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Stimulation des défenses naturelles de la tomate et de l'olivier et induction de la résistance vis-à-vis de la verticilliose par les poly/oligosaccharides d'algues marines des côtes marocaines

Meriem Aitouguinane

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**Stimulation des défenses naturelles de la tomate et de
l'olivier et induction de la résistance vis-à-vis de la
verticilliose par les poly/oligosaccharides d'algues marines
des côtes marocaines**

par :

Meriem AITOUGUINANE

(Dernier diplôme : Master Microbiologie Appliquée)

soutenue le 17 Novembre 2022 devant la commission d'examen :

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LISTE DES ABBREVIATIONS

¹H NMR : Proton Nuclear Magnetic Resonance
4CL : 4-Coumarate : Coenzyme A Ligase
ABA : Acide Abscissique
ACC : Acide 1-Aminocyclopropane-1-Carboxylique
AG : Acide Guluronique
AGB : Arabinogalactane sulfaté extrait de *Codium decorticum* (Extrait brut)
AGP : Arabinogalactane sulfatés extrait de *Codium decorticum* (Extrait purifié)
AGPs : Arabinogalactanes Riche En Protéines
AJ : Acide Jasmonique
ALCM : Alginate extrait de *Cystoseira myriophylloides*
ALGC : Alginate commercial
ALSM : Alginate extrait de *Sargassum muticum*
AM : Acide Mannuronique
AMF : Champignons Mycorhiziens Arbusculaires
AMM : Autorisation de Mise Sur Le Marché
AMV : Alfalfa Mosaic Virus
ANE : Extrait d'*Ascophyllum nodosum*
ANOVA : Analyse de Variance
AOS : Allene Oxyde Synthase
AOSs : Oligoalginates
ArMV : Arabis Mosaic Virus
AS : Acide Salicylique
AU : Uronic Acid
AUDPC : Area Under the Disease Progress Curve
BBSA : Alginate extrait de *Bifurcaria bifurcata*
BCA : Agents de Biocontrôle
BSA : Albumine de Sérum Bovin
BSTFA : N, O-Bis (Trimethylsilyl) Trifluoroacetamide
C3H : *p*-Coumarate 3-Hydroxylase
C4H : Cinnamate 4-Hydroxylase
CAD : Alcool Cinnamylique Déshydrogénase
CAT : Catalase
CCoAOMT : Caféoyl-Coa O-Méthyltransférase
CCR : Cinnamoyl-Coa Réductase
CD : *Codium decorticum*
CERK1 : Chitin Elicitor Receptor Kinase 1
CEVd : Citrus Exocortis Viroid
CLRV : Cherry Leafroll Virus
CLVd : Columnea Latent Viroid
CM : *Cystoseira myriophylloides*
CMV : Cucumber Mosaic Virus
COMT : Acide Caféique O-Méthyltransférase
COS : Chitooligosaccharide
COV : Composés Organiques Volatils
CpCDV : Chickpea Chlorotic Dwarf Virus
CRD : Carbohydrate-Recognition Domain
CRDO : Oligosaccharide de Curdlane
CSE : Caféoyl Shikimate Estérase

CYP : Cytochrome
D : Polydispersity Index
DAMP : Damage-Associated Molecular Patterns
DI : Dwarfing Index
DMDS : Disulfure De Diméthyle
DO : Densité Optique
DP : Degree of Polymerization
DW : Dry Weight
ECD : Ligand Binding Ectodomain
EDTA : Ethylenediaminetetraacetic Acid
EGF : Epidermal Growth Factor
EMDV : Eggplant Mottled Dwarf Virus
ESM : Epithiospecifer Modifier
ET : Ethylène
ETI : Effector-Triggered Immunity
EU : European Union
F5H : Férulate 5-Hydroxylase
FAO : Organisation des Nations Unies pour l'Alimentation et l'Agriculture
FC : Folin–Ciocalteu
FMS : Final Mean Severity
FTIR/ATR-FTIR : Attenuated Total Reflectance- Fourier Transform Infrared
FUCCM : Fucoïdane extrait de *Cystoseira myriophylloides*
FW : Fresh Weight
GC-EIMS : Gas Chromatography–Cold Electron Ionization–Mass Spectrometry
GM : Cultures Génétiquement Modifiées
GP : Glycoprotein
GRP : Protéines Riches en Glycine
GST : Glutathion S-Transférase
HAMP : Herbivore-Associated Molecular Patterns
HCT : Hydroxycinnamoyl-CoA Shikimate
HPAEC-PAD : High-Performance Anion-Exchange Chromatography-Pulsed Amperometric Detection
HPSEC-MALLS/ SEC-MALS : High-Performance Size-Exclusion Chromatography-on-line Multi-Angle Laser Light Scattering
HRGP : Glycoprotéines Riches en Hydroxyproline
IAA : Auxines-Acide Indole-3-Acétique
IPP : Isopentenyl Diphosphate
ISR : Résistance Systémique Induite
LAC : Laccases
LMW : Low Molecular Weight
LOX : Lipoxygénases
LPS : Lipopolysaccharides
LysMs : Lysine Motifs
MAMP : Microbe-Associated Molecular Patterns
MAPK : Mitogen-Activated Protein Kinases
MeJA : Methyl Jasmonate
m-HDP : m-Hydroxydiphenyl
MIS : Maximum Intensity Symptoms
M_n : Number Average Molecular Weight
MS : Microscélérotés

MSE : Methanolic Seaweed Extract
M_w : Weight Average Molecular Weight
NB-LRR : Nucleotide Binding site-Leucine Rich Repeat
NO : Oxyde Nitrique
OACM : alginate de faible poids moléculaire obtenu des alginates de *Cystoseira myriophylloides*
OASM : alginate de faible poids moléculaire obtenu des alginates de *Sargassum muticum*
OC : Oligocarraghénane
OF : Oligofucane
OG : Oligogulonate
OGA : oligogalacturonide
OGAB : Oligoalginates obtenu des alginates de *Bifurcaria bifurcata*
OLRV : Olive Latent Ringspot Virus
OLS : Olive Leaf Spot
OLV : Olive Latent Virus
OLYaV : Olive Leaf Yellowing-Associated Virus
OM : Oligomannuronate
OMMV : Olive Mild Mosaic Virus
ON : Neutral Sugar
ONSSA : Office National de Sécurité Sanitaire des Produits Alimentaires
OQDS : Syndrome de Déclin Rapide de l'Olivier
OSLV : Olive Semilatif Virus
OU : Oligoulvane
OVYaV/OVYV : Olive Vein Yellowing Associated Virus
OXGL : Oligoxyloglucane
OYMDaV : Olive Yellow Mottle and Decline-Associated Virus
pAGS : Pyruvylated Arabinogalactan Sulfates
PAL : Phénylalanine Ammonia-Lyase
PAMP : Pathogen Associated Molecular Pattern
PCD : Mort Programmée des Cellules
PDA : Potato Dextrose Agar
PDF/Def : Défensine de la Plante
PepMV : Pepino Mosaic Virus
PGN : Peptidoglycane
PGPF : Champignons Favorisant la Croissance des Plantes
PGPR : Plant Growth-Promoting Rhizobacteria
PMoV : Parietaria Mottle Virus
POD : Peroxydases
PPase : Peptidyl Prolyl *cis-trans* Isomérase
PPO : Polyphénol-Oxydases
PPP : Produits Phytopharmaceutiques
PR : Pathogenesis-Related Protein
PRP : Protéines Riches en Proline
PRR : Pattern Recognition Receptor
PSTVd : Potato Spindle Tuber Viroid
PTI : PAMP-Trigged Immunity
PVPP : Polyvinylpolypyrrolidone
PVX : Virus X de la Pomme de Terre
PVY : Potato Virus Y
PZSV : Pelargonium Zonate Spot Virus

RH : Réponse d'Hypersensibilité
RLKs : Receptor-Like Kinases
RMT : Réseau Mixte Technologique
ROS : Reactive Oxygen Species
SAR : Résistance Systémique Acquise
SDP : Stimulateurs des Défenses Naturelles des Plantes
SE : Standard Error
SEs : Extraits Bruts
SLRSV : Strawberry Latent Ringspot Virus
SM : *Sargassum muticum*
SOD : Superoxyde Dismutase
SPs : Polysaccharides Sulfatés
STS : Stilbène Synthase
STV : Southern Tomato Virus
TAL : Tyrosine Ammonia-Lyase
TASVd : Tomato Apical Stunt Viroid
TCDVd : Tomato Chlorotic Dwarf Viroid
TFA : Trifluoroacetic Acid
TI : Protéine Inhibitrice de la Trypsine
TICV : Tomato Infectious Chlorosis Virus
TMV : Tobacco Mosaic Virus
TNV : Tobacco Necrosis Virus
ToBRFV : Tomato Brown Rugose Fruit Virus
ToCV : Tomato Chlorosis Virus
ToLCNDV : Tomato Leaf Curl New Delhi Virus
ToMV : Tomato Mosaic Virus
ToTV : Tomato Torrado Virus
TSWV : Tomato Spotted Wilt Virus
TYLCSV : Tomato Yellow Leaf Curl Sardinia Virus
TYLCV : Tomato Yellow Leaf Curl Virus
VWO : Verticilliose de l'Olivier
WAK : Wall-Associated Kinases

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RESUME

Ce travail repose sur les connaissances fondamentales acquises sur les interactions plante-parasite en matière d'induction des mécanismes de défense des plantes en réponse aux agents phytopathogènes afin de développer une nouvelle génération de molécules biosourcées susceptibles de stimuler les défenses naturelles des plantes dans un contexte de développement durable. Il est ainsi admis que le déclenchement du système de défense de la plante résulte d'une reconnaissance par la cellule-hôte d'un éliciteur souvent produit par le pathogène (éliciteur exogène) ou par la plante-hôte (éliciteur endogène). La plupart des éliciteurs sont des polysaccharides et particulièrement des oligosaccharides. Compte tenu de leur richesse en polymères de glucides, les algues constituent une source naturelle pouvant être valorisée en matière de recherche d'éliciteurs des défenses naturelles des plantes. L'objectif principal de ce travail consiste en la recherche et la caractérisation des poly/oligosaccharides d'algues marines, susceptibles de stimuler les défenses naturelles (SDPs) de la tomate et de l'olivier et d'induire la résistance vis-à-vis de la verticilliose.

Le travail a permis tout d'abord l'extraction et les caractérisations chimique et structurale des polysaccharides non sulfatés (alginates) à partir des algues brunes marocaines (*Cystoseira myriophylloides*, *Sargassum muticum* et *Bifurcaria bifurcata*) et la production de leurs dérivés de faibles poids moléculaires, ainsi que les polysaccharides sulfatés (arabinogalactanes et fucoïdanes) à partir des algues verte et brune (*Codium decorticatum* et *C. myriophylloides*). Le pouvoir éliciteur de l'ensemble de ces molécules a été évalué sur les plants de tomate et les disques foliaires de l'olivier à travers le suivi des principaux mécanismes de défense reconnus par leur implication dans la résistance des plantes aux agents phytopathogènes notamment, la phénylalanine ammonia-lyase (PAL) et la tyrosine ammonia-lyase (TAL), enzymes clés du métabolisme phénolique et l'accumulation des polyphénols solubles et de la lignine. Cette étude a permis d'opérer les principaux paramètres conduisant à des réponses optimales (structure des molécules, mode d'application et synergie entre différents motifs éliciteurs). Le traitement des plants de tomate et les disques foliaires de l'olivier par les fractions extraites des algues brunes et verte ainsi que leurs dérivés a révélé un potentiel éliciteur remarquable. Toutefois, les activités électriques ont montré une variabilité en fonction de la durée de l'incubation, la structure des molécules et le mode de traitement. En outre, ces motifs saccharidiques ont montré un potentiel protecteur des plants de tomate vis-à-vis du développement de la verticilliose causée par *Verticillium dahliae* avec une réduction considérable de l'incidence et de la sévérité de la maladie et de la mortalité, comparée à la laminarine qui représente la substance active du SDP commercial Iodus 40[®]. L'ensemble des résultats obtenus ouvre de nouveaux horizons pour la gestion des phytopathogènes vasculaires à travers une stratégie compatible avec les principes d'une agriculture durable respectueuse de l'environnement.

Mots clés : Polysaccharides, Oligosaccharides, Algues marines, SDPs, *Verticillium dahliae*, Agriculture durable.

ABSTRACT

This work is based on the principles of plant-parasitic interactions in terms of the induction of defense mechanisms in order to develop a new generation of biosourced molecules capable of stimulating the natural defenses of plants in a context of sustainable development. It is known that the induction of plant responses is a result of the recognition of an elicitor by the host cells. This elicitor could be exogenous (produced by the pathogen) or endogenous (produced by the host plant). Most of these molecules are polysaccharides, and particularly oligosaccharides. Due to their richness in carbohydrate polymers, seaweed constitutes a natural resource that can be used to hunt for bioactive compounds with eliciting potential. The main aim of this work is the research and characterization of poly/oligosaccharides from seaweed, capable to stimulate the natural defenses (SDPs) of tomato and olive trees, and to induce the resistance against *Verticillium* wilt.

First, this study enabled the extraction, chemical and structural characterization, and production of low molecular weight derivatives of non-sulfated polysaccharides (alginates) from Moroccan brown algae (*Cystoseira myriophylloides*, *Sargassum muticum* and *Bifurcaria bifurcata*), as well as sulfated polysaccharides (arabinogalactans and fucoidans) from green and brown algae (*Codium decorticatum* and *C. myriophylloides*). The elicitor activities of these molecules were evaluated on tomato plants and olive tree leaves by monitoring the main defense mechanisms involved in plant resistance to phytopathogenic agents, such as the phenylalanine ammonia-lyase (PAL) and the tyrosine ammonia-lyase (TAL), the key enzymes of phenolic metabolism, as well as the accumulation of soluble polyphenols and lignin. These were performed with an assessment of the main factors influencing the induced responses (structure of the molecules, mode of application, and synergy between different saccharide molecules). The treatment of tomato seedlings and olive tree leaves with fractions from brown and green algae and their derivatives revealed a considerable elicitor potential. The elicitor activities showed variability depending on the duration of the incubation, the structure of the molecules and the mode of treatment. In addition, these saccharide molecules showed a protective potential against the development of verticillium wilt caused by *Verticillium dahlia* in tomato seedlings, with a considerable reduction of disease incidence and severity and mortality, compared to laminarin, the active substance of commercial SDP Iodus 40®. These results open up a new orientation to managing vascular phytopathogens using an approach that is in accordance with the principles of eco-sustainable agriculture.

Keywords: Polysaccharides, Oligosaccharides, Seaweed, SDPs, *Verticillium dahliae*, Sustainable agriculture.

ملخص

يعتمد هذا العمل على مبادئ التفاعلات النباتية والطفيليات من حيث تحريض آليات الدفاع من أجل تطوير جيل جديد من الجزيئات الحيوية القادرة على تحفيز الدفاعات الطبيعية للنباتات في سياق التنمية المستدامة. من المعروف أن بدء تشغيل نظام دفاع النبات هو نتيجة لتعرف الخلايا النباتية على جسيمات خارجية (منتجة من طرف العامل الممرض) أو داخلية (منتجة من طرف النباتات نفسها). معظم هذه الجسيمات عبارة عن متعدد السكاريد، وخاصة سكريات قليلة التعدد. نظرًا لغناها بالبوليمرات الكربوهيدراتية، تشكل الأعشاب البحرية موردًا طبيعيًا يمكن استخدامه للبحث عن المركبات النشطة بيولوجيًا ذات الإمكانيات المعززة لمناعة النباتات. تتمثل الأهداف الرئيسية لهذا العمل في البحث وتشخيص البولي/أوليغوساكاريد من الأعشاب البحرية، كمحفزات الدفاعات الطبيعية (SDPs) للطماطم وأشجار الزيتون، ومحفزات المقاومة ضد ذبول الفريسيليوم.

أولاً، مكنت هذه الدراسة من استخلاص وتشخيص كيميائي وتركيبى وإنتاج مشتقات ذات كتلة جزيئية منخفضة من السكريات غير الكبريتية (alginates) من الطحالب البنية المغربية (*Cystoseira myriophylloides*, *Sargassum muticum*) و (*Bifurcaria bifurcata*)، بالإضافة إلى السكريات الكبريتية (arabinogalactans و fucoidans) من الطحالب الخضراء والبنية (*Codium decortcatum* و *C. myriophylloides*). تم تقييم الأنشطة التحفيزية لهذه الجزيئات على نباتات الطماطم وأوراق شجرة الزيتون من خلال مراقبة آليات الدفاع الرئيسية المشاركة في مقاومة النبات للعوامل المسببة للأمراض النباتية كالفيينيل ألانين أمونيا-لياز (PAL) و التيروزين أمونيا-لياز (TAL)، الإنزيمات الرئيسية في التمثيل الغذائي الفينولي، وتراكم مادة البوليفينول القابلة للذوبان واللجنين. تم إجراء هذه الدراسة مع تقييم العوامل الرئيسية التي تؤثر على الاستجابات المناعية (بنية الجزيئات، وطريقة المعالجة، والخلط بين جزيئات السكاريد المختلفة). أظهرت جميع الجزيئات البولي / قليل السكاريدية المستخلصة من الطحالب البنية والخضراء ومشتقاتها إمكانية كبيرة لتحفيز الاستجابات المناعية لشتلات الطماطم وأوراق شجر الزيتون. كما أظهرت هذه الأنشطة تباينًا اعتمادًا على مدة الحضارة وبنية الجزيئات وطريقة العلاج. بالإضافة إلى ذلك، فإن هذه الجزيئات السكاريد المختلفة بينت قدرة وقائية ضد تطور ذبول الفريسيليوم الناجم عن *Verticillium dahlia* في شتلات الطماطم، مع انخفاض كبير في شدة المرض والوفيات، مقارنة باللامينارين، المادة الفعالة في ال SDP التجاري Iodus40®. تفتح هذه النتائج توجهاً جديداً لإدارة مسببات الأمراض النباتية الوعائية باستخدام نهج يتوافق مع مبادئ الزراعة المستدامة التي تحترم البيئة.

الكلمات المفتاحية: السكريات، السكريات ذات كتلة جزيئية منخفضة، الأعشاب البحرية، SDPs ، *Verticillium dahliae*، الزراعة المستدامة.

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INTRODUCTION GÉNÉRALE

Introduction générale

Introduction générale

Actuellement, la population mondiale augmente avec un taux de 1,05 % par an [1]. Elle devrait passer de 8 milliards actuellement à 9,2 milliards d'ici 2050 [2]. Cette croissance a provoqué une énorme production agricole qui devrait augmenter de 60 à 70 % pour subvenir aux besoins de la population [3]. Toutefois, les cultures sont constamment exposées à de nombreuses attaques de bioagresseurs, provoquant d'importantes pertes de récoltes à hauteur de 40 milliards de dollars dans le monde [4]. Les pratiques agricoles actuelles de gestion des maladies des plantes reposent essentiellement sur l'utilisation des pesticides chimiques. Environ un tiers des produits agricoles sont produits en fonction de l'application de pesticides [5]. La non-utilisation de ces produits entraînerait des pertes de 78 % en production fruitière, 54 % en production maraîchère et 32 % en production céréalière [5]. De plus, la production mondiale de pesticides augmente d'environ 11 % par an, avec trois milliards de kilogrammes utilisés chaque année dans le monde [5]. Toutefois, cette utilisation excessive a engendré des dommages environnementaux potentiels, affectant les sols, les nappes phréatiques, les organismes cibles (pression sélective sur les bioagresseurs) et non cibles, ainsi que la santé humaine [5]. Par conséquent, il est aujourd'hui indispensable de développer une agriculture plus respectueuse de l'environnement, des agriculteurs et des consommateurs tout en réduisant le recours aux intrants chimiques. Pour cela, l'attention de la communauté scientifique s'est détournée vers la recherche de nouvelles stratégies alternatives. L'utilisation des molécules biologiques susceptibles de stimuler les défenses naturelles des plantes, en mimant l'attaque parasitaire, constitue l'une des alternatives la plus prometteuse de par son efficacité, et son aspect durable et écologique. Cette alternative biologique repose sur l'application des molécules dites éliciteurs ou stimulateurs des défenses naturelles (SDP). Ces substances sont caractérisées par : (i) la non-toxicité (ii) la biodégradabilité et (iii) le spectre d'action large.

