brasileira**, Brasília, v. 29 (suplemento), p. 107-107, 2004.

MACKEY, D.; BELKHADIR, Y.; ALONSO, J.M.; ECKER, J.R.; DANGL, J.L. *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. **Cell**, v. 112, p. 379-389, 2003.

MACKEY, D.; MCFALL, A. J. MAMPs and MIMPs: proposed classifications for inducers of innate immunity. **Molecular Microbiology**, v. 61, p. 1365-1371, 2006.

MAEDA, K.; HOUJYOU, Y.; KOMATSU, T.; HORI, H.; KODAIRA, T.; ISHIKAWA, A. AGB1 and PMR5 contribute to PEN2-mediated preinvasion resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*. **Molecular Plant-Microbe Interactions**, v. 22, p. 1331-1340, 2009.

MARUTA, T.; INOUE, T.; TAMOI, M.; YABUTA, Y.; YOSHIMURA, K.; ISHIKAWA, T.; SHIGEOKA, S. *Arabidopsis* NADPH oxidases, AtrbohD and AatrbohF, are essential for jasmonic acid-induced expression of genes regulated by MYC2 transcription factor. **Plant Science**, v. 180, p. 655-660, 2011.

MEINKE, D. W.; CHERRY, M. J.; DEAN, C.; ROUNSLEY, S. D.; KOORNNEEF, M. *Arabidopsis thaliana*: a model plant for genome analysis. **Science**, v. 282, p. 662-682, 1998.

MÉNARD, R.; ALBAN, S.; DE RUFFRAY, P.; JAMOIS F.; FRANZ, G.; FRITIG, B.; YVIN, J. C.; KAUFFMANN, S. B-1,3 Glucan sulfate, but not B-1,3 glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis. **The Plant Cell**, v. 16, p. 3020-3032, 2004.

MÉNARD, R.; DE RUFFRAY, P.; FRITIG, B.; YVIN, J. C.; KAUFFMANN, S. Defense and resistance-inducing activities in tobacco of the sulfated B-1,3 glucan PS3 and its synergistic activities with the unsulfated molecule. **Plant Cell Physiology**, v. 46, p. 1964-1972, 2005.

MERCIER, L.; LAFITTE, C.; BORDERIES, G.; BRIAND, X.; ESQUERRÉ-TUGAYÉ, M. T.; FOURNIER, J. The algal polysaccharide carrageenans can act as an elicitor of plant defense. **New Phytologist**, v. 149, p. 43-51, 2001.

MEYEROWITZ, E. M. *Arabidopsis thaliana*. **Annual Reviews of Genetics**, v. 21, p. 93-111, 1987.

MITTLER, R.; VANDERAUWERE, S.; GOLLERY, M.; VAN BREUSEGEM, F. Reactive oxygen gene network of plants. **Trends in Plant Science**, v. 9, p. 490-498, 2004.

MOGHADDAM, M. R. B.; VAN DEN ENDE, W. Sugars and plant innate immunity. **Journal of Experimental Botany**, v. 63, p. 3989-3998, 2012.

MORKUNAS, I.; MARCZAK, Q.; STACHOWIAK, J.; STOBIECKI, M. Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. **Plant Physiology and Biochemistry**, v. 43, p. 363-373, 2005.

MUKHTAR, S.; NISHIMURA, M.T.; DANGL, J. NPR1 in plant defense: it's over 'till it's turned over. **Cell**, v. 137, p. 804-806, 2009.

NAKAO, M.; NAKAMURA, R.; KITA, K.; INUKAI, R.; ISHIKAWA, A. Non-host resistance to penetration and hyphal growth of *Magnaporthe oryzae* in Arabidopsis. **Scientific Reports**, 1, 171, 2011.

NARUSAKA, Y.; NARUSAKA, M.; PARK, P.; KUBO, Y.; HIRAYAMA, T.; SEKI, M.; et al., *RCH1*, a locus in *Arabidopsis* that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. **Molecular Plant-Microbe Interaction**, v. 17, p. 749-762, 2004.

NOWICKI, M.; NOWAKOWSKA, M.; NIEZGODA, A.; KOZIK, E. U. Alternaria black spot of crucifers: symptoms, importance of disease and perspectives of resistance breeding. **Vegetable Crop Research Bulletin**, v. 76, p. 5-19, 2012.