Les SDPs peuvent assurer une résistance systémique et durable à la plante sans apparition de phénomènes de résistance chez les agents pathogènes, générés par la pression sélective. Divers éliciteurs biotiques, tels que les polysaccharides, les lipides, les (glyco)peptides et les (glyco)protéines, ont été signalés comme inducteurs du système immunitaire de la plante contre un large éventail de bioagresseurs [6]. Ces molécules agissent comme des modèles moléculaires PAMP (Pathogen Associated Molecular Pattern) ou MAMP (Microbe-Associated Molecular Patterns) et sont reconnus par les récepteurs de type PRR (Pattern Recognition Receptor) pour déclencher des réactions de défense appelées PTI (PAMP-Triggered Immunity) [6].

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Divers polysaccharides et leurs dérivés de masses molaires plus faibles ont montré un potentiel à stimuler les défenses naturelles des plantes, ils pourraient être des candidats idéaux pour une formulation stable, biocompatible et adaptées aux produits phytosanitaires [3,6]. Les algues marines constituent des sources importantes de polysaccharides chimiquement polyvalents, avec de nombreux groupes fonctionnels réactifs (amino, carboxyle et hydroxyle). Ceci rend ces molécules propices à des réactions chimiques pour l'introduction de nouveaux groupes dans leur structure, ainsi qu'à la formulation durable de produits phytosanitaires biosourcés [1].

Certaines des algues sont abondamment disponibles quelle que soit la zone géographique, bien que certaines d'entre elles soient spécifiques à certaines régions. Récemment, des énormes afflux d'espèces algales envahissantes comme des espèces de *Sargassum* sp. qui ont atteint de nombreuses régions de l'Amérique, des Caraïbes et de l'Afrique de l'Ouest [7]. Les quantités massives d'algues déposées sur les côtes créent fréquemment des crises économiques et environnementales [7]. La recherche et l'extraction des composés biologiquement actifs, particulièrement les polysaccharides ayant un potentiel SDP, pourrait être une voie de valorisation de cette biomasse algale. Elle pourrait ainsi répondre aux préoccupations des scientifiques et des différents acteurs de l'agriculture moderne par sa durabilité et sa contribution à la production d'agents phytosanitaires respectueux de l'environnement.

Le présent travail s'insère dans ce contexte général et a pour objectif de rechercher des molécules SDPs d'algues marines dotées d'un pouvoir SDP susceptibles de constituer une alternative aux intrants chimiques. Il explore les potentialités des polysaccharides de trois algues marines marocaines (*Cystoseira myriophylloides*, *Sargassum muticum*, *Bifurcaria bifurcata* et *Codium decorticum*) et leurs dérivés oligosaccharidiques, à stimuler les mécanismes de défense naturelle de la tomate et de l'olivier. Notre travail a permis également l'optimisation des principaux paramètres de bio élévation notamment, la structure des molécules (degré de polymérisation, nature des oses constitutifs, présence des groupement sulfatés, et la présence de protéines), la synergie entre différents motifs éléivateurs, le mode d'application (foliaire ou racinaire), et la polyvalence de l'activité SDP (tomate, olivier).

Le présent manuscrit est ainsi structuré en 5 chapitres.

Le chapitre I : aborde une synthèse bibliographique relatant les principales connaissances acquises sur le sujet de thèse traité.

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Les chapitres II, III, IV et V exposent les principaux résultats de la thèse présentés sous forme de publications acceptées ou soumises.

Le chapitre II : porte sur l'évaluation de l'effet éliciteur des alginates (polysaccharides non sulfatés) extraits d'algues brunes marocaines et leurs dérivés oligosaccharidiques sur les plants de tomate. Il est représenté par deux articles :

- ✓ **Article 1 (accepté)** : Induction of natural defenses in tomato seedlings by using alginate and oligoalginates derivatives extracted from Moroccan brown algae. *Mar. Drugs* **2020**, 18, 521, doi : 10.3390/md18100521. **Impact factor (IF = 6.085)**.
- ✓ **Article 2 (en préparation pour la soumission)** : Elicitor activity of low molecular weight alginates obtained by oxidative degradation of alginates extracted from *Sargassum muticum* and *Cystoseira myriophylloides*.

Le chapitre III : traite la caractérisation et l'évaluation de l'effet éliciteur des polysaccharides sulfatés (arabinogalactanes) extraits d'algue verte marocaine *Codium decorticum*, sur les plants de tomate. Il est représenté par un article :

- ✓ **Article 3 (accepté)** : A novel sulfated glycoprotein elicitor extracted from the moroccan green seaweed *Codium decorticum* induces natural defenses in tomato. *Appl. Sci.* **2022**, 12(7) : 3643, doi : 10.3390/app12073643. **Impact factor (IF = 2.838)**.

Le chapitre IV : aborde l'évaluation de l'effet éliciteur des polysaccharides sulfatés et non sulfatés extraits d'algues verte et brune sur les feuilles de l'olivier. Il est représenté par un article :

- ✓ **Article 4 (accepté)** : Polysaccharides from Moroccan green and brown seaweed and their derivatives stimulate natural defenses in olive tree leaves. *Appl. Sci.* **2022**, 12(17), 8842, doi : 10.3390/app12178842. **Impact factor (IF = 2.838)**.

Le chapitre V : consacré à l'évaluation de l'efficacité des mécanismes de défense induits par les différents éliciteurs sur la réduction de l'impact de la verticilliose sur les plants de tomate traités. Il est représenté par un article :

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- ✓ **Article 5 (en préparation pour la soumission)** : Saccharide fractions extracted from marine seaweed induce immunity against *Verticillium dahliae* in tomato: new orientation for verticillium wilt management.

Le manuscrit se termine par une discussion générale et des perspectives.



CHAPITRE I

ANALYSE BIBLIOGRAPHIQUE



Chapitre I : Analyse bibliographique

I. Vers une agriculture durable : induction des défenses naturelles des plantes, une approche durable pour la protection des cultures

1. Aperçu sur les pesticides

Le développement d'une agriculture durable, productive et plus écologique est devenu une préoccupation majeure ces dernières décennies. En raison de la propagation des maladies, la production agricole est confrontée à des pertes considérables évaluées à hauteur de 40 milliards de dollars à l'échelle mondiale [4]. Pour limiter ces dégâts, le traitement par les intrants chimiques constitue actuellement le principal moyen efficace pour garantir un meilleur rendement et une bonne protection des cultures. Mais il est désormais avéré que ces produits chimiques ont des effets néfastes toxiques et durables sur les écosystèmes et la santé humaine. En outre, l'usage continu des pesticides entraîne également des impacts négatifs sur la faune agricole bénéfique (auxiliaires, ver de terre, etc.) et des pressions sélectives sur les microorganismes cibles conduisant à l'apparition de populations de bioagresseurs résistantes à ces produits chimiques et virulentes pour les cultures [8]. De ce fait, cette problématique constitue un défi majeur à relever pour l'agriculture de demain.

Selon la FAO (Organisation des nations unies pour l'alimentation et l'agriculture), la consommation mondiale des pesticides ne cesse de croître depuis 1990, passant de 2,3 à 4,1 millions de tonnes. L'Amérique est le continent où ces produits chimiques sont les plus utilisés (45,5 %), suivie par l'Asie (28,5 %), l'Europe (20,7 %) et l'Afrique (3,4 %) [9]. En Europe, la France est actuellement l'un des trois premiers consommateurs de pesticides, aux côtés de l'Espagne et de l'Italie, avec une production annuelle de 75000 tonnes et une consommation de 66600 tonnes, soit un tiers du tonnage utilisé en Europe. La France est ainsi le 2^{ème} marché européen et le 7^{ème} pays consommateur des intrants chimiques dans le monde [9].

Bien que les pays en développement représentent un quart de l'utilisation mondiale des pesticides, ils enregistrent quant à eux 99 % des problèmes de santé humaine et de l'environnement. Selon l'ONSSA (Office national de sécurité sanitaire des produits Alimentaires), le Maroc compte parmi les pays utilisateurs des produits phytopharmaceutiques (PPP) en agriculture. Au total, 1280 PPPs sont autorisés. L'absence d'unités de fabrication de ces produits, fait que 95 % sont importés prêts à l'emploi (en moyenne 15425 tonnes/an). La consommation des pesticides au Maroc varie d'une culture à l'autre et d'une année à l'autre. Les céréales d'automne, telles que le blé tendre, blé dur et l'orge, se placent en premier lieu sur

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le plan d'utilisation des PPPs (18 %), suivies de la tomate sous serre (11 %) et de la betterave à sucre et des agrumes (9 %). A ce jour, la tomate compte parmi les cultures utilisant 500 différents pesticides.

Au regard de cela, la réduction de l'usage et des impacts de ces intrants chimiques demeure l'objectif principal d'un développement agricole mondial durable qui préserve la santé publique et la biodiversité. De nombreux pays ont renforcé leurs efforts pour minimiser l'utilisation de ces produits chimiques en mettant en œuvre des programmes tels que le plan Ecophyto pris par le gouvernement français. Ce programme a pour objectif de réduire les usages des PPPs de 50 % d'ici 2025, tout en maintenant une production agricole en quantité et en qualité. Ceci encourage l'introduction des méthodes alternatives plus écologiques afin de limiter la dépendance aux pesticides.

2. Principales méthodes alternatives à la lutte chimique

Parmi les principales techniques alternatives préconisées nous retrouvons :

- **La lutte génétique** : consiste à créer des variétés de plantes plus résistantes ou tolérantes aux bioagresseurs. Cette approche est l'une des applications biotechnologiques la plus performante pour la gestion de plusieurs maladies, mais elle représente certaines contraintes telles que la réduction de la résistance au cours du temps, le coût élevé pour l'approbation des cultures génétiquement modifiées (GM) par les organismes de réglementation et le problème d'acceptation par les consommateurs dans plusieurs pays [10].
- **La lutte biologique** : dite aussi biocontrôle, consiste à utiliser des organismes vivants, appelés agents de biocontrôle (BCA), en se basant sur l'exploitation des interactions naturelles telles que le parasitisme, l'antibiose ou la compétition [4]. Ceci dans le but de réduire les impacts négatifs des pathogènes tout en favorisant la croissance des cultures, des insectes et des microorganismes bénéfiques. Plusieurs PPPs à base de BCA sont sur le marché pour lutter contre divers agents pathogènes [11]. Cette stratégie est certes efficace, mais elle représente certaines restrictions à savoir le coût de production élevé pour la plupart des agents de biocontrôle pour des applications à grande échelle, l'impact négatif sur les microorganismes non-ciblés de la microflore endogène, les problèmes d'adaptation à la niche écologique, et surtout les effets directs conduisant au développement de la résistance dans les communautés de pathogènes ciblés. En outre,

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une compréhension plus approfondie des relations entre les plantes et les phytopathogènes ainsi que les facteurs environnementaux prévalant dans une zone spécifique est nécessaire avant l'introduction de ces BCAs.

Compte tenu de ces méthodes de gestion des bioagresseurs, les avancées des connaissances sur les mécanismes de résistance chez les plantes sont mises à contribution pour développer de nouvelles et meilleures techniques. La stimulation des défenses naturelles des plantes par des éliciteurs exogènes (SDPs) se positionne parmi les alternatives les plus prometteuses.

3. Stimulateurs des défenses naturelles des plantes (SDPs) : une nouvelle alternative aux pesticides

3.1. Définition et caractéristiques

Les stimulateurs des défenses naturelles (SDPs), appelés également SDP (stimulateurs des défenses des plantes) ou inducteurs de résistance, ont été définis en 2013 par le Réseau Mixte Technologique (RMT) Elicitra : « *Toute substance ou tout micro-organisme vivant non pathogène capable d'induire (ou de préparer à l'induction) des réponses de défense chez une plante traitée, conduisant à une meilleure résistance contre le stress biotique* » [12].

Le concept d'utilisation des SDPs dans la protection des plantes a été énoncé pour la première fois par Peter Albersheim et Anderson-Prouty en 1975. Ces deux collaborateurs ont découvert qu'un polysaccharide issu du pathogène *Colletotrichum lindemuthianum* était capable de déclencher la production des phytoalexines après son application sur les surfaces coupées de cotylédons et de l'hypocotyles du haricot (*Phaseolus vulgaris*) [13]. En 1990, Cosio et al. a également mis en évidence le pouvoir des β -glucanes libérés, par hydrolyse acide, à partir des parois mycéliennes de *Phytophthora megasperma* f. sp. *Glycinea*, à induire la synthèse des phytoalexines chez les graines du soja [14].

Il est toutefois nécessaire de distinguer entre les deux grandes catégories des SDPs, les éliciteurs et les potentialisateurs. Les éliciteurs sont des molécules qui entraînent, une fois appliquées sur la plante, une activation directe des réactions de défense en présence ou en absence de pathogènes. En revanche, les potentialisateurs permettent l'activation des premières étapes des défenses mettant la plante dans un état d'alerte. Ils sensibilisent la plante à réagir plus rapidement et plus efficacement suite à une attaque immédiate de bioagresseur (priming).

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Leurs propriétés et leurs multiples rôles bénéfiques font de ces produits une nouvelle génération d'intrants verts, Parmi celles-ci :

- Le mode d'action indirect sur les bioagresseurs : les SDPs agissent sur la plante, comme un message d'alerte, ce qui limite les phénomènes de résistance et de sélectivité vu la diversité des défenses actives déclenchées ;
- Le spectre d'action large : les SDPs sont susceptibles de protéger contre une large gamme de bioagresseurs, car l'éventail des défenses activées n'est pas spécifique d'un bioagresseur donné ;
- Leur origine naturelle : les SDPs sont des extraits ou composés purifiés d'organismes divers, des microorganismes non pathogènes. Cependant, ils peuvent être aussi des molécules synthétiques, qui agissent directement sur les défenses des plantes ;
- La biodégradabilité : les SDPs ne représentent aucune toxicité sur les êtres vivants et sur l'environnement. En effet, les espèces ciblées sont atteintes à travers la plante et généralement aucun cas d'écotoxicité n'a pu être révélé.

3.2. Origine, nature et homologation des SDPs

Les SDPs peuvent être de plusieurs origines, ils sont souvent d'origine naturelle : minérale (poudres de roche : calcite, silicate), végétale (extraits de plantes : algues, plantes supérieures), hormones (brassinostéroïdes), microbienne (extraits de microorganismes : champignons, bactéries, etc.) ou animale. Certains sont d'origine synthétique obtenus par synthèse chimique des analogues de substances naturelles. Les SDPs sont soit des extraits bruts (extraits bruts d'algues, de sauge, de prêle, d'ortie, de bourdaine, etc.), soit des molécules plus ou moins purifiées du produit d'origine, ou des composés de fermentation des extraits bruts [15].

Quelle que soit leur nature, les SDPs sont tous soumis à la réglementation concernant la mise sur le marché (AMM) des PPPs (Règlement CE n°1107/2009). Certains composés sont homologués en tant que SDP, tandis que de nombreuses autres substances se retrouvent sous des statuts réglementaires différents (Biocontrôle, engrais ou fertilisant) même si elles possèdent la capacité à stimuler les mécanismes de défenses des plantes [15]. La différence des allégations d'utilisation est souvent liée à l'absence des informations concernant le mode d'action, les risques toxicologiques potentiels, les propriétés physicochimiques et biologiques détaillées de la matière active et son efficacité dans des conditions climatiques variées et dans des régions géographiques différentes. Le coût et la durée d'une telle procédure sont également

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des barrières empêchant l'homologation de nouvelles substances en tant que SDP. Pour contourner ces contraintes, les produits sont donc commercialisés comme des fertilisants ou des engrais, puisque ces derniers peuvent être mis sur le marché sans homologation préalable. Des renseignements supplémentaires relatifs aux autres effets du produit peuvent ensuite être fournis aux producteurs [15].

Pour le moment, il n'existe pas de données spécifiques sur le marché et les ventes des SDPs. Cependant, quelques SDPs sont actuellement disponibles sur le marché (**Tableau 1**).

Tableau 1. Liste des produits disposant d'une AMM en tant que SDP [15,16].

Substances actives	Produits	Compagnies	Usages/cultures autorisées	Maladies/pathogènes	Formulations	Années
Cerevisane (941 g/kg)	Actileaf	Agrauxine	Chicorées, Cucurbitacées	<i>Bremia lactucae</i> Oïdium Pourriture grise Mildiou	Poudre mouillable	2017
	Romeo	Agrauxine				
	Romeo garden	Agrauxine	Fraisier Laitue			
	Cervitis	Soralys	Poivron			
	Cezane 941 PM	Saga	Tomate Aubergine Vigne			2020
	Caromait	M. Cazorla				
Phosphonates de potassium (755 g/L)	Epatan	De Sangosse	Vigne	—	Concentré soluble	2019
	Facinan					2020
	Alucinan					
	Etonan					
	Pertinan		Chicorées Cucurbitacées			2010
	LBG-01F34	Luxembourg Pamol	Laitue Poivron			
	Kutaisi	Unisem	Tomate Aubergine Vigne			2020
	Alamode	Hmwc				2021
	Phostim Pro	Saga				
Acibenzolar-S-méthyle (500 g/kg)	Bion 50 WG	Syngenta France	Cucurbitacées	Cladosporiose	Granulé dispersable	1999
	Peon WG	Agri Canigou	Cultures florales et plantes vertes Kiwi			2015
	Azcenzae	M. Cazorla	Tomate Aubergine			2017

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	Fytosave garden		Emploi autorisé dans les jardins Cultures légumières	Oïdium		2017
Chito-oligosaccharides et oligogalacturonides (COS-OGA) (12.5 g/L)		Fytofend		<i>Sphaerotheca fuliginea</i>		2019
	Fytosave		Bananier Céréales à paille Cultures légumières	Oïdium neolycopersici <i>Leveillula taurica</i>	Concentré soluble	
	Galopin+	Phyto Service	Cultures ornementales Fruits à pépins Houblon	Oïdium		2017
	Messenger	Cerience	Riz Tabac			2015
	Performer SL	Saga	Vigne	Oïdium Mildiou		2020
	Vacazoteli	M. Cazorla				2017
Laminarine (37 g/L)	Iodus 2 cereales		Céréales à paille	Oïdium Septoriose Helminthosporiose		2002
Laminarine (45 g/L)		Laboratoires Goëmar	Fraisier Fruits à pépins Laitue Vigne	Pourriture grise Oïdium Tavelure Gloeosporiose Feu bactérien Mildiou	Concentré soluble	2008
	Plantvax	Arysta Lifescience	Vigne	Oïdium		2021
Chitosane (4%)	Elexa™	GlycoGenesys	<i>Pennisetum glaucum</i>	—	—	—
Harpine (Peptide produit par la bactérie <i>Erwinia amylovora</i>)	Messenger®	Eden Biosciences	Coton Tomate Concombre Poivron Tabac Fraise Riz, Blé	—	—	2000

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Extraits de graines de fenugrec	Stifénia®	Société occitane de fabrication de technologies	Vigne	Oïdium Cloque du pêcher Mildiou	—	2005
Extraits de <i>Reynoutria sachalinensis</i> (5%)	Milsana	HH BioSci	Cultures maraîchères et ornementales	Oïdium	—	—
Silicate de potassium	Sil-MATRIX™	PQ Corporation	Melon Cerise Fraise Riz Canne à sucre Céréales	—	—	—
Phosphonate disodique (500 g/L)	Delire+	Phyto Service	Vigne	—	Concentré soluble	2017
<i>Bacillus amyloliquefaciens</i> souche MBI600 (22×10^9) UFC/mL	Integral pro	Basf France	Crucifères oléagineuses Lin	<i>Leptosphaeria maculans</i> <i>Psylliodes chrysocephala</i> <i>Phyllotreta</i> sp.	Suspension concentrée pour traitement des semences	2018
Virus de la mosaïque du pépino isolat VC1 (15×10^{10}) et VX1 (75×10^{10}) Corps viraux/L	V10	Valto BV	Tomate Aubergine	Cirus de la mosaïque du pépino (PepMV)	Suspension concentrée	2019
Composants du lysat de la souche DDSF623 de <i>Saccharomyces cerevisiae</i> (ABE IT 56) (325.6 g/L)	Belvine	Cerience	Vigne	Mildiou	Suspension concentrée	2021

3.3. Principes et mécanismes d'action des SDPs

Dans leur environnement, les plantes représentent plus au moins une sensibilité aux molécules étrangères qui peuvent être d'origine endogène provenant de la plante hôte (protéines, hormones, acide jasmonique, acide salicylique) ou exogène (pathogènes, extraits de plante, etc.). Lorsqu'une famille de molécules caractéristiques est détectée par les récepteurs de la

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plante, une cascade de signalisation adéquate est déclenchée aboutissant à l'activation de divers mécanismes de défense. Ce concept est à la base de l'émergence du principe de la vaccination des plantes en utilisant des agents exogènes comme les SDPs. Il repose sur l'immunisation préalable du système défensif de la plante par des molécules (glucides, protéines, lipides, peptides, etc.), permettant de mimer l'attaque subséquente d'un bioagresseur.

Pour comprendre le rôle de ces éliciteurs dans la défense, il est indispensable de comprendre les mécanismes d'induction du système immunitaire des végétaux qui consiste en trois phases principales : (i) une phase de reconnaissance, (ii) une phase de transduction de signal et (iii) une phase d'induction de mécanismes de résistance.

3.3.1. Phase de reconnaissance

La reconnaissance des bioagresseurs est une étape clé dans la mise en place de l'immunité végétale. Les plantes sont capables de détecter les éliciteurs dérivés des agents pathogènes et qui peuvent être classés en deux catégories :

- **Eliciteurs généraux** : ils sont essentiellement présents dans les parois cellulaires des agents pathogènes en tant que composants structurels, par exemple le glucane, la chitine, la flagelline et les lipopolysaccharides (LPS) [17]. Ces éliciteurs sont reconnus par les récepteurs PRR (Pattern Recognition Receptor), situés dans la membrane plasmique ou dans le cytoplasme de la plante, en tant que PAMPs (Pathogen-Associated Molecular Pattern), MAMPs (Microbe-Associated Molecular Patterns), HAMPs (Herbivore-Associated Molecular Patterns) ou DAMPs (Damage-Associated Molecular Patterns) (**Figure I. 1**). Les PAMPs, les MAMPs et les HAMPs sont des constituants de la paroi cellulaire des microorganismes et des insectes, tandis que les DAMPs sont des signaux associés à la plante hôte [17]. Ils permettent d'induire le premier niveau de la défense, la résistance basale, connue aussi sous le nom « résistance non spécifique ou non hôte », « résistance quantitative » ou PTI (PAMP-Triggered Immunity) [18,19].
- **Eliciteurs spécifiques** : il s'agit d'un deuxième niveau de reconnaissance des plantes. Contrairement aux PAMPs, les éliciteurs spécifiques sont des molécules dites effectrices secrétées par les pathogènes agissant en tant que déterminants de la virulence. Ils permettent de franchir la barrière de la reconnaissance non-spécifique de la plante et de mettre en place une résistance spécifique dite « résistance gène pour gène

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», « résistance qualitative », « résistance race-cultivar-spécifique » ou encore ETI (Effector-Triggered Immunity) (**Figure I. 1**) [17]. La plupart des effecteurs ont été identifiés comme des produits d'un gène d'avirulence (Avr) spécifique à une certaine souche microbienne qui sont reconnus par un produit de gène de résistance (R) correspondant spécifique à un certain cultivar de plante [19]. Contrairement au PRR, les protéines réceptrices reconnaissant les effecteurs sont développées par la plante suite à l'infection. Elles se trouvent principalement dans le cytoplasme, bien que quelques-unes soient des récepteurs de surface [19]. Depuis le développement de ce concept, de nombreux gènes Avr ont été isolés chez les champignons, les oomycètes et les bactéries [20-23].

Les récepteurs végétaux peuvent être divisés en récepteurs de surface (PRR) et intracellulaires. Ces derniers sont connus sous le nom de NB-LRR (Nucleotide Binding site-Leucine Rich Repeat) et qui fonctionnent exclusivement dans la reconnaissance des effecteurs (**Figure I. 1**) [19]. En revanche, les récepteurs de surface PRR sont connus pour détecter à la fois les PAMPs, et les effecteurs. Ils comprennent les RLKs (Receptor-Like Kinases), les RLPs (Receptor-Like Proteins) et les protéines de liaison extracellulaires [19]. A ce jour, l'identification des récepteurs des éliciteurs représente un défi majeur, seuls quelques récepteurs ont été caractérisés pour des molécules élicitrices spécifiques [17].

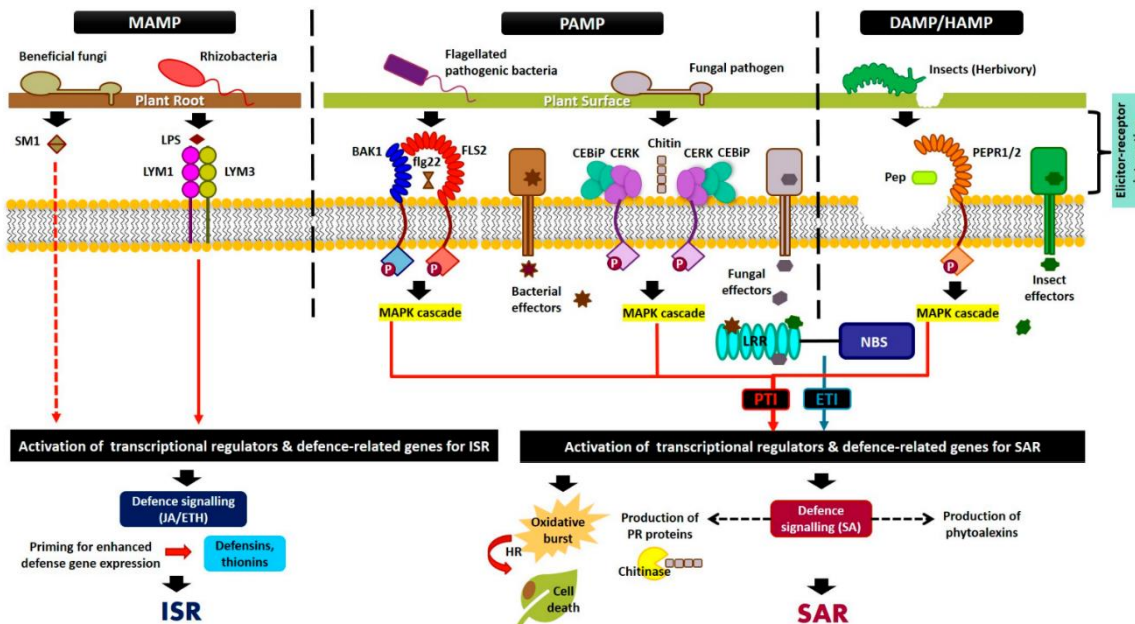


Figure I. 1. Activation de la résistance systémique acquise (SAR) et induite (ISR) par les différents types d'éliciteurs produits par des microorganismes et des insectes pathogènes (PAMP, HAMP) et non pathogènes (MAMP, DAMP) après leur reconnaissance par les récepteurs de surface et intracellulaire de la plante [17].

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3.3.2. Phase de transduction de signal

L'interaction éliciteur-récepteur permet de déclencher des cascades de signalisation précoce et tardive afin d'induire le système de défense d'une plante. La modification de la perméabilité ionique de la membrane plasmique est l'élément précoce de la signalisation. Elle se traduit d'abord par l'ouverture des canaux ioniques engendrant des influx de Ca^{2+} , H^+ et des efflux de K^+ et Cl^- dans les cellules végétales, ce qui active ensuite les protéines MAPK (Mitogen-Activated Protein Kinases) et la production massive d'espèces réactives de l'oxygène (ROS, Reactive Oxygen Species) (**Figure I. 1**) [17,24]. L'accumulation des ROS autour du site d'infection peut agir directement comme un mécanisme de défense ou initier l'activation d'autres voies métaboliques [24]. Les ROSs, l' H_2O_2 , les influx d'ions et l'oxyde nitrique (NO) sont des seconds messagers essentiels pour la transduction du signal. L'éthylène (ET), l'acide jasmonique (JA) et acide salicylique (SA) constituent une autre classe importante de molécules de signalisation impliquées dans les mécanismes de résistance des plantes [17,25].

3.3.3. Phase d'induction des mécanismes de résistance

La perception de l'agent pathogène par les deux systèmes de reconnaissance de la plante, décrite précédemment, conduit à la mise en place de divers mécanismes de défense. Selon la spécificité de l'interaction, la plante développe des réponses locales et systémiques. Les réponses locales se traduisent par le déclenchement des réactions d'hypersensibilité (RH), entraînant la mort programmée des cellules (PCD) autour du point d'infection créant un environnement défavorable au développement du parasite [25,26]. En plus des réponses locales, les plantes peuvent également développer une résistance systémique aboutissant à l'activation des mécanismes de défense physiques et chimiques. [27]. En fonction de la nature de l'éliciteur et de la voie de régulation, deux types de résistances systémiques ont été identifiées et largement étudiées : la résistance systémique acquise (SAR) et la résistance systémique induite (ISR).

3.3.3.1. Résistance systémique acquise (SAR)

C'est un type de défense des plantes induite suite à une infection par des bioagresseurs virulents, avirulents et non pathogènes ou par l'application des molécules élicitrices biotiques et abiotiques. L'induction de SAR est liée à la production de SA endogène en tant que molécule de signalisation, qui intervient dans l'activation des gènes codant pour les protéines PR (Pathogenesis-Related Protein) (**Figure I. 1**) [28].

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En réponse, les plantes mettent en place divers mécanismes de défense notamment, le renforcement de la paroi cellulaire, l'accumulation des enzymes de défense (enzymes de la voie des phénylpropanoïdes et de la voie oxydative), ainsi que la synthèse des métabolites secondaires.

- **Renforcement de la paroi cellulaire** : le renforcement de la barrière mécanique naturelle des cellules végétales, la paroi pectocellulosique, constitue un élément important des défenses des plantes. L'accumulation des macromolécules telles les glycoprotéines riches en hydroxyproline (HRGP) [29], de protéines riches en glycine (GRP) ou riches en proline (PRP), et des dépôts de callose (polymère de D-glucose) [30], interviennent dans ces processus de défense mécanique. Des dépôts de la lignine peuvent également s'effectuer au niveau des plasmodesmes apportant une rigidité à la paroi cellulaire afin de bloquer la perméabilité aux phytopathogènes [31].
- **Accumulation des enzymes de défense** : les enzymes les plus étudiées au cours des interactions plante-pathogène sont :
 - ✓ **Les enzymes de la voie des phénylpropanoïdes** : la phénylalanine ammonia-lyase (PAL) est l'enzyme clé de cette voie, elle catalyse la première réaction par la conversion de la L-phénylalanine (Phe) en acide *trans*-cinnamique. La formation ultérieure du coumarate implique une hydroxylation directe du cycle aromatique par la *trans*-cinnamate 4-hydroxylase (C4H) (**Figure I. 2**). La tyrosine ammonia-lyase (TAL) est une autre enzyme impliquée dans cette voie. Chez certaines graminées, la tyrosine (Tyr) est convertie directement en coumarate sous l'action de la TAL [32] (**Figure I. 2**). La Phe et la Tyr sont formés via la voie des shikimates à partir d'arogénate. Ce composé est transformé en Phe par déshydratation (arogénate déhydratase) ou en Tyr par déshydrogénation (arogénate déshydrogénase) (**Figure I. 3**). Néanmoins, la PAL est l'enzyme la plus étudiée [32], elle est impliquée dans la production des molécules servant comme précurseurs pour synthétiser la majorité des métabolites secondaires (**Figure I. 4**) [33].

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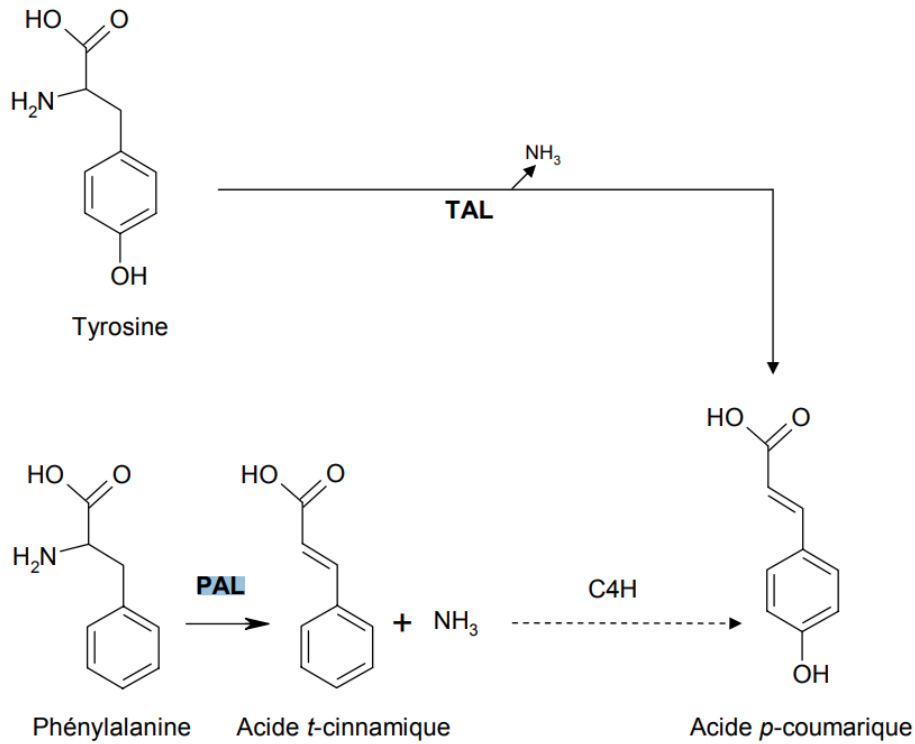


Figure I. 2. Voie de formation de l'acide p-coumarique par la phénylalanine ammonia-lyase (PAL) et la tyrosine ammonia-lyase (TAL). C4H, cinnamate 4-hydroxylase.

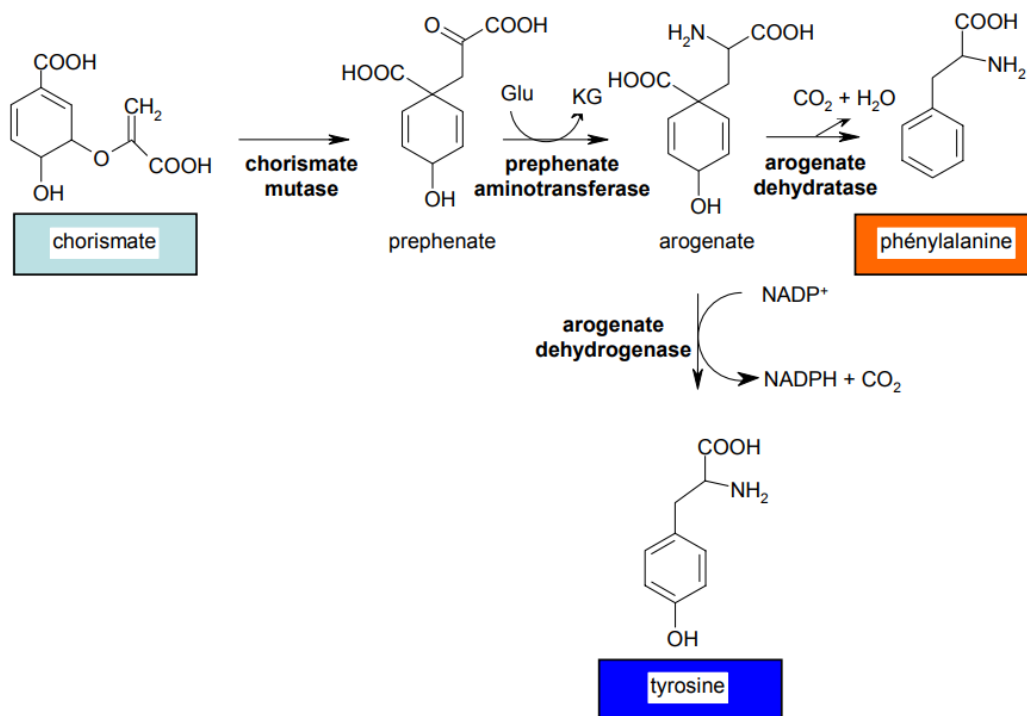


Figure I. 3. Voie de synthèse de la phénylalanine et la tyrosine à partir de l'arogène

- ✓ **Les enzymes de la voie oxydative** : il s'agit des polyphénol-oxydases (PPO), des peroxydases (POD), des laccases (LAC), des lipoxygénases

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(LOX), la catalase (CAT) et la superoxyde dismutase (SOD). Ces enzymes oxydoréductases sont impliquées dans de nombreux processus physiologiques tels que la détoxification des cellules du H₂O₂ et des ROSs, le catabolisme de l'auxine, la lignification, la synthèse de la subérine, l'élaboration de liens intermoléculaires entre les polysaccharides pariétaux, l'insolubilisation des protéines structurales et l'oxydation des métabolites secondaires [34,35].

- ✓ **Les enzymes hydrolytiques** : telles que les chitinases, les pectinases, les glucanases, etc. Ces enzymes jouent un rôle essentiel comme molécules antifongiques. elles agissent sur la paroi cellulaire des bioagresseurs entraînant une dégradation et une perte du contenu de la cellule [36].
- **Production des métabolites secondaires** : différents composés antimicrobiens sont produits par les plantes leur permettent d'assurer une protection contre les agents pathogènes (**Figure I. 4**).

Deux catégories de métabolites secondaires sont impliquées dans l'immunité végétale, les phytoanticipines et les phytoalexines. Ces dernières, sont des molécules synthétisées *de Novo* et accumulées dans le site d'infection en réponse à un stress biotique ou abiotique [37]. La plupart des phytoalexines sont des composés phénoliques dérivés des voies de biosynthèse des phénylpropanoïdes, d'autres sont issus de la voie de l'acétyl-CoA, de l'isopentenyl diphosphate (IPP), des sesquiterpènes et des shikimates (Coumarines) [38]. En revanche, les phytoanticipines sont des substances préformées dans les plantes de façon constitutive avant même l'attaque d'un bioagresseur [39]. Cependant, la distinction entre les phytoalexines et les phytoanticipines n'est pas toujours évidente. Certains composés peuvent être des phytoalexines chez une espèce et des phytoanticipines chez d'autre [38]. C'est le cas de la sakuranétine qui s'accumule de manière constitutive dans les glandes foliaires du Cassissier, mais qui est un métabolite antimicrobien inductible majeur dans les feuilles de riz [38].