O'BRIEN, J. A.; DAUDI, A.; BUTT, V.S.; BOLWELL, G. P. Reactive oxygen species and their role in plant defense and cell wall metabolism. **Planta**, v. 236, p. 765-779, 2012.

O'CONNELL, R. J.; HERBERT, C.; SREENIVASAPRASAD, S.; KHATIB, N.; ESQUERRÉ-TUGAYÉ, M. T.; DUMAS, B. Anovel *Arabidopsis-Colletotrichum* pathosystem for the molecular dissection of plant-fungal interactions. **Molecular Plant-Microbe Interactions**, v. 17, p. 272-282, 2004.

O'CONNELL, R. J.; PERFECT, S.; HUGHES, B.; CARZANIGA, R.; BAILEY, J.; GREEN, J. Dissecting the cell biology of *Colletotrichum* infection processes. In: PRUSKY, D.; FREEMAN, W.; DICKMAN, M. B. **Colletotrichum: Host Specificity, Pathology and Host-Pathogen Interaction**. St. Paul: APS Press, 2000, p. 57-77.

O'CONNELL, R. J.; THON, M. R.; HACQUARD, S.; AMYOTTE, S. G.; KLEEMANN, J.; TORRES, M. F.; et al. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. **Nature Genetics**, v. 44, p. 1060-1065, 2012.

OERKE, E. C. Crop losses to pests. **Journal of Agricultural Science**. v. 144, p. 31-43, 2006.

PAULERT, R.; SMANIA JÚNIOR, J.; STADNIK, M. J.; PIZZOLATTI, M. G. Antimicrobial properties of extracts from the green seaweed *Ulva fasciata* Delile against pathogenic bacteria and fungi. **Algological Studies**, v. 123, p. 123-130, 2007.

PAULERT, R.; EBBINGHAUS, D.; URLASS, C.; MOERSCHBACHER, B. M. Priming of the oxidative burst in rice and wheat cell cultures by ulvan, a polysaccharide from green macroalgae, and enhanced resistance against powdery mildew in wheat and barley plants. **Plant Pathology**, v. 59, p. 634-642, 2010.

PAULERT, R.; TALAMINI, V.; CASSOLATO, J. E. F.; DUARTE, M. E. R.; NOSEDA, M. D.; SMANIA JÚNIOR, A.; STADNIK, M. J. Effects of sulfated polysaccharide and alcoholic extracts from Green seaweed *Ulva fasciata* on anthracnose severity and growth of common bean (*Phaseolus vulgaris* L.). **Journal of Plant Diseases and Protection**, v. 116, p. 263-270, 2009.

PERCHEPIED, L.; BALAGUÉ, C.; RIOU, C.; CLAUDEL-RENARD, C.; RIVIÈRE, N.; GRZES-BRESSET, B.; ROBY, D. Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. **Molecular Plant-Microbe Interactions**, v. 23, p. 846-860, 2010.

PERFECT, S. E.; BLEDDYN HUGHES, H.; O'CONNELL, R. J.; GREEN, J. R. *Colletotrichum*: a model genus for studies on pathology and fungal-plant interactions. **Fungal Genetics and Biology**, v. 27, p. 186-198, 1999.

PERUCH, L.A.M.; MEDEIROS, A.M.; BRUNA, E.D.; STADNIK, M.J. Biomassa cítrica, extrato de algas, calda bordalesa e fosfitos no controle do míldio da videira, cv. Niágara Branca. **Revista de Ciências Agroveterinárias**, v. 6, p. 143-148, 2007.

QI, H.; ZHANG, Q.; ZHAO, T.; CHEN, R.; ZHANG, H.; NIU, X.; LI, Z. Antioxidant activity of different sulfate content derivatives of polysaccharides extracted from *Ulva pertusa* (Chlorophyta) in vitro. **International Journal of Biological Macromolecules**, v. 37, p. 195-199, 2005.

QI, H.; HUANG, L.; LIU, X.; LIU, D.; ZHANG, Q.; LIU, S. Antihyperlipidemic activity of high sulfate content derivative of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). **Carbohydrate Polymers**, v. 87, p. 1637-1640, 2012.

RADOTIC, K.; DUCIE, T.; MUTAVDZIC, D. Changes on peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium. **Environmental and Experimental Botany**, v. 44, p. 105-113, 2000.