Les phénylpropanoïdes sont les principaux métabolites secondaires, impliqués dans différentes fonctions telles que la pigmentation, la lignification, la signalisation et la protection contre les stress biotiques et abiotiques [39,40]. Ils sont dérivés principalement de la voie des shikimates à partir de l'acide *trans*-cinnamique par une succession d'étapes d'hydroxylation, de méthylation et de réduction (**Figure I. 4**). Les

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phénylpropanoïdes contiennent tous un cycle phényle et une chaîne latérale de trois carbones (C6-C3). En plus des phénols simples, ils comprennent des flavonoïdes, des stilbènes, des tanins, des lignanes et de la lignine. Ils sont également des composants de la subérine et de la cutine [40] (**Figure I. 4**). La lignine et l'un des métabolites secondaires les plus importants, c'est le deuxième biopolymère le plus abondant, après la cellulose. Elle représente 30% de la teneur en carbone organique de la biosphère [41]. La biosynthèse de la lignine se divise en trois processus (i) la biosynthèse des monomères de lignine, (ii) le transport et (iii) la polymérisation. Après une série d'étapes impliquant la désamination, l'hydroxylation, la méthylation et la réduction, les monolignols sont produits dans le cytoplasme et transportés vers l'apoplaste. En présence des peroxydases (POD) et des laccases (LAC), la lignine est formée par la polymérisation de trois principaux types de monolignols, l'alcool sinapylique (unité S), l'alcool coniférylique (unité G) et alcool *p*-coumarylique (unité H) [41,42] (**Figure I. 5**). Ce polymère phénolique, joue un rôle essentiel dans la croissance et le développement des plantes. Il permet d'améliorer la rigidité de la paroi cellulaire et de favoriser le transport des minéraux à travers les faisceaux vasculaires de la plante [43]. La lignine contribue également à la résistance en réponse aux stress biotiques et abiotiques [41].

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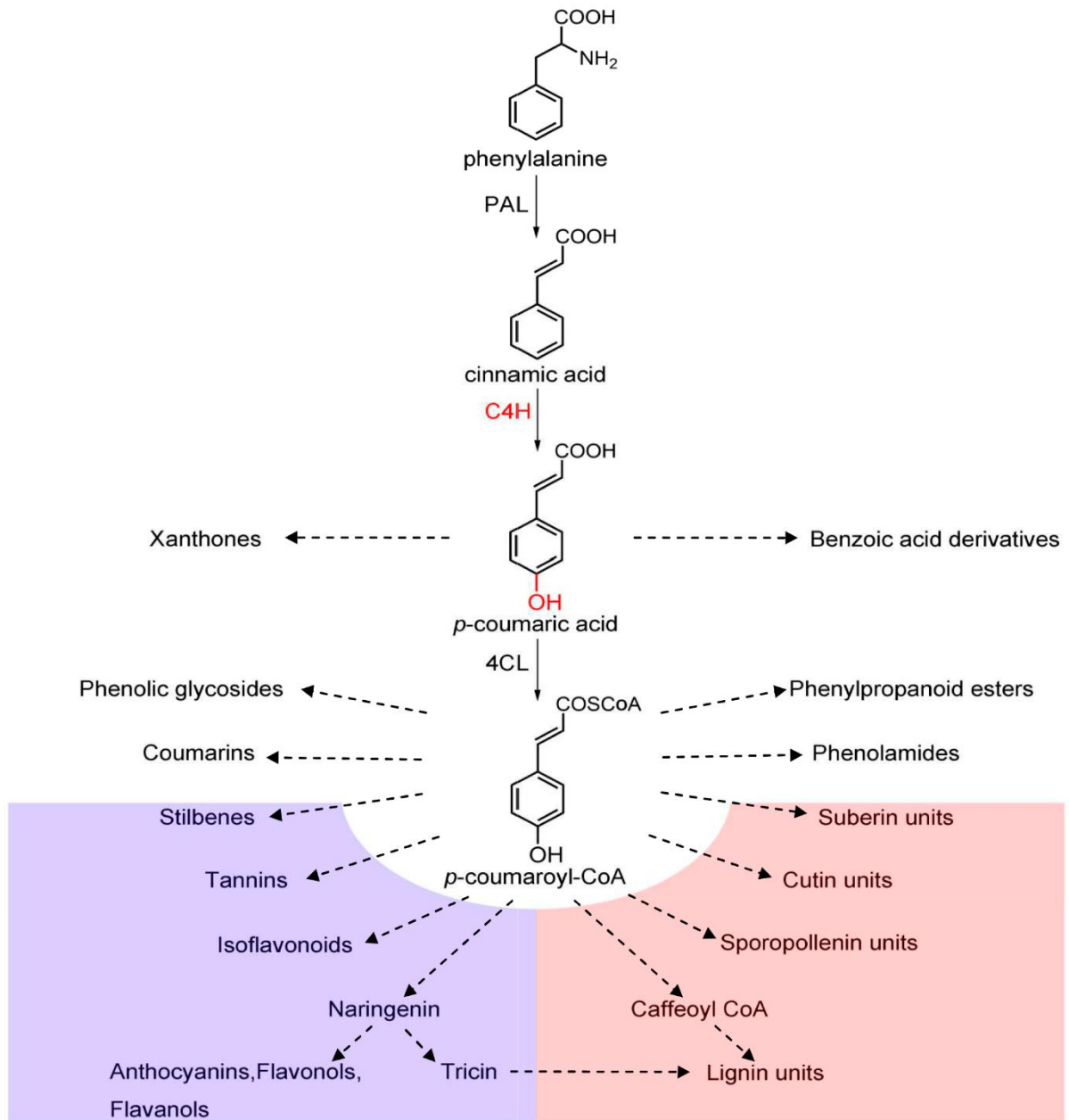


Figure I. 4. Quelques métabolites secondaires dérivant du métabolisme des phénylpropanoïdes (voie des shikimates) [44]. PAL : phenylalanine ammonia-lyase ; C4H : cinnamate 4-hydroxylase ; 4CL : 4-coumarate : coenzyme A ligase.

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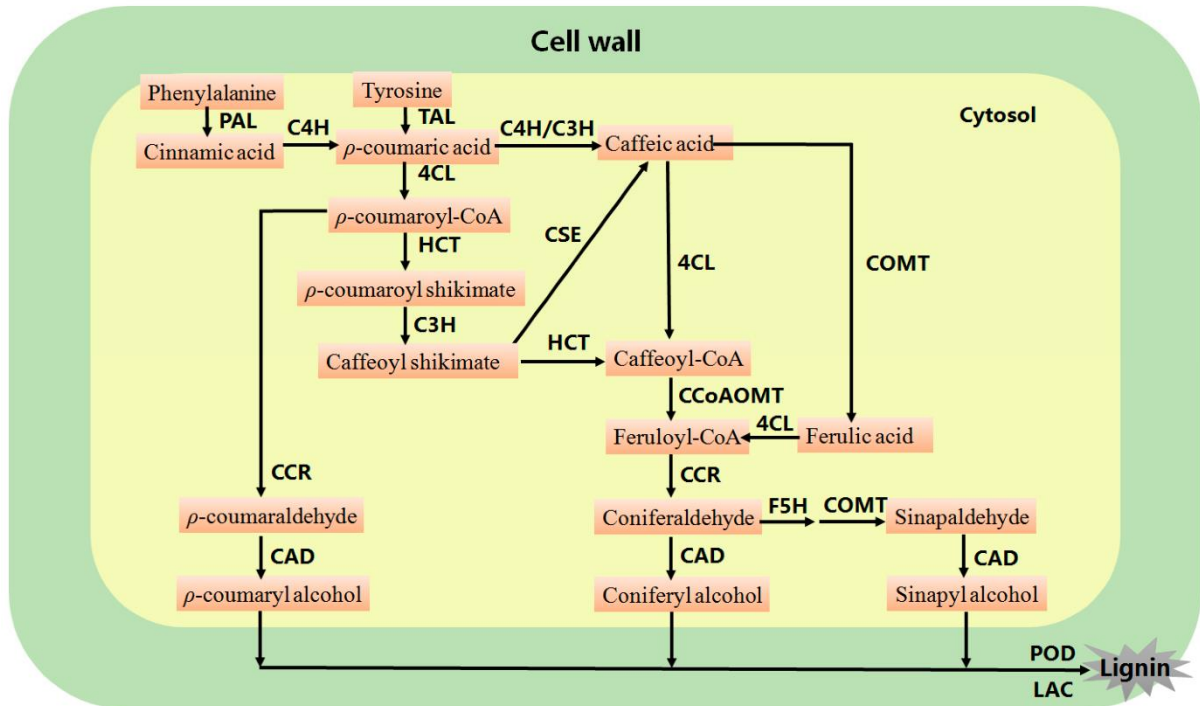


Figure I. 5. La voie de la synthèse de la lignine chez les végétaux [41]. PAL : phénylalanine ammonia-lyase ; TAL : tyrosine ammonia-lyase ; C4H : cinnamate 4-hydroxylase ; 4CL : 4-coumarate : CoA ligase ; CCR : cinnamoyl-CoA réductase ; HCT : hydroxycinnamoyl-CoA shikimate ; C3H : p-coumarate 3 hydroxylase ; CCoAOMT : caféoyl-CoA O-méthyltransférase ; F5H : férulate 5-hydroxylase ; CSE : caféoyl shikimate estérase ; COMT : acide caféique O-méthyltransférase ; CAD : alcool cinnamylique déshydrogénase ; LAC : laccase ; POD : peroxydase.

3.3.3.2. Résistance systémique induite (ISR)

Contrairement à la SAR, la ISR est déclenchée suite à une interaction bénéfique entre les plantes et les microorganismes de type PGPR (Plant Growth-Promoting Rhizobacteria). Les déterminants rhizobactériens qui permettent d'activer la ISR sont les protéines flagellaires, les lipopolysaccharides, les antibiotiques, les molécules signal du quorum sensing, les composés organiques volatils (COV) et les sidérophores. Ce type de résistance est indépendante de l'acide salicylique (SA) et n'entraîne pas des changements majeurs dans l'expression des gènes de défense. Cependant, la ISR est liée essentiellement à la synthèse de l'acide jasmonique (JA) ou de l'éthylène (ET) (**Figure I. 1**) [28].

Compte tenu de ce qui précède, les SDPs sont donc considérés comme des éliciteurs généraux agissant comme des PAMPs/MAMPs capable d'induire une résistance non spécifique (PTI).

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Comme décrit précédemment, ces molécules comprennent les saccharides, les lipides, les peptides, les glycolipides et les glycoprotéines.

Chez les plantes, les saccharides sont connus pour leur rôle essentiel en tant que sources vitales d'énergie et de molécules de signalisation pour la régulation des gènes de défense [45]. Ils sont ainsi considérés comme des acteurs intervenant dans la croissance et dans l'immunité des végétaux [46]. Ceci a permis d'exploiter ces molécules dans le contrôle des maladies des plantes. Divers saccharides/hydrates de carbone et leurs dérivés provenant de différentes sources sont actuellement étudiés et expérimentés pour leur rôle éventuel en tant que SDP [6]. Récemment, une attention particulière est portée à l'utilisation des polysaccharides et des oligosaccharides dérivés des algues marines comme des candidats idéaux pour la protection des cultures.

II. Macroalgues marines : une importante source de molécules SDP

Les algues marines constituent une source très importante de substances hautement bioactives telles que les protéines (1 à 50 %), les lipides (0,3 à 5 %) et les polysaccharides (15 à 76 %) [47]. Les progrès de la biotechnologie algale ont révélé un énorme potentiel des extraits bruts et des produits purifiés des algues, tels que les polysaccharides et leurs dérivés oligosaccharidiques, à améliorer les systèmes de production végétale et renforcer la défense des plantes contre les ravageurs et les maladies [48,49].

1. Les polysaccharides

Les algues marines produisent une vaste famille de polysaccharides spécifiques (**Figure I. 6**), au sein de laquelle on distingue :

- **Les polysaccharides de réserve** (stockés à l'intérieur de la cellule) : Chez les algues brunes, il s'agit de la laminarine (polymère du 1,3- β -glucopyranose), chez les algues rouges, de l'amidon floridéen (polymère du 1,4- α -glucose), et chez les algues vertes, du glucane (polymère du 1,4- β -glucose) [50].
- **Les polysaccharides de faibles poids moléculaires** : Solubles dans le milieu, ils passent au travers de la membrane pour réguler la pression osmotique. Chez les algues rouges, on distingue le floridoside et l'isofloridoside. Chez les algues brunes, un type particulier de saccharide sous forme de polyols, le D-mannitol, qui représente jusqu'à

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25% du poids sec de l'algue. Enfin, les algues vertes comportent des traces de monosaccharides tels que le fructose ou le saccharose [50].

➤ **Les polysaccharides de structure** : Appelés aussi polysaccharides pariétaux (de la paroi), ils comprennent : une phase squelettique ou fibrillaire, constituée de polysaccharides insolubles (cellulose, mannanes, xylanes), et une phase matricielle contenant des polysaccharides de structure plus hydrosolubles noyés dans la matrice (zone amorphe), dans laquelle ont lieu les échanges cationiques. La nature des polysaccharides matriciels est spécifique à chaque type d'algue.

- **Chez les algues vertes** : leurs polysaccharides matriciels ont été considérés comme des hétéropolysaccharides ramifiés sulfatés [51] : extraits des Ulvales ;
- **Chez les algues brunes** : Trois principaux types de polysaccharides matriciels sont extraits des Phéophycées : les fucanes, les ascophylanes et les alginates, qui représentent le mucilage matriciel majeur des algues brunes [52] ;
- **Chez les algues rouges** : les carraghénanes et agarocolloïdes sont les principaux polysaccharides matriciels.

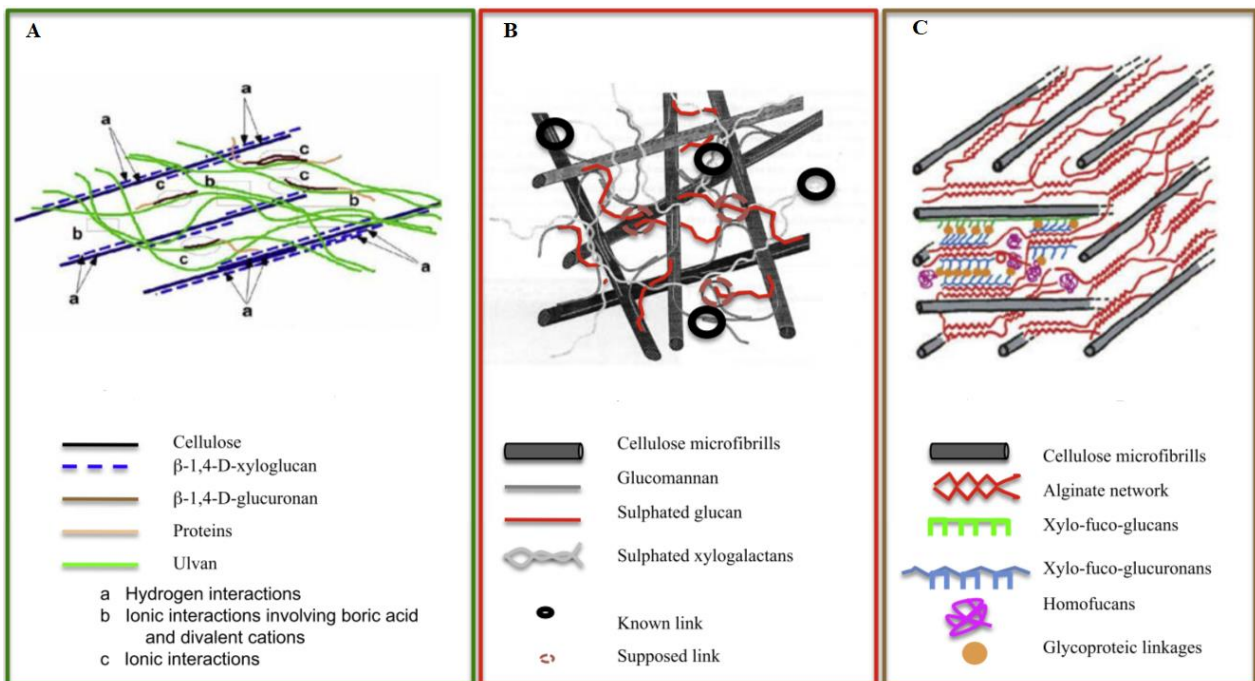


Figure I. 6. Distribution des polysaccharides caractéristiques dans la paroi cellulaire des algues vertes (A), des algues rouges (B) et des algues brunes (C) [53].

En raison de leur abondance, les polysaccharides algaux sont les plus exploités dans les secteurs agroalimentaire [54], cosmétique [55], pharmacologique [56], et plus récemment celui de

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l'agriculture [57]. Dernièrement, ces macromolécules ainsi que leurs dérivés oligosaccharidiques ont attiré l'attention des chercheurs pour leurs activités élicitrices, permettant d'induire les mécanismes de résistance des plantes contre un large éventail de phytopathogène. Actuellement, les carraghénanes issues des algues rouges, les ulvanes purifiés des algues vertes, les alginates, les fucanes/fucoidanes et les laminarines extraits des algues brunes, sont les molécules les plus utilisées en tant qu'éliciteurs des défenses des plantes [58].

1.1. Les ulvanes

Les ulvanes sont des hétéropolysaccharides polyanioniques hydrosolubles sulfatés de la paroi cellulaire des algues vertes du genre *Ulva*, représentant généralement 8 à 29 % de leur biomasse [59]. Ils sont principalement composés de rhamnose (16,8-45,0 %), de xylose (2,1-12,0 %), de glucose (0,5-6,4 %), d'acide glucuronique (6,5- 19,0 %), de l'acide iduronique (1,1-9,1 %) et de sulfate (16,0-23,2 %). Ces polysaccharides sont souvent constitués de monosaccharides liés en α - et β -(1,4) avec une succession d'unités disaccharidiques caractéristiques [60] (**Figure I. 7**). Les ulvanes ont un poids moléculaire moyen allant de 189 à 8200 kDa [61]. Ils sont généralement obtenus par extraction acide ou enzymatique. L'extraction acide provoque une désulfatation totale de l'ulvane tandis que les méthodes enzymatiques maintiennent un niveau significatif de substituant SO_3 avec un rendement et une pureté élevés [62]. L'extraction à l'eau chaude a également été utilisée dans différentes études [63-65].

Les ulvanes ont été largement exploitées dans les domaines agroalimentaires, pharmaceutique et biomédicale. Elles sont reconnues par leurs activités antioxydante, anti-inflammatoire, antivirale, anticoagulante, antibactérienne et antihyperlipidémique [60]. Récemment, diverses études ont révélé que les ulvanes peuvent être appliqués pour stimuler la croissance et la défense des plantes contre une large gamme de phytopathogène [66-69] (**Tableau 2**). Les ulvanes extraits des algues vertes *U. fasciata* et *U. armoricana* ont montré leurs capacités à protéger les plants du haricot contre *Fusarium oxysporum* f. sp. *phaseoli* (Fop) [70], *Uromyces appendiculatus* [71,72], *Colletotrichum lindemuthianum* [73-76] et *Erysiphe poligoni* [77]. L'effet des ulvanes à contrôler les maladies a été étudié sur d'autres espèces végétales telles que le blé [69,78,79], le riz [78,80,81], les pommes [67,82-86], l'olivier [87], le tabac [88], la luzerne [81,88,89], les tomates [66], *Arabidopsis thaliana* [68,90,91], l'orge [78], le raisin de table [92], le melon [93], la papaye [94], le basilic et le persil [95].

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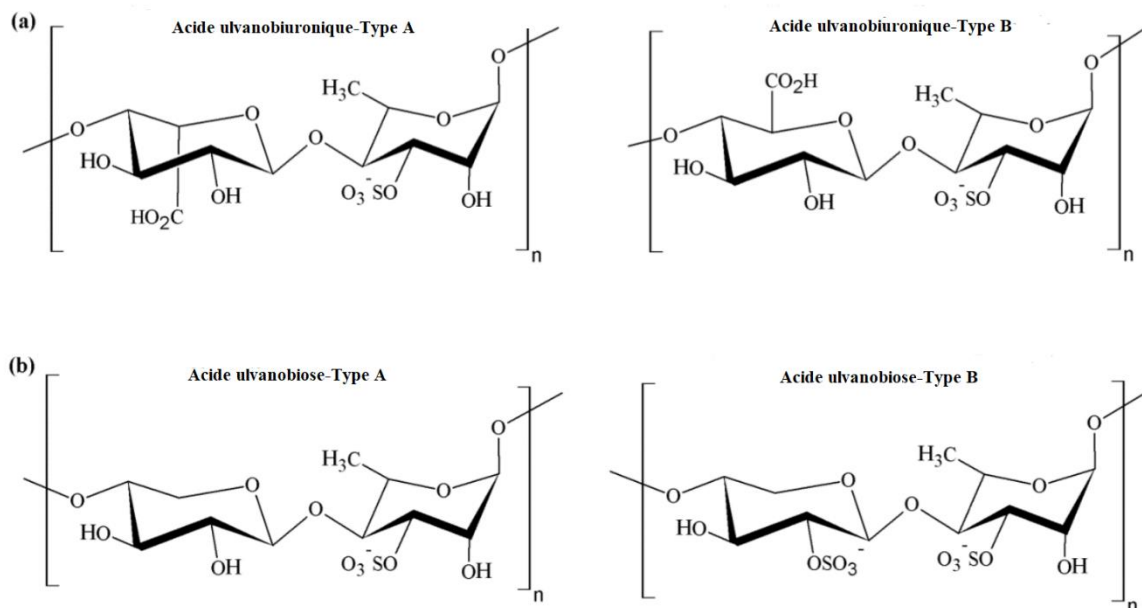


Figure I. 7. Structure des principales unités disaccharidiques répétitives de l'ulvane isolé d'*Ulva* sp. (a) Acide ulvanobiuronique du type A et B ; (b) acide ulvanobiose du type A et B.

Tableau 2. Application des ulvanes extraits de *Ulva* spp. pour contrôler les maladies dans différentes interactions hôte-pathogène.

Sources	Plantes hôtes	Pathogènes	Concentrations	Modes de traitement	Références
<i>Ulva lactuca</i>	Tomate	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	3 g/L	Injection (20 µL)	[66]
	Pomme	<i>Botrytis cinerea</i> et <i>Penicillium expansum</i>	5 g/L	Trou au niveau des fruits (5 mm de diamètre ; 3 mm de profondeur ; 20 µL)	[67]
	Olivier	<i>Verticillium dahliae</i>	2 g/L	Trempage des rameaux de 10 cm	[87]
<i>U. fasciata</i>	Haricot	<i>Colletotrichum lindemuthianum</i>	0,1, 1 et 10 g/L	Pulvérisation (5 mL)	[76]
			10 g/L	Pulvérisation (3 mL)	[74]
			10 g/L	Pulvérisation (5 mL)	[75]
		10 g/L	Pulvérisation (5mL)	[73]	
		<i>Uromyces appendiculatus</i>	10 g/L	Pulvérisation (2 mL)	[71]
			1 et 10 g/L	Pulvérisation	[72]

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		<i>F. oxysporum</i> f. sp. <i>Phaseoli</i>	—	Trempe des graines (200 µL), irrigation du sol (5 mL) et pulvérisation (4 mL)	[70]
	Blé et orge	<i>Blumeria graminis</i>	0,1 et 1 g/L	Pulvérisation	[78]
	Pommier	<i>C. gloeosporioides</i>	1, 5, 10 et 20 g/L	Pulvérisation	[86]
	Pommier		10 g/L	Pulvérisation (4 mL)	[82-84]
	<i>Arabidopsis thaliana</i>	<i>Alternaria brassicicola</i>	1 g/L	Pulvérisation (0,9 mL)	[91]
	Blé	<i>Blumeria graminis</i> f. sp. <i>Tritici</i>	1 g/L	Pulvérisation (10 mL)	[79]
<i>Ulva</i> spp.	<i>Medicago truncatula</i>	<i>C. trifolii</i>	1 g/L	Infiltration dans les feuilles et pulvérisation (250 µL)	[89]
	Pommier	<i>B. cinerea</i>	1 g/L	Pulvérisation	[85]
<i>U. armoricana</i>	Haricot, vigne et concombre	<i>Erysiphe polygoni</i> , <i>E. necator</i> et <i>Sphareotheca fuliginea</i>	—	Pulvérisation	[77]
<i>U. rigida</i>	<i>Vitis vinifera</i>	<i>B. cinerea</i>	1 g/L	Trempe des fruits	[92]
Commercial (Elicityl), <i>U. armoricana</i>	Papaye	<i>C. gloeosporioides</i>	2 g/L	Trou au niveau des fruits (3 mm de diamètre ; 20 µL)	[94]
Commercial (Elicityl), <i>U. armoricana</i>	Melon	<i>F. proliferatum</i>	5 g/L	Trou au niveau des fruits (3 mm de diamètre ; 20 µL)	[93]

1.2. Les carraghénanes

Les carraghénanes sont des polysaccharides sulfatés linéaires, partiellement hydrophiles présents dans la paroi des algues rouges (30 à 70 % du poids sec) [96]. Leur poids moléculaire moyen varie de 100 à 1000 kDa [97]. Les carraghénanes sont constituées principalement de deux unités alternées, β -D-galactopyranose et α -D-anhydrogalactopyranose, liées par des liaisons α -1,3 et β -1,4-glycosidiques (**Figure I. 8**). Selon le degré et la position des groupements sulfates, ils sont classés en six grandes formes : iota (ι -), kappa (κ -), lambda (λ -), mu (μ -), nu (ν -) et thêta (θ -) carraghénanes [98]. Les ι , κ et λ - carraghénanes, sont les principaux types présentant entre 20 à 41 % de sulfates [98]. Leurs principales sources sont les algues rouges : *Sarcothalia crispata*, *Gigartina skottsbergii*, *Gigartina pistillata*, *Mastocarpus stellatus*, *Eucheuma denticulatum*, *Kappaphycus alvarezii*, *Chondrus crispus* et *Hypnea musciformis*

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[99,100], dont *Eucheuma* sp. et *Kappaphycus* sp. couvrant plus de 90 % de la production totale des carraghénanes [101]. En plus de leurs applications comme additifs dans plusieurs procédés industriels et produits alimentaires et pharmaceutiques [102,103], ces polysaccharides ont été rapportés dans plusieurs études comme promoteurs de la croissance et éliciteurs des défenses naturelles des plantes. Ils ont permis d'induire les différentes voies de signalisation, notamment SA, JA et ET [98] (**Figure I. 9**). L'efficacité élicitrice des carraghénanes dépend de leur type de liaison et leur degré de sulfatation [104-106]. Les plantes d'*Arabidopsis* traitées avec les ι - et λ -carraghénanes (1 g/L) ont manifestées différents niveaux de résistance à l'infection par *Sclerotinia sclerotiorum* selon le degré de sulfatation. Le λ -carraghénane avec une teneur en sulfate de 35 % était plus efficace pour réduire le développement des lésions et la propagation de l'infection. En revanche les plantes traitées par ι -carraghénane (30 % de sulfates) ont montré une sensibilité accrue à *S. sclerotiorum*, entraînant une propagation rapide de la maladie [104]. En outre, en réponse aux iota(ι -), lambda(λ -) et kappa(κ -)carraghénanes à 1 g/L, le λ -carraghénane avec un taux de sulfate élevé (35 %) a montré une efficacité à supprimer l'expression des symptômes du viroïde TCDVd (Tomato Chlorotic Dwarf Viroid) chez les plants de tomates, comparé aux autres traitements [105].

Le prétraitement foliaire des plants de tomates par les κ/λ -carraghénanes a révélé une induction du métabolisme oxydatif et l'expression des protéines et les gènes de défense contre *Septoria lycopersici* [107,108] et le viroïde TCDVd [105]. Par ailleurs, les κ/β -carraghénanes extraits de *Hypnea musciformis*, *Tichocarpus crinitus* ont permis de fournir une meilleure protection contre le virus de la mosaïque du tabac (TMV) [109-111] et le virus X de la pomme de terre (PVX) [109]. Bien que les potentiels éliciteurs de ces polysaccharides contre les virus et les viroïdes soient bien documentés, il existe d'autres études qui ont évalué leur capacité à contrôler les insectes et les champignons chez *A. thaliana* [104,112], le blé [113], *Capsicum annuum* [114] et *Hevea brasiliensis* [115].

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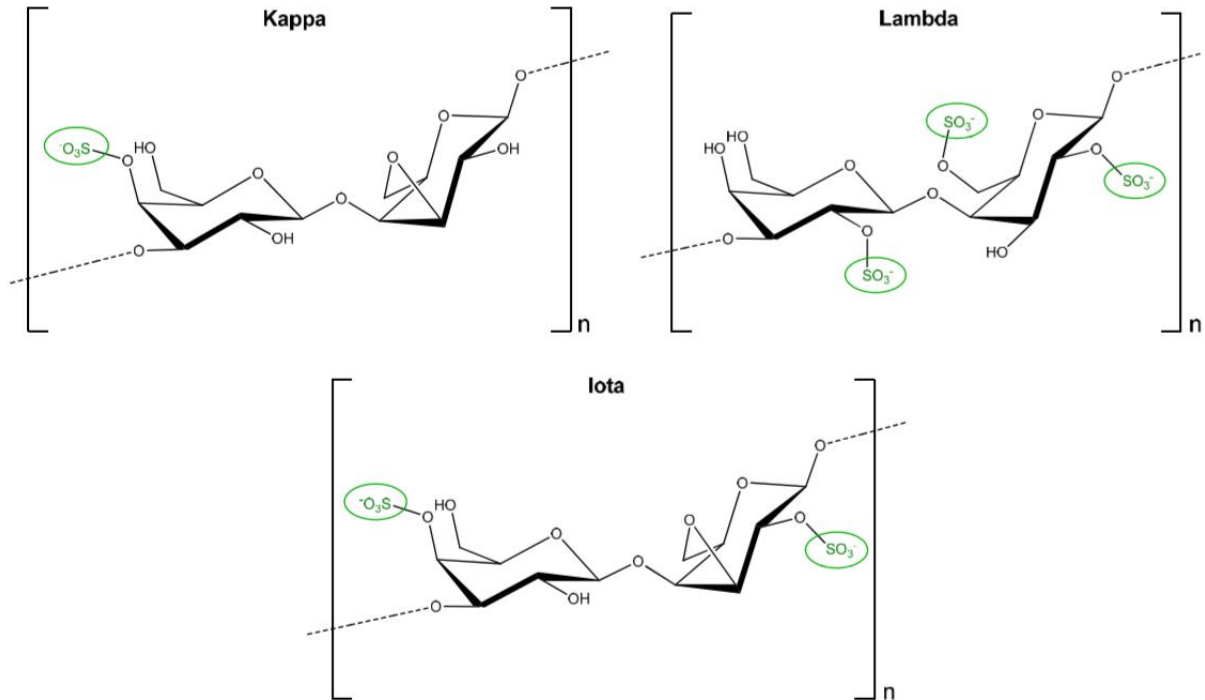


Figure I. 8. Structures chimiques des trois principaux groupes du carraghénane.

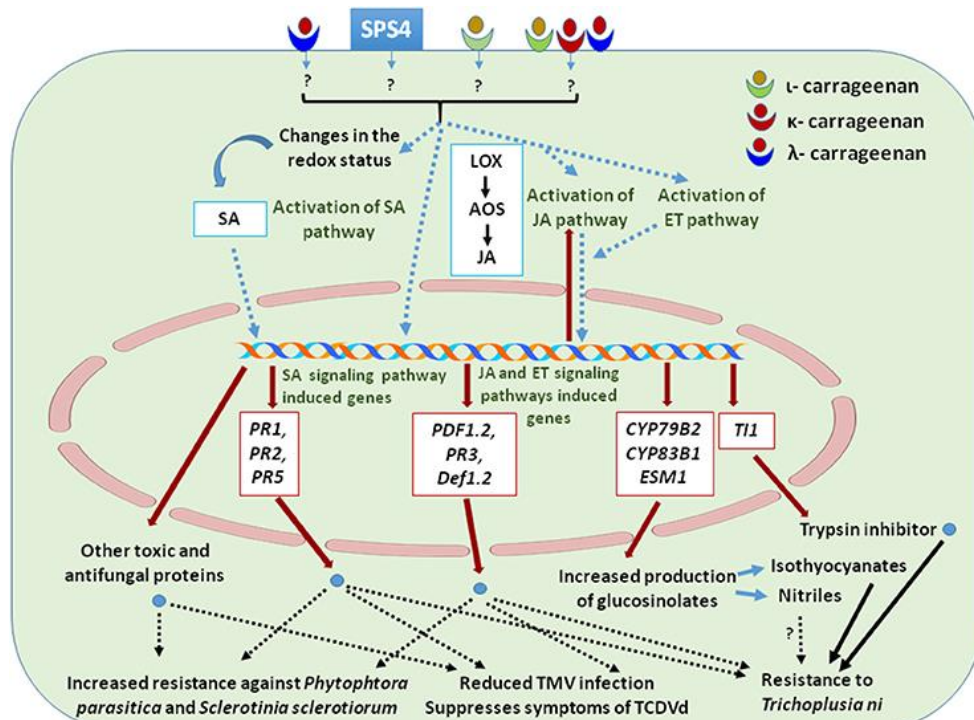


Figure I. 9. Voie de signalisation impliquée dans les mécanismes de défense induits par les différents types de carraghénanes [98]. SA : acide salicylique ; LOX : lipoxygénase ; AOS : allene oxyde synthase ; JA : acide jasmonique ; ET : éthylène ; PR : pathogenesis related protein ; PDF/Def : défensine de la plante ; CYP : cytochrome ; ESM : Epithiospecifer Modifier ; TI : protéine inhibitrice de la trypsine.

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1.3. Les fucanes et fucoïdanes

Les fucanes et les fucoïdanes sont des polysaccharides sulfatés présents dans la paroi des algues brunes, représentant 2 à 20 % de leur poids sec. Leurs poids moléculaires varient entre 3 et 950 kDa [97]. Les fucanes sont des homopolymères composés d'un seul type de monosaccharide qui est le L-fucose. Ils sont constitués généralement de deux principaux squelettes, α -L-Fuc_p lié en 3-O ou α -L-Fuc_p lié en 3 et 4-O [116] (**Figure I. 10**). Les fucoïdanes sont des hétéropolymères contenant du fucose et des proportions variées de galactose, de glucose, de mannose, de xylose, de rhamnose et des acides uroniques [116]. Les fucoïdanes sont les plus étudiés pour leurs activités biologiques, notamment antioxydantes, antibactériennes, antivirales, antitumorales, anticoagulantes et anti-inflammatoires [55,116-119]. Cependant, leur effet en tant que SDP n'est exploré que dans une mesure très limitée. Les fucoïdanes extraits de *Bifurcaria bifurcata*, *Fucus spiralis* et *Lessonia vadosa* ont montré une induction des métabolismes oxydatif et phénolique dans les racines du palmier dattier [120] et au niveau des plants de tabac [121]. En outre, le traitement des feuilles de tabac par des fucoïdanes isolés de *F. evanescens* (1 g/L) a révélé une réduction de plus de 90 % des lésions nécrotiques causées par le virus de la mosaïque du tabac [122].

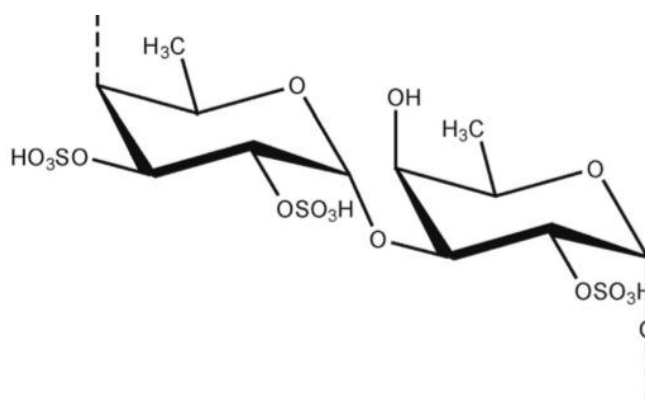


Figure I. 10. Unité répétitive du fucoïdane.

1.4. Les laminarines

Les laminarines sont des homopolysaccharides linéaires de réserve isolées des algues brunes représentant 0 à 18 % de leur poids sec. Leur poids moléculaire moyen est de 5 kDa [59,123]. Elles sont principalement constituées de 25 à 50 d'unités de glucose liées par des liaisons β -(1,3) et β -(1,6) glycosidiques avec des ramifications en position O-6 [124] (**Figure I. 11**). Il

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existe deux types de laminarine, soluble et insoluble. Le premier présente une solubilité dans l'eau froide, tandis que le second n'est hydrosoluble qu'à des températures élevées [125]. Actuellement, ce polysaccharide est commercialisé sous forme de trois concentrés solubles (SL), le Vacciplant[®], l'Iodus[®] 2 (37 g/L) et l'Iodus[®] 40. Le Vacciplant[®] est recommandé pour contrôler toutes les maladies de toutes les céréales à pailles (avoine, blé tendre, blé dur, épeautre, orge, seigle, sarrasin, triticale), particulièrement la septoriose du blé et l'helminthosporiose de l'orge. L'Iodus[®] 2 (45 g/L) est utilisé pour le traitement des pommiers contre la tavelure, l'oïdium et les maladies de conservation, et l'Iodus[®] 40 (37 g/L) pour contrôler les maladies fongiques des céréales (blé, épeautre et l'orge) [48,126]. Les laminarines et les laminarines sulfatées (issues d'une sulfatation chimique) ont montré leur efficacité d'induire la production des organiques volatiles (COV) et des espèces réactives de l'oxygène (ROS) ainsi que l'expression des gènes de défenses (**Figure I. 12**), permettant de protéger contre *E. necator*, *B. cinerea* et *Plasmopara viticola* de la vigne [127-135]. L'application de ces produits a reconnu une extension d'usage majeur pour le traitement et la stimulation des réactions de défense de diverses cultures notamment, le tabac [136-140], *A. thaliana* [138], le théier [141], la fraise [142], le framboisier [143], l'olivier [144], la pomme de terre [140,145,146] et la tomate [140].

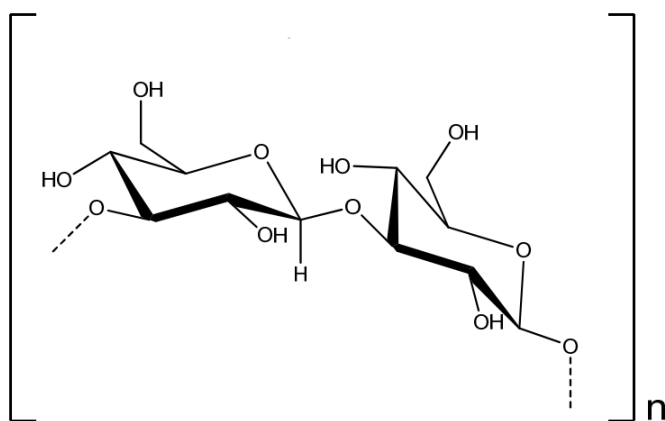


Figure I. 11. Unités de glucose de la laminarine.