RAY, B.; LAHAYE, M. Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (Ulvales, Chlorophyta). Chemical structure of ulvan. **Carbohydrate Research**, v. 274, p. 313-318, 1995.

REIGNAULT, P.; COJAN, A.; MUCHEMBLED, L.; SAHOURI, A. L.; DURAND, R.; SANCHOLLE, M. Trehalose induces resistance to powdery mildew in wheat. **New Phytologist**, v. 149, p. 519-529, 2001.

ROBIC, A.; GAILLARD, C.; SASSI, J. F.; LERAT, Y.; LAHAYE, M. Ultrastructure of ulvan: a polysaccharide from green seaweed. **Biopolymers**, v. 91, p. 652-664, 2009.

ROBIC, A.; SASSI, J. F.; LAHAYE, M. Impact of stabilization treatments of the green seaweed *Ulva rotundata* (Chlorophyta) on the extraction yield, the physico-chemical and rheological properties of ulvan. **Carbohydrate Polymers**, v. 74, p. 344-352, 2008.

ROLLAND, F.; BAENA-GONZALEZ, E.; SHEEN, J. Sugar sensing and signaling in plants: conserved and novel mechanisms. **Annual Reviews in Plant Biology**, v. 57, 675-709, 2006.

ROTEM, J. **The genus *Alternaria***. APS Press, 1998, 326p.

SANGHA, J. S.; RAVICHANDRAN, S.; PRITHIVIRAJ, H.; CRITCHLEY, A. T.; PRITHIVIRAJ, B. Sulfated macroalgal polysaccharides λ -carrageenan and ι -carrageenan differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*. **Physiological and Molecular Plant Pathology**, v. 75, p. 38-45, 2010.

SCHONS, R. F.; DE FREITAS, M.B.; STADNIK, M. J. Durabilidade da resistência induzida por ulvana e efeito da concentração de inóculo no controle da antracnose do feijão. **Bioscience Journal**, v. 27, p. 544-551, 2011.

SHARMA, H. S. S.; FLEMING, C.; SELBY, C.; RAO, J.R.; MARTIN, T. Plant biostimulants: a review on the processing of macroalgae and use

of extracts for crop management to reduce abiotic and biotic stress. **Journal of Applied Phycology**, v. 26, p. 465-490, 2014.

SHEEN, L.; ZHOU, L.; JANG, J. C. Sugars as signaling molecules. **Current Opinion in Plant Biology**, v. 2, p. 410-418, 1999.

SHAO, F.; MERRITT, P.M.; BAO, Z.; INNES, R.W.; DIXON, J.E. A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. **Cell**, v. 109, p. 575-588, 2002.

SHAO, F.; GOLSTEIN, C.; ADE, J.; STOUTEMEYR, M.; DIXON, J.E.; INNES, R.W. Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. **Science**, v. 301, p. 1230-1233, 2003.

SMEEKENS, S. Sugar-induced signal transduction in plants. **Annual Reviews of Plant Physiology and Plant Molecular Biology**, v. 51, p. 49-81, 2000.

SPOEL, H. S.; DONG, X. How do plants achieve immunity? Defense without specialized immune cells. **Nature Reviews**, v. 12, p. 89-100, 2012.

SPOEL, H.S.; JOHNSON, J.S.; DONG, X. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. **Proceedings of the National Academy of Sciences**, v. 104, p. 18842-18847, 2007.

STADNIK, M. J.; DE FREITAS, M.B. Algal polysaccharides as source of plant resistance inducers. **Tropical Plant Pathology**, v. 39, p. 111-118, 2014.

STADNIK, M. J.; MARASCHIN, M. Indução de resistência de plantas a fitopatógenos. In: STADNIK, M. J.; TALAMINI, V. (Eds.). **Manejo ecológico de doenças de plantas**. Florianópolis: CCA/UFSC, 2004, p. 221-244.

STADNIK, M. J.; PAULERT, R. Uso de macroalgas marinhas na agricultura. **Museu Nacional de Rio de Janeiro**, v. 30, p. 267-279, 2008.

STADNIK, M.J.; STEINHAUSER, C.; DE FREITAS, M.B.; POGANY, M.; GOESSL, L.; DURNER, J. Ulvan-induced resistance to *Alternaria brassicicola* in *Arabidopsis* is salicylic acid-independent but characterized by PR-1 accumulation. **Tropical Plant Pathology (supl.)**, v. 34, S258, 2009.