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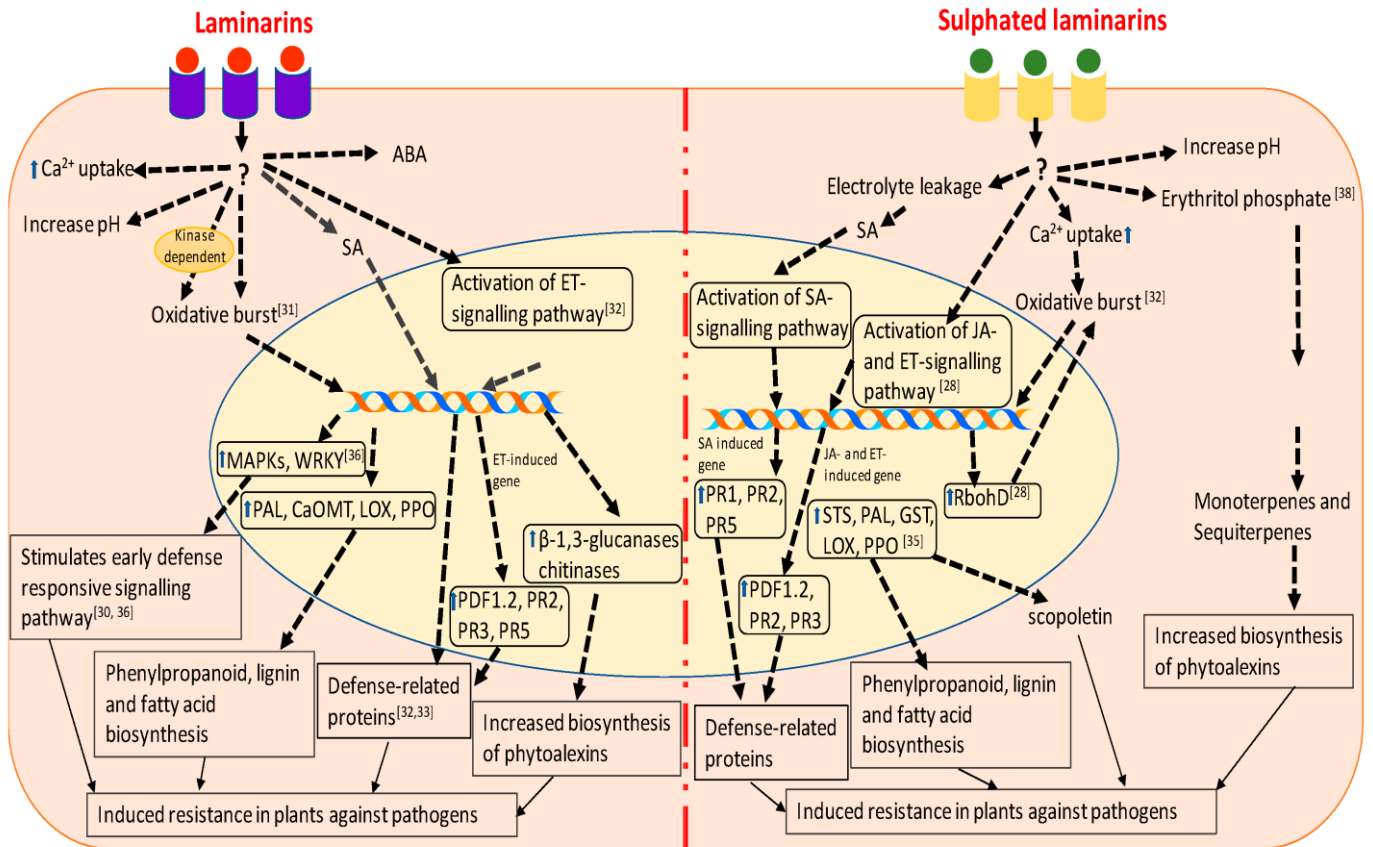


Figure I. 12. Voies de signalisation impliquées dans les mécanismes de défense induits par la laminarine et la laminarine sulfatée [48]. ABA : acide abscissique ; SA : acide salicylique ; JA : acide jasmonique ; ET : éthylène ; PR : Pathogenesis Related Protein ; PDF : défensine de la plante ; MAPK : Mitogen-Activated Protein Kinases ; PAL : phénylalanine ammonia-lyase ; LOX : lipoxygénase ; PPO : polyphénol oxydase ; GST : glutathion S-transférase ; STS : stilbène synthase.

1.5. Les alginate

Les alginate sont des polysaccharides anioniques linéaires de la paroi cellulaire des algues brunes, représentant 17 à 45 % de leur poids sec [147]. Ils sont composés de deux isomères β -D-mannuronate (M) et α -D-guluronate (G) liés par des liaisons de type β -(1,4) glycosidiques, constituant des blocs homogènes (MM-GG) et/ou hétérogènes (MG, GM) [147] (**Figure I. 13**). Les alginate sont largement utilisés dans les domaines agroalimentaire [148], pharmaceutique et biomédical [149]. Récemment, ces polymères ont été utilisés comme des engrais [3,150] et des éliciteurs du système immunitaire inné des plantes pour résister aux stress biotique [3,151,152] et abiotique [3,153]. Bien qu'il existe des études traitant de l'utilisation de ces polysaccharides en agriculture, leur utilisation comme éliciteur reste limitée. Les alginate

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extraits de *B. bifurcata* ont révélé leur efficacité à induire l'activité PAL et les polyphénols chez le palmier dattier [151]. Le traitement des plantules de palmier dattier par ces molécules a déclenché l'expression des gènes de défense impliqués dans les voies phénolique (PAL) et oxydative (POX and SOD) contre le pathogène tellurique *Fusarium oxysporum* f. sp. *albedinis* [152]. Une autre étude a révélé le rôle majeur des alginates à stimuler la biosynthèse de stilbènes et de flavonoïdes, ainsi que l'expression de gènes impliqués dans leur biosynthèse (PAL, CoA ligase, stilbène synthase et chalcone synthase) au niveau des cultures cellulaires de la vigne. [154]. Les alginates constituent également d'excellents composants de matrices d'hydrogels destinés à encapsuler des microorganismes bénéfiques [155] et des éliciteurs de nature protéique [156] pour optimiser la durabilité de leurs activités et la viabilité dans le sol. Cette technique, qui fait partie des récentes tendances biotechnologiques, assure une protection contre les facteurs externes indésirables et limite les interactions avec la microfaune du sol. Ceci permet en outre de faciliter les interactions directes avec les plantes [28,155]. Popletaeva et al. (2018) ont révélé que l'encapsulation du peptidyl prolyl cis-trans isomérase (PPIase) dans des microparticules d'alginate de sodium, a conservé sa capacité élicitrice inchangeable après l'exposition aux irradiations UV à long terme et à la protéinase K, contrairement à la PPIase libre (propriétés élicitrices perdues après 8 h) [156]. Par ailleurs, l'application du complexe alginate-albumine de sérum bovin (BSA) (ALG-BSA), a décelé une synergie d'action réduisant le développement de *Stagonospora nodorum* dans le blé [156].

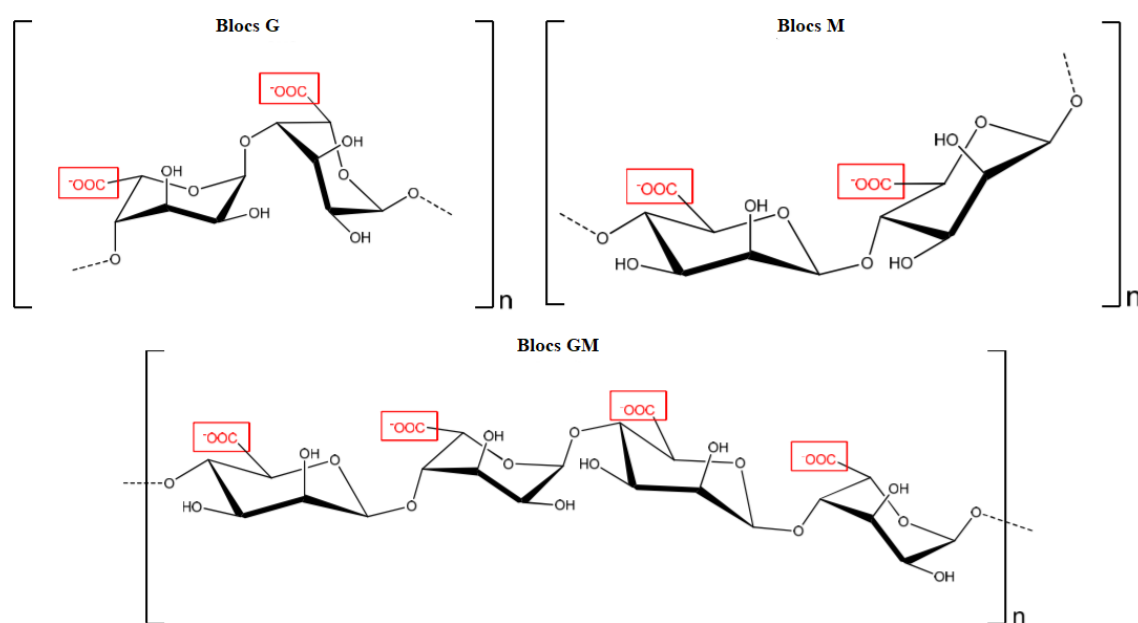


Figure I. 13. Unités d'acide D-glucuronique (blocs G), d'acide poly D-mannuronique (blocs M) et d'unités alternées (blocs GM) de l'alginate.

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2. Autres polysaccharides

Les glucuronanes, ou l'acide β -(1,4) D-polyglucuronique, sont des homopolysaccharides anioniques extraits de l'algue verte *Ulva* sp. Peu d'études ont été réalisées pour étudier l'effets éliciteur de ce polysaccharide. Les plants de tomates et les pommes traités par les glucuronanes dérivés de *Ulva lactuca* ont révélé leur capacité d'induire les mécanismes de résistance contre *Penicillium expansum*, *Botrytis cinerea* et *Fusarium oxysporum* f. sp. *lycopersici* [66,157].

Les algues vertes du genre *Codium* constituent une importante source de polysaccharides sulfatés. Leur paroi cellulaire est principalement composée de mannanes, d'arabinanes sulfatés, de galactanes sulfatés et de protéines riches en arabinogalactanes (AGPs) [158]. Ces polysaccharides sont particulièrement utilisés à des fins médicales et pharmaceutiques en vue de leurs activités anticoagulantes, antiangiogéniques, antivirales et immunostimulantes [158]. Cependant, leur utilisation en tant que SDP n'est pas encore suffisamment explorée et pourrait ouvrir la voie à l'exploitation des polysaccharides extraits de *Codium* sp. à des fins agricoles.

3. Les oligosaccharides

Les polysaccharides subissent des modifications chimiques, physiques ou enzymatiques pour générer des nouveaux dérivés fonctionnels avec une excellente biodisponibilité et une bonne solubilité tels que les oligosaccharides. Plusieurs méthodes sont appliquées pour produire ces oligomères y compris :

- ✓ **Des méthodes chimiques** : elles comprennent l'hydrolyse acide, alcaline et la dégradation oxydative. L'hydrolyse acide est actuellement la plus simple, la plus rapide et la plus efficace pour la production des oligomères. Les acides utilisés sont généralement l'acide chlorhydrique (HCl), l'acide oxalique, l'acide formique et l'acide sulfurique [159]. Dans un milieu acide, les liaisons glycosidiques sont facilement clivées pour former des fragments de faible poids moléculaire. Cependant, la dégradation acide est aléatoire et sans spécificité, elle permet d'obtenir un mélange d'oligosaccharides avec différents degré de polymérisation [160]. La dépolymérisation oxydative consiste à dégrader le polysaccharide en ajoutant des agents oxydants tels que l' H_2O_2 , NaClO, KMnO_4 et NaNO_2 [160].
- ✓ **Des méthodes physiques** : elles reposent principalement sur le traitement par micro-ondes, rayons gamma et ultrasons [159]. Ces techniques présentent les avantages d'un

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système technologique simple à faible coût sans avoir recours à des réactifs chimiques. Cependant, elles exigent des doses d'irradiations élevées et un appareillage adapté et parfois coûteux [160,161].

- ✓ **Des méthodes enzymatiques** : elles consistent à dégrader les polysaccharides par des enzymes telles que les alginates lyases, les carraghénases, les ulvanes lyases, et les fucoïdanases, etc. dans des conditions de réaction spécifiques [159]. Ces enzymes sont généralement dérivées de bactéries marines, de champignons, d'algues, de mollusques et de microorganismes du sol [162-165]. Les alginates lyases dégradent les alginates par un mécanisme d'élimination de type β -(1,4) et produisent des oligosaccharides insaturés avec des doubles liaisons à l'extrémité non réductrice. Selon leurs spécificités au substrat, elles peuvent être classées en 3 groupes, la lyase spécifique au bloc G (lyase polyG), la lyase spécifique au bloc M (lyase polyM) et les lyases bifonctionnelles [159]. Selon leur mode d'action, les alginates lyases sont regroupées en alginate lyase endolytique et exolytique. Les enzymes endolytiques permettent de cliver les liaisons glycosidiques à l'intérieur de de l'alginate et libérer des oligosaccharides insaturés (tels que les di-, tri- et tétra-saccharides), tandis que les exolytiques agissent sur les extrémités réductrices ou non réductrices des oligoalginates ou des alginates pour libérer les unités monomères insaturées [159] (**Figure I. 14**). Les carraghénases sont regroupées en k-carraghénase, ι -carraghénase et λ -carraghénase, en fonction de leurs spécificités vis à vis des substrats et de leurs séquences primaires [164]. Ce sont toutes des enzymes endolytiques qui coupent les liaisons β (1-4) internes des carraghénanes [164]. Bien que les méthodes enzymatiques soient plus efficaces pour générer des oligosaccharides plus actifs, uniformes et de tailles contrôlées, elles sont limitées par le coût parfois élevé de production et les longues durées des réactions et de purifications sélectives.

Actuellement, l'hydrolyse radicalaire utilisant le peroxyde d'hydrogène constitue un processus vert et écologique produisant des molécules à faibles masses molaires avec un rendement élevé sans générer d'impuretés toxiques. Ces oligomères dérivés des polysaccharides algaux ont suscité une attention particulière en raison de leurs propriétés PAMPs similaires à celles des phytopathogènes, en termes d'induction du système immunitaire des plantes.

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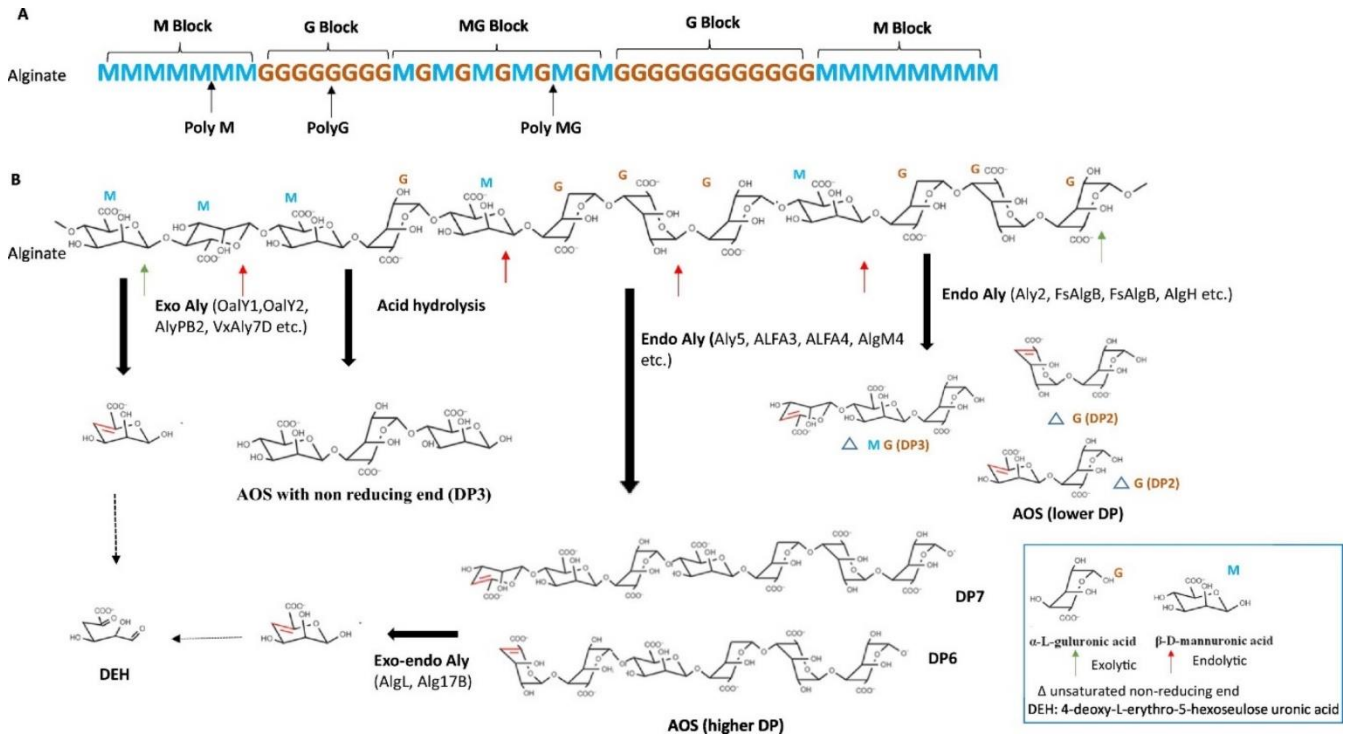


Figure I. 14. Structure de l'alginate (A) ; Préparation d'oligoalginates (AOS) par des méthodes enzymatiques (alginates lyases endolytiques et exolytiques) fournissent à l'AOS des extrémités non réductrices insaturées et par des méthodes chimiques libèrent des oligomères avec des extrémités réductrices saturées (B) [161].

3.1. Les oligoalginates

Plusieurs études ont montré la capacité des oligoalginates (OAS) à déclencher la résistance des plantes contre les agents pathogènes. Cependant, leurs mécanismes d'action n'ont pas été élucidés. Plusieurs études ont démontré que le pouvoir éliciteur de ces oligomères dépend de leur degré de dépolymérisation et de leur composition structurale. Des OAS obtenus par hydrolyse enzymatique ont induit l'activité PAL et la production des phytoalexines dépendamment de leur DP [166]. En outre, les graines de soja traitées par des OAS ont révélé une stimulation de la production de glycéoline. Cette production était non seulement liée aux DPs, mais également à la composition en unité guluronate et unité mannuronate (ratio G/M) [167]. Les OAS enrichis en acide mannuronique (OM) ont été plus actifs que ceux enrichis en acide guluronique (OG) en terme d'induction de l'activité PAL et les peroxydases (POD) dans les plants de blé [168]. De même, les OM produits par hydrolyse acide, ont révélé une capacité plus élevée que les OG à activer les enzymes de défenses permettant d'induire une résistance contre le TMV dans les plants de tabac [169]. L'application des OAS sur d'autres cultures telles que le riz [170] et *A. Thaliana* [171] a permis de déclencher les mécanismes de la voie oxydative

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et phénolique et de protéger contre Pst DC3000 et *Magnaporthe grisea*. En plus de leur pouvoir à stimuler les défenses contre les bioagresseurs, les oligoalginates induisent également la tolérance aux stress abiotiques notamment, le stress hydrique, la salinité et les métaux lourds [3].

3.2. Les oligocarraghénanes

Peu de recherches ont été menées pour examiner le pouvoir SDP des oligocarraghénanes (OCs). Les κ -OCs obtenu par hydrolyse acide ont augmenté l'activité glucanase dans les cellules de *Rubus fruticosus*. La meilleure activité élicitrice a été observée en réponse à des oligomères avec un DP = 6 [172]. Une autre étude a évalué l'effet des (κ)-, (ι)- et (λ)-OCs sur la suppression des infections causées par le TMV, *B. cinerea* et *Pectobacterium carotovorum* dans les plants de tabac. Ces oligomères ont également induit l'activité PAL, le taux de SA et l'accumulation des composés phénylpropanoïdes tels que l'acide vanillique, l'acide caféïque, le scopolétol, l'esculétine et la quercétine [173]. Les OCs ont ainsi démontré leur capacité à améliorer la tolérance au stress hydrique dans *Cymbopogon flexuosus* (Lemongrass), en augmentant les caractéristiques biochimiques, et les constituants actifs notamment le citral et le géraniol [174].

3.3. Les oligoulvanes et oligofucanes

Les oligofucanes (OFs) et les oligoulvanes (OUs) sont rarement explorés pour leur effet SDP. Les pommes et les plants de tomates traités par les OUs (DP = 2) et les oligoglucuronanes (DP = 3), issus de *Ulva lactuca*, ont montré une stimulation des enzymes de défense et les métabolites secondaires, induisant ainsi une résistance contre *Penicillium expansum*, *B. cinerea* [67,157] et *Fusarium oxysporum* f. sp. *lycopersici* [66]. Les OUs préparés par dégradation acide ont permis également de déclencher le burst oxydatif dépendamment de l'activité de la NADPH oxydase dans les cellules végétales de la luzerne, le tabac et *Rubus fruticosus* [88]. Dans les cultures cellulaires de tabac, les OFs ont rapidement provoqué une libération de l' H_2O_2 et une alcalinisation du milieu extracellulaire suivi par une forte induction de l'activité PAL et les lipoxygénases (LOXs). En outre, les feuilles de tabac ont manifesté une accumulation locale de SA, le scopolétol et les protéines PR, renforçant la résistance contre le TMV [175]. Une autre étude a révélé la capacité des fractions des fucoïdanes, partiellement hydrolysées par l' H_2O_2 , à déclencher l'activité des enzymes de défense, notamment la PAL, la LOX et la glutathion-S-transférase (GST) [121].

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4. Extraits bruts des macroalgues

En plus du rôle des polysaccharides purifiés de différentes algues, les extraits bruts (SEs) des macroalgues ont également montré leur efficacité à renforcer les défenses des plantes contre les agents pathogènes fongiques, bactériens et viraux [176,177]. Leur richesse en polysaccharides, oligosaccharides, peptides, protéines et phytohormones, fait des SEs de bons candidats pour améliorer la tolérance des plantes aux maladies. En 2016, les SEs représentaient 33 % (483 millions d'euros) du marché mondial des biostimulants. Actuellement, il est estimé que le marché des SEs atteindrait une valeur de 894 millions d'euros d'ici fin 2022, avec une majeure domination des extraits d'algues brunes [49]. Les études antérieures ont révélé le pouvoir de ces extraits à induire l'expression des gènes liés à diverses voies métaboliques à savoir la voie oxydative, et antimicrobienne ainsi que les voies liées à la synthèse des protéines PR, SA et JA [49]. Il a été rapporté que les extraits aqueux, acides et alcalins de l'algue brune *Ascophyllum nodosum* (ANE), protègent les plants de carottes, les oranges, le soja, le concombre, le fraisier, le pois, *Arabidopsis thaliana*, les tomates et les poivrons contre une gamme de phytopathogènes, notamment *Alternaria radicina*, *Botrytis cinerea*, *Alternaria cucumerinum*, *Didymella applanata*, *Fusarium oxysporum*, *Phytophthora melonis*, *Pseudomonas syringae*, *Xanthomonas campestris* pv. *Vesicatoria*, *Rhizoctonia solani*, *Podosphaera aphanis*, *Penicillium digitatum*, *Meloidogyne javanica* et *Alternaria solani* [178-188]. D'autres extraits des algues brunes *Sargassum polycystum* [189], *S. tenerrimum* [190], *S. wightii* [191], *S. liebmannii* [192], *S. vulgare* [193], *S. filipendula* [194], *Padina gymnospora* [192], *P. pavonia* [195], *Cystoseira myriophylloides* [196,197], *Laminaria digitata* [196,197], *Durvillaea potatorum* [186] et *Fucus spiralis* [196,197], des algues rouges *Kappaphycus alvarezii* [49] *Kappaphycus* sp. [198,199], *Euclima* sp. [198], *Gelidium serrulatum* [194] et *Acanthophora spicifera* [193,195] et des algues vertes *Ulva armoricana* [77], *U. lactuca* [192,194,195] et *Caulerpa sertularioides* [192] ont également induit les mécanismes de résistances de nombreuses plantes.

5. Macroalgues utilisées comme source de molécules SDP

Pour extraire les composés bioactifs, il est aujourd'hui important de sélectionner les ressources algales en se basant sur leur abondance et leur richesse en molécules d'intérêt. Actuellement, l'algue brune *Ascophyllum nodosum* est la plus utilisée comme source de biostimulants industriels et commerciaux. Actuellement, plus de 47 entreprises produisent et commercialisent divers extraits d'algues à usage agricole dont la majorité des formulations sont issues de A.

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nodosum [200]. Comme décrit ci-dessus, divers extraits commerciaux de cette algue améliorent la croissance des plantes et atténuent certains stress biotiques et abiotiques tout en renforçant les mécanismes de résistance des plantes. D'autres espèces algales sont également utilisées comme matière première durable, telles que *Ecklonia Maxima*, *Sargassum* sp., *Kappaphycus Alvarezii*, *Gracilaria* sp. et *Ulva* sp.

➤ *Ascophyllum nodosum*

Ascophyllum nodosum, également appelée Goémon noir, est une algue brune intertidale abondamment répartie sur la côte nord-ouest de l'Europe (du Svalbard au Portugal), y compris l'est du Groenland et sur la côte nord-est de l'Amérique du Nord [201] (**Figure I. 15**). Cette algue est considérée comme une source importante d'alginate (28 %), de fucoïdanes (11 %), de laminarines (4,5 %) et d'autres composés bioactifs (mannitol, ascophyllan, stérols, phlorotanins, etc.) [201]. Ces caractéristiques uniques font d'*A. nodosum* l'une des sources les plus importantes de matières premières pour la production commerciale de molécules d'intérêt. Plus de 20 extraits préparés à partir de la biomasse de cette algue sont commercialisés et ont été désignés pour améliorer la croissance des plantes et la tolérance aux stress biotique et abiotique [48,178].



Figure I. 15. Morphologie de l'algue brune *Ascophyllum nodosum*

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➤ *Sargassum* sp.

En raison de leur abondance, *Sargassum* est l'un des plus grands genres d'algues brunes qui fait partie de la plupart des écosystèmes marins. Cependant, certaines espèces sont de nature envahissante, principalement *S. muticum* (**Figure I. 16**). Cette algue, dite aussi la sargasse japonaise, est une macroalgue brune endémique du Japon, mais qui a été introduite dans les côtes pacifiques de l'Amérique du Nord dans les années 1940 (Colombie-Britannique, Canada) et les côtes atlantiques de l'Europe depuis 1960 (France). Actuellement, sa répartition est étendue de l'Alaska au Mexique dans le Pacifique nord-est et de la Norvège au Maroc dans l'Atlantique [202]. Depuis son introduction, elle est maintenant l'une des espèces de Sargassaceae les plus utilisées sur les marchés européens en vue de sa richesse en molécules bioactives notamment les phlorotannins, les laminarines, les alginates, les composés phénoliques, les antioxydants, les caroténoïdes et les composés anticancéreux tels que la fucoxanthine [48].



Figure I. 16. Morphologie de l'algue brune *Sargassum muticum*

➤ *Ecklonia maxima*

Ecklonia est un genre d'algue brune distribué principalement dans l'Asie de l'Est, l'Afrique du Sud, l'Australie et la Nouvelle-Zélande, avec des petites populations à Oman et le nord-ouest de l'Afrique [203] (**Figure I. 17**). *E. maxima*, est l'espèce la plus utilisée pour la fabrication de

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biostimulants commerciaux en raison de sa richesse en source de polyamines, de phlorotanins et d'acide 1-aminocyclopropane-1-carboxylique (ACC) [48]. Kelpak[®] est un extrait commercialisé de cette algue contenant divers composés qui favorise la croissance et améliore la productivité des plantes dans des conditions de stress. Il a été rapporté que ce produit protège les plantes contre divers agents pathogènes [204,205].



Figure I. 17. Morphologie de l'algue brune *Ecklonia maxima*

➤ *Kappaphycus Alvarezii*

K. alvarezii est une algue rouge, industriellement importante en vue de sa richesse en carraghénanes et certains composés d'ammonium quaternaire [206] (**Figure I. 18**). La culture de cette algue a commencé dans le Sud aux Philippines et elle s'est étendue à l'Indonésie, les Fidji, la Micronésie, le Vietnam, la Chine, l'Afrique du Sud [206]. K-sap est un produit commercialisé obtenu par broyage mécanique de *K. alvarezii* et qui contient un mélange de phytohormones facilitant la promotion de la croissance des plantes en cas de stress. L'application foliaire de K-sap (5 %) permet de stimuler l'expression des gènes de défense (PR-1, PR-3 et PR-5) ainsi que des phytohormone végétales (ABA, IAA, SA et zéatine) chez les plants de tomate contre *Macrophomina phaseolina* [207].



Figure I. 18. Morphologie de l'algue rouge *Kappaphycus alvarezii*

➤ *Gracilaria* sp.

Gracilaria est un genre des algues rouges, reparti principalement dans l'Indo-Pacifique et dans l'Atlantique Ouest (**Figure I. 19**). C'est une bioressource marine importante exploitée principalement pour la production de 80 % d'agar [208]. Les espèces de ce genre sont utilisées ainsi dans la médecine traditionnelle et dans l'alimentation humaine dans de nombreuses régions du monde [208]. Il a été rapporté que les extraits aqueux de *Gracilaria* sp. favorisent la croissance et réduisent l'incidence des maladies des plantes [48,208].



Figure I. 19. Morphologie de l'algue rouge *Gracilaria* sp.

➤ *Ulva* sp.

Ulva, appelé aussi la laitue de mer, est le genre d'algue verte le plus abondant au monde (**Figure I. 20**). C'est une source riche en ulvane. Plusieurs études ont rapporté le pouvoir des extraits des espèces de ce genre à induire les mécanismes de résistance des plantes. Heliosol® est un produit commercial dérivé de l'extrait d'*U. armoricana*, qui permet de stimuler les défenses naturelles chez le haricot, la vigne et le concombre contre *E. polygona*, *E. necator* et *Spherotheca fuliginea* [207].



Figure I. 20. Morphologie de l'algue verte *Ulva* sp.

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Comme décrit précédemment, d'autres algues ont également été utilisées comme source de composés bioactifs pour le contrôle des maladies des plantes. Toutefois, elles ne sont pas encore exploitables sur le plan économique.

III. Modèles d'étude : Tomate (*Solanum lycopersicum L.*) et olivier (*Olea europaea L.*)

1. Pathosystème tomate/*Verticillium dahliae*

La tomate (*Solanum lycopersicum L.*) est l'une des cultures horticoles les plus consommées et cultivées dans le monde. Il s'agit de la deuxième culture importante après la pomme de terre avec une production mondiale estimée à environ 186,8 millions de tonnes de fruits frais pour 5,05 hectares (ha), en 2020 [209]. Selon l'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO), la Chine est le premier producteur mondial avec 64,8 millions de tonnes de fruit frais produits en 2020 (35 % de la production mondiale) sur 1,11 ha, suivie par l'Inde (20,5 millions de tonnes) et la Turquie (13,2 millions de tonnes) [209]. En Europe, la tomate est classée parmi les cultures les plus productives avec une production de 22,8 millions de tonnes de fruits par 424449 ha, dont deux tiers proviennent de l'Italie (37,8 %) et de l'Espagne (26,1 %) [209]. La France est le 7^{ème} pays de l'Europe couvrant 5 950 ha et produisant 670600 millions de tonnes de tomates [209]. Le Maroc compte parmi les principaux producteurs du bassin méditerranéen de tomates. C'est l'un des principaux produits agricoles après les céréales, atteignant 1,39 million de tonnes en 2020 sur une superficie de 14781 ha cultivée, avec un rendement de 9,46 kilos/m² [209]. Les tomates n'ont pas seulement une importance économique mondiale, mais elles constituent également une des espèces les mieux étudiées comme modèle pour les recherches scientifiques. Elles se caractérisent par une biologie pratique et un génome facile à manipuler qui permet d'apporter certains éléments de compréhension sur le fonctionnement de certaines Solanacées tels que la caractérisation des gènes, la maturation des fruits, la fonction hormonale, et la biosynthèse des vitamines [210].

Malgré les intérêts qu'elle présente, la culture de tomate est sujette à diverses maladies bactériennes et fongiques ainsi que les ravageurs qui entravent sa productivité, en causant des pertes de rendement et de qualité des fruits [183] (**Tableau 3**, et **Figure I. 21-23**).

La maladie bactérienne causée par *Xanthomonas campestris* pv. *vesicatoria*, l'alternariose causée par *Alternaria solani*, le mildiou causé par *Phytophthora infestans* et l'oïdium causé par

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Leveillula taurica sont considérés comme les maladies foliaires les plus désastreuses de la tomate [183,211]. Parmi les pathogènes vasculaires, *Fusarium oxysporum* f. sp. *lycopersici* (Fusariose) et *Verticillium dahliae* (verticilliose) sont les champignons telluriques les plus répandus, affectant la productivité et la durabilité économique des plants de tomates dans le monde [212,213]. *V. dahliae* est l'un des champignons les plus destructeur qui touche de nombreuses autres cultures notamment plusieurs solanacées (tabac, pomme de terre, poivron, aubergine, etc.) dans différentes régions tempérées et tropicales du monde [214]. Il s'agit d'une espèce de champignon ascomycète qui survit dans le sol pendant des années dans des conditions difficiles sous forme de microsclérotites [215]. Il cause des réductions du rendement qui peuvent atteindre 20 à 50 % [216] avec un impact plus important à des températures entre 21 à 30 °C (température optimale pour la croissance du champignon). Une fois que les plantes sont infectées par ce pathogène, aucun fongicide n'est disponible pour atténuer la maladie [213]. Ceci rend *V. dahliae* extrêmement dangereux et difficile à gérer.

Tableau 3. Liste des différents agents pathogènes des plants de tomate.

Types de pathogènes	Noms de pathogènes	Références
Champignons foliaires et oomycètes	<i>Alternaria solani</i> , <i>Botrytis cinerea</i> , <i>Cladosporium fulvum</i> , <i>Leveillula taurica</i> , <i>Oidium lycopersici</i> , <i>Pseudoidium neolycopersici</i> , <i>Rhizoctonia solani</i> , <i>Septoria lycopersici</i> , <i>Sclerotinia sclerotiorum</i> , <i>Sclerotium rolfsii</i> , <i>Stemphylium</i> spp, <i>Phytophthora infestans</i> , <i>Phytophthora nicotianae</i>	[216,217]
Champignons telluriques et vasculaires	<i>Colletotrichum coccodes</i> , <i>Fusarium oxysporum</i> , <i>Fusarium clavum</i> , <i>Pyrenochaeta lycopersici</i> , <i>Verticillium dahliae</i> , <i>Pythium debaryanum</i> , <i>Pythium sylvaticum</i> , <i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	[216-218]
Bactéries et phytoplasmes	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> , <i>Erwinia carotovora</i> subsp. <i>carotovora</i> , <i>Pseudomonas corrugata</i> , <i>Pseudomonas mediterranea</i> , <i>Pseudomonas syringae</i> pv. <i>tomato</i> , <i>Ralstonia solanacearum</i> , <i>Xanthomonas axonopodis</i> pv. <i>Vesicatoria</i> , <i>Phytoplasma solani</i> , <i>Candidatus Phytoplasma</i> spp.	[216-218]
Virus	Alfalfa mosaic virus (AMV), Chickpea chlorotic dwarf virus (CpCDV), Cucumber mosaic virus (CMV), Eggplant mottled dwarf virus (EMDV), Parietaria mottle virus (PMoV), Pelargonium zonate spot virus (PZSV), Pepino mosaic virus (PepMV), Potato virus Y (PVY), Southern tomato virus (STV), Tobacco mosaic virus (TMV), Tomato brown rugose fruit virus (ToBRFV), Tomato chlorosis virus (ToCV), Tomato infectious chlorosis virus (TICV), Tomato leaf curl New Delhi virus (ToLCNDV), Tomato mosaic virus (ToMV), Tomato spotted wilt virus (TSWV), Tomato torrado	[216,217,219]

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	virus (ToTV), Tomato yellow leaf curl virus (TYLCV), Tomato yellow leaf curl Sardinia virus (TYLCSV)	
Viroïdes	Potato spindle tuber viroid (PSTVd), Tomato apical stunt viroid (TASVd), Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), Tomato chlorotic dwarf viroid (TCDVd)	[217]
Nématodes	<i>Globodera</i> spp., <i>Meloidogyne</i> spp., <i>Pratylenchus</i> spp.	[217,218]
Insectes	Aleurodes, Cochenilles, Mouche des fruits, Mouches mineuses, Papillons de nuit, Pucerons, Punaises, Thrips, <i>Tuta absoluta</i>	[218]

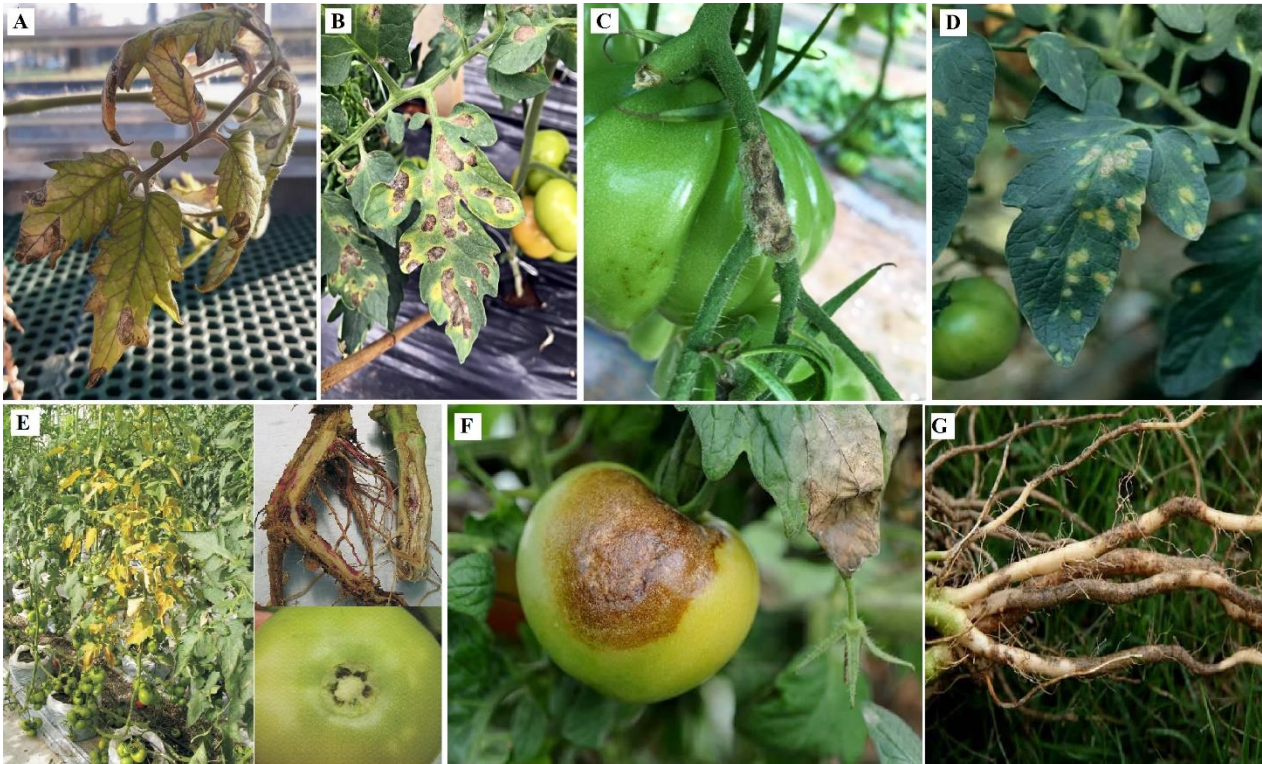


Figure I. 21. Symptômes des principales maladies fongiques de la tomate. Alternariose causés par *Alternaria solani* (A) ; Tache septorienne, causée par *Septoria lycopersici* (B) ; Pourriture grise causée par *Botrytis cinerea* (C) ; Fusariose causée par *Fusarium oxysporum* f. sp. *lycopersici* (E) ; Mildiou causé par *Phytophthora infestans* (F) ; Anthracnose causée par *Colletotrichum coccodes* (G).