STEIN, M.; DITTGEN, J.; SÁNCHEZ-RODRÍGUEZ, C.; HOU, B.H.; MOLINA, A.; SCHULZE-LEFERT, P.; LIPKA, V.; SOMMERVILLE, S. *Arabidopsis* PEN3/ PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. **The Plant Cell**, v. 18, p. 731-746, 2006.

SUZUKI, N.; MILLER, G.; MORALES, J.; SHULAEV, V.; TORRES, M. A.; MITTLER, R. Respiratory burst oxidases: the engines of ROS signaling. **Current Opinion in Plant Biology**, v. 14, p. 691-699, 2011.

TAYEFI-NASRABADI, H.; DEGHAN, G.; DAEIHASSANI, B.; MOVAFEGI, A.; SAMADI, A. Some biochemical properties of guaiacol peroxidases as modified by salt stress in leaves of salt-tolerant and salt-sensitive safflower (*Carthamus tinctorius* L.cv.) cultivars. **African Journal of Biotechnology**, v. 10, p. 751-763, 2011.

TAYEH, C.; RANDOUX, B.; VINCENT, D.; BOURDON, N.; REIGNAULT, P. Exogenous trehalose induces defenses in wheat before and during a biotic stress caused by powdery mildew. **Phytopathology**, v. 104, p. 293-305, 2014.

THIBAUD, M. C.; GINESTE, S.; NUSSAUME, L.; ROBAGLIA, C. Sucrose increases pathogenesis-related PR-2 gene expression in *Arabidopsis thaliana* through an SA-dependent but NPR1-independent signaling pathway. **Plant Physiology and Biochemistry**, v. 42, p. 81-88, 2004.

THOMMA, B. P. H. J. *Alternaria* spp.: from general saprophyte to specific parasite. **Molecular Plant Pathology**, v. 4, p. 225-236, 2003.

TORRES, M. A.; DANGL, J. L.; JONES, J. D. G. *Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. **Proceedings of the National Academy of Sciences**, v. 99, p. 517-522, 2002.

TROUVELOT, S.; HÉLOIR, M. C.; POINSSOT, B.; GAUTHIER, A.; PARIS, F.; GUILLIER, C.; COMBIER, M.; TRDÁ, L.; DAIRE, X.; ADRIAN, M. Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. **Frontiers in Plant Science**, v.5, 592, 2014.

WALTERS D. R. Introduction. In: WALTERS, D. **Disease Control in Crops**. Wiley-Blackwell, 2009, p. 1-5.

WALTERS, D. R. Induced resistance: destined to remain on the sidelines of crop protection? **Phytoparasitica**, v. 38, p. 1-4, 2010.

WALTERS, D. R.; RATSEP, J.; HAVIS, N. D. Controlling crop diseases using induced resistance: challenges for the future. **Journal of Experimental Botany**, v. 64, p. 1263-1280, 2013.

WANG, Y.; WISNIEWSKI, M.; MEILAN, R.; CUI, M.; WEBB, R.; FUCHIGAMI, L. Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. **Journal of the American Society for Horticultural Science**, v. 130, p. 167-173, 2005.

WILLEKENS, H.; CHAMNONGPOL, M.; DAVEY, M.;SCHRAUDNER, M.; LANGEBARTELS, C.; VAN MONTAGU, M.; INZÉ, D.; VAN CAMP, W. Catalase is a sink for H₂O₂ and is indispensable for stress defence in C3 plants. **The EMBO Journal**, v. 16, p. 4806-4816, 1997.

WORDELL FILHO, J.A.; MARTINS, D.A.; STADNIK, M.J. (2007) Aplicação foliar de tratamentos para o controle do míldio e da podridão-de-escamas de bulbos de cebola. *Horticultura Brasileira* 25:544-549.

YABUTA, Y.; MOTOKI, T.; YOSHIMURA, K.; TAKEDA, T.; ISHIKAWA, T.; SHIGEOKA, S. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. **Plant Journal**, v. 32, p. 915-925, 2002.

ZHOU, J.; SUN, A.; XING, D. Modulation of cellular redox status by thiamine-activated NADPH oxidase confers *Arabidopsis* resistance to *Sclerotinia sclerotiorum*. **Journal of Experimental Botany**, v.11, p. 3261-3272, 2013.