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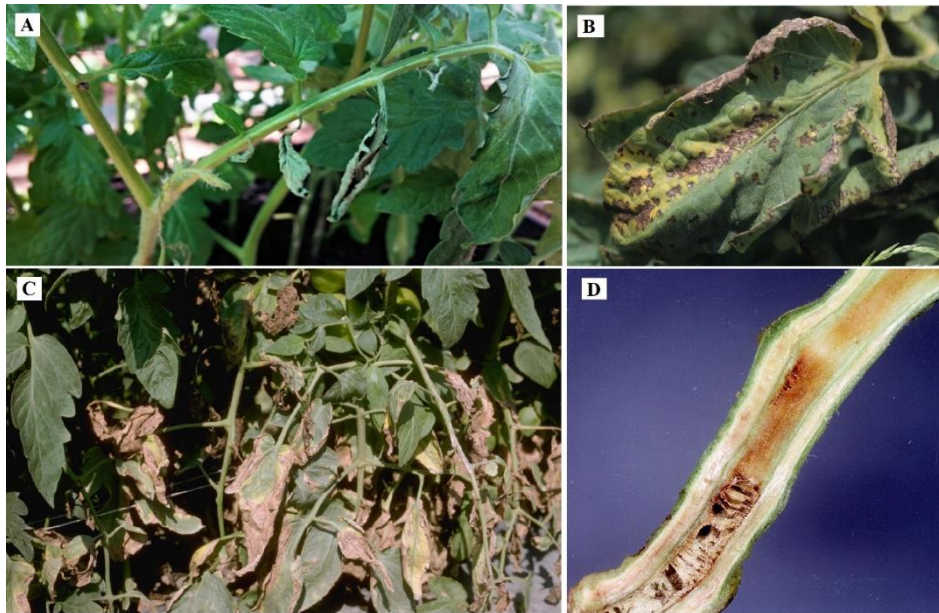


Figure I. 22. Symptômes des principales maladies bactériennes de la tomate. Chancre bactérien causé par *Clavibacter michiganensis* subsp. *michiganensis* (A) ; Tache bactérienne causée par *Pseudomonas syringae* pv. *tomate* (B) ; Tache bactérienne causée par *Xanthomonas campestris* pv. *vesicatoria* (C) ; Flétrissement bactérien causé par *Ralstonia solanacearum* (D).

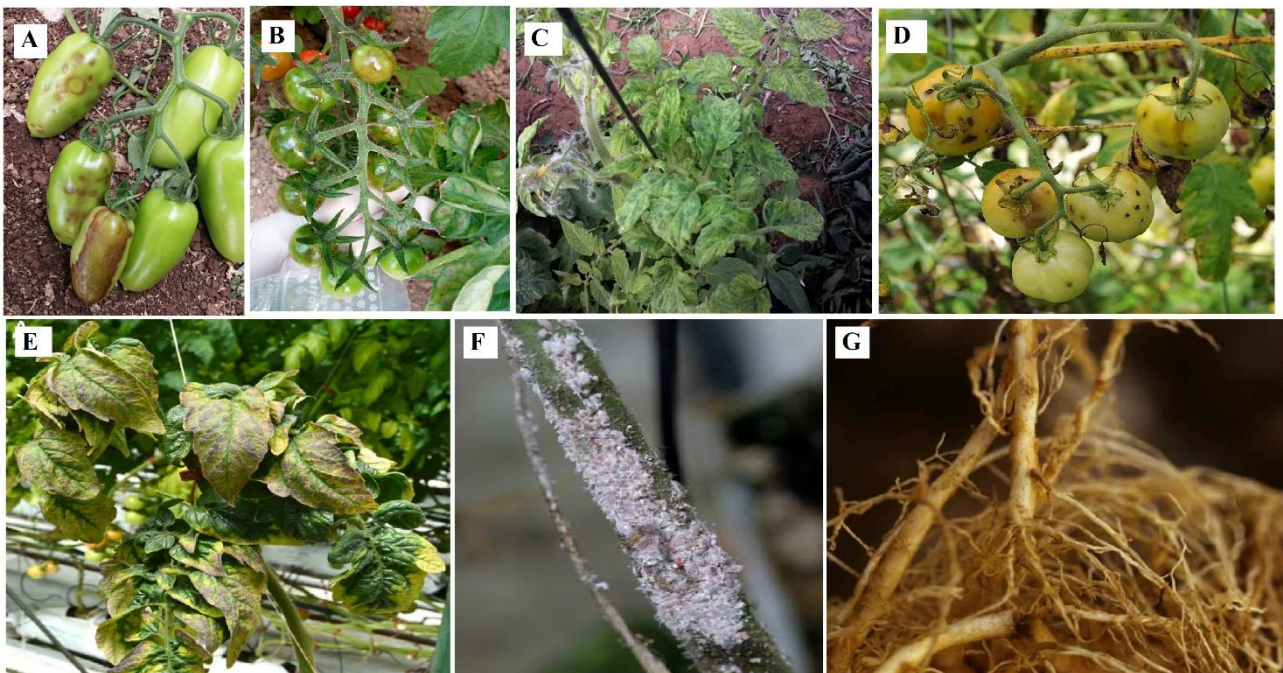


Figure I. 23. Symptômes des principales maladies causées par des virus, des viroïdes, des nématodes et des insectes. Virus de la maladie bronzée de la tomate (TSWV) (A) ; Virus de la marbrure pariétaire (PMoV) (B) ; Virus de la mosaïque de la tomate (ToMV) (C) ; Fruits affectés par *Neoceratitis cyanescens* (mouche des fruits) (D) ; Viroïde nain chlorotique de la tomate (TCDVd) (E) ; Cochenilles sur la tige de tomate (*Pseudococcus viburni*) (F) ; Lésions racinaires causées par le nematode *Pratylenchus* spp. (G).

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1.1. Cycle de vie de *V. dahliae* et symptômes chez les tomates

Verticillium dahliae provoque une maladie monocyclique, ce qui signifie qu'un seul cycle de maladie et de production de mycélium se produit pendant une saison de croissance [215]. Le cycle de vie de *V. dahliae* peut être divisé en une phase dormante, parasitaire et saprophyte. La première phase commence par des microsclérotés (MS) dormants, qui sont répartis dans le sol ou incrustés dans des débris végétaux. En l'absence d'hôte, les MS peuvent rester viables jusqu'à 14 ans [220]. En présence des exsudats racinaires des plantes hôtes et non hôtes [221], les MS produisent un à plusieurs hyphes qui s'étendent vers les racines de l'hôte. Le pathogène entre ensuite dans le stade parasitaire en infectant les plantes sensibles soit à l'extrémité des racines, soit aux sites de formation des racines latérales [215]. Une fois pénétré dans l'endoderme, les conidies sont produites dans le xylème et transportées avec le flux de sève pour coloniser les éléments vasculaires adjacents [215]. Ceci correspond au développement des symptômes de la maladie. Au cours de la nécrose des tissus ou de la sénescence des plantes, le champignon entre dans un stade saprophyte (**Figure I. 24**).

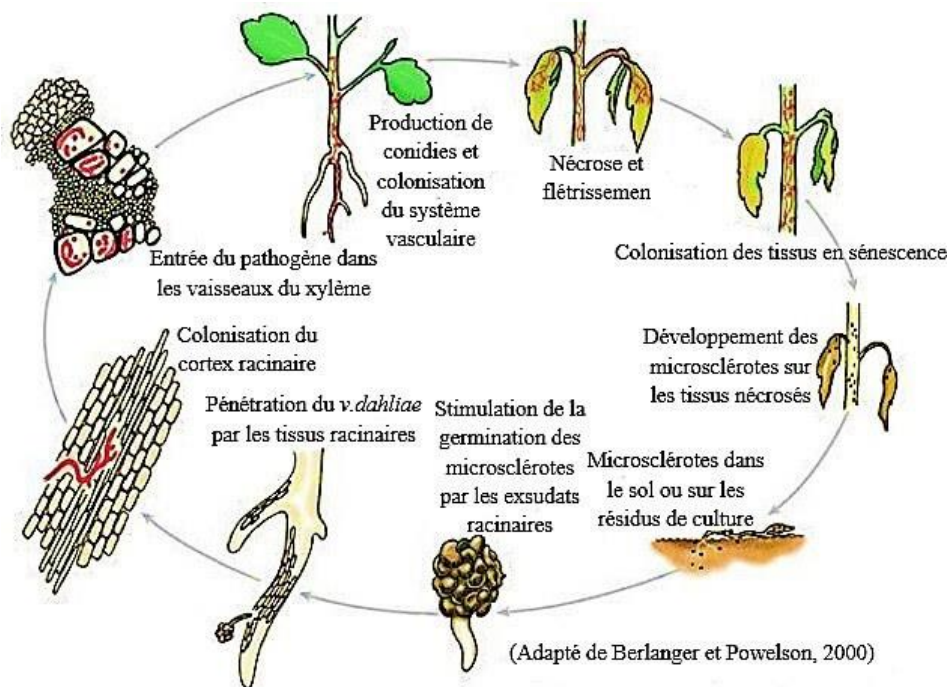


Figure I. 24. Cycle de vie de *Verticillium dahliae* [222].

Chez la tomate, les symptômes typiques commencent par un jaunissement des feuilles inférieures, un flétrissement sur la moitié des feuilles infectées, une chlorose et des lésions en forme de V sur les bords des feuilles (**Figure I. 25**). Au fur et à mesure que la maladie progresse, une nécrose commence à se développer dans les lésions, entraînant une défoliation sévère et la

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mort des plantes [220]. L'apparition des symptômes chez un cultivar de tomate sensible dépend de la virulence de *V. dahliae* et des facteurs environnementaux. Actuellement, deux races physiologiques sont connues sur la tomate [213]. La race 1 portant le gène d'avirulence Ave1 et la race 2 qui en est dépourvu [216]. Au cours d'une infection, le gène responsable de la résistance à la race 1 chez les tomates est désigné sous le nom de gène Ve1. Il code pour un récepteur de surface cellulaire qui reconnaît l'effecteur Ave1 du pathogène [223]. Cependant le gène de résistance à la race 2 est actuellement inconnu.

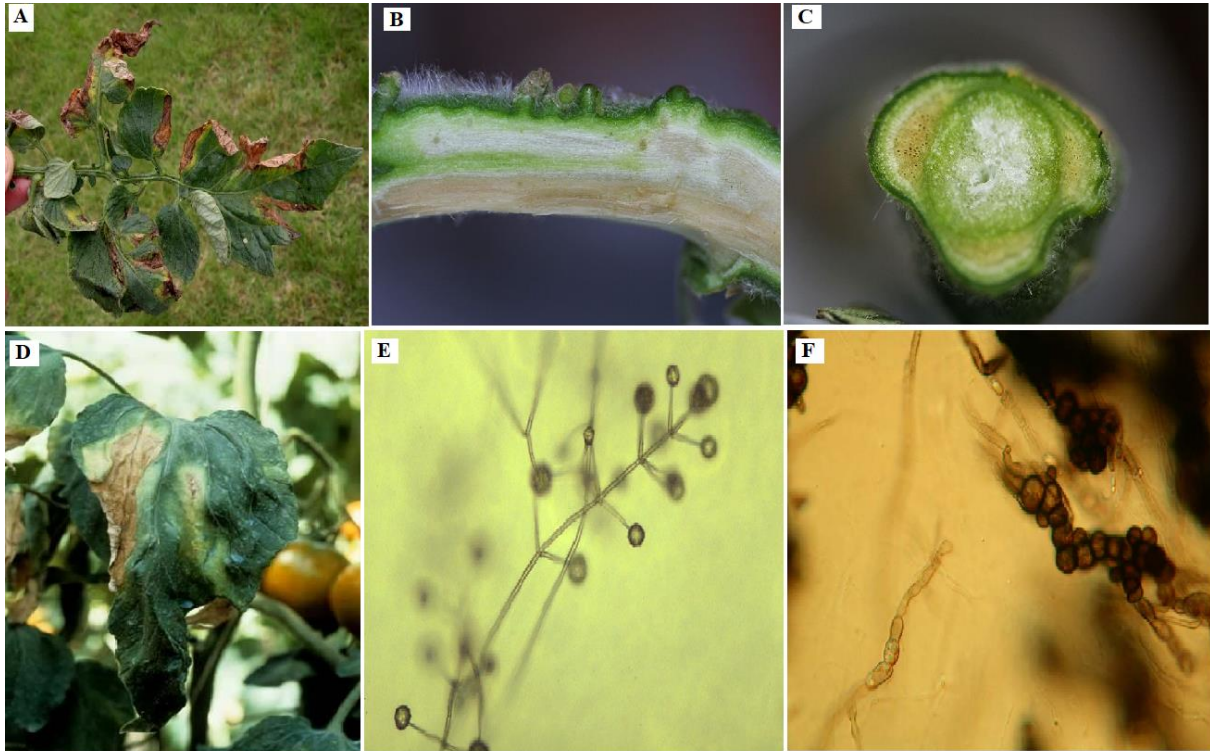


Figure I. 25. Symptôme et caractéristiques de *Verticillium Dahliae* sur les plants de tomate. Feuille d'un plant de tomate présentant des lésions nécrotiques en forme V (A) ; Coupe longitudinale de la tige présentant des vaisseaux grisâtres à légèrement bruns (B) ; Coupe transversale de la tige présentant des vaisseaux grisâtres à légèrement bruns (C) ; Feuille d'un plant de tomate présentant des symptômes de jaunissement et de nécrose (D) ; Observation microscopique du mycélium de *V. dahliae* montrant la disposition «verticillée» des conidiophores (E) ; Microsclérotés de *V. dahliae* en culture (F).

1.2. Mesures actuelles pour la gestion de la verticilliose de tomate

La gestion de la verticilliose est très difficile en raison de la longue survie du pathogène dans le sol sous des formes de résistance (MS), sa grande diversité génétique et sa capacité à infecter une large gamme d'hôtes. Une identification précise des espèces et des races est recommandée

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pour le contrôle de cette maladie. La lutte contre la verticilliose repose actuellement sur des mesures préventives, car aucun traitement fongicide curatif efficace n'est disponible pour le moment, surtout après la pénétration de l'agent pathogène dans le xylème des plantes. Parmi les mesures de contrôle de la verticilliose de la tomate :

- **Le greffage** : le greffage de variétés végétales est l'une des méthodes alternatives les plus populaires qui ont été largement mises en œuvre au cours des deux dernières décennies pour le contrôle des pathogènes telluriques [224]. Le succès à long terme d'un porte-greffe donné avec une résistance spécifique à la race dépendra d'un dépistage généralisé sur la nature de l'agent pathogène sur un système agricole précis [225]. Cependant, aucun succès à long terme n'a été trouvé en utilisant une diversité de porte-greffes pour gérer la verticilliose de la tomate. Étant donné que les races de *V. dahliae* ne peuvent être discernées que lorsque les gènes de résistance sont déployés, la durabilité d'un seul gène est incertaine. Par conséquent, la dépendance excessive à l'égard de porte-greffes spécifiques dans les systèmes de production a conduit à l'émergence de nouvelles races d'agents pathogènes ou à des changements dans la spécificité d'hôte [225].
- **La fumigation du sol** : les fumigants à base de dichloropropène, de chloropicrine, de dazomet, de disulfure de diméthyle (DMDS), de métam (y compris le sodium et le potassium) et leurs combinaisons, sont les plus efficaces dans la gestion de *V. dahliae* de la tomate [226-228]. Cependant, leur utilisation est limitée en raison des effets néfastes de ces produits chimiques sur les propriétés physicochimiques et le microbiome du sol.
- **La lutte biologique** : en utilisant les BCAs et les produits biologiques. Les endophytes bactériens, les champignons et les champignons mycorhiziens arbusculaires (AMF) ainsi que les oomycètes ont été largement étudiés en tant que BCA pour leur potentiel contre la verticilliose [229]. Des extraits de plantes et les huiles essentielles ont également été utilisés en tant qu'agents antimicrobiens contre *V. dahliae* [230,231]. D'autres mesures de gestion de la maladie ont été explorées incluant la rotation des cultures, l'utilisation des cultivars de tomates résistants, la désinfestation non-chimique des sols (solarisation, traitement à la vapeur du sol, désinfestation anaérobie, inondation et biofumigation) et l'utilisation des amendements organiques [213]. Bien que ces méthodes soient écologiques, leur

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utilisation reste limitée en raison de la variabilité de leur efficacité selon les champs et les saisons.

La stimulation des défenses naturelles des plantes par des éliciteurs naturels dérivés des algues marines peut également être une alternative prometteuse aux méthodes conventionnelles ouvrant une nouvelle orientation pour la gestion de la verticilliose.

2. Olivier

L'olivier (*Olea europaea* L.) est l'une des espèces végétales les plus cultivées et la seule de la famille des Oléacées qui produit des fruits comestibles destinés à la production de l'huile et des olives de table dans diverses zones géographiques du monde. L'olivier joue un rôle socio-économique et écologique clé surtout dans les pays méditerranéens [232]. Au cours des deux dernières décennies, la demande internationale croissante d'huile d'olive et d'olives de table a conduit à l'expansion de la culture de l'olivier dans d'autres pays tels que l'Australie, le Chili, l'Argentine ou le Pérou [233]. Actuellement, la superficie totale des oliviers récoltés dans le monde est de 12,76 millions d'ha avec une production de 23,64 millions de tonnes d'olives et 3,09 millions de tonnes d'huile d'olive [234]. Aujourd'hui, environ 73,1 % d'huile d'olive et 65,8 % des olives sont produits en Europe. L'Espagne, l'Italie et la Grèce étant les plus grands producteurs [234]. Le Maroc, après l'Espagne, l'Italie, la Grèce et la Turquie, est le 5^{ème} producteur d'olive (1,4 million de tonnes) et le 7^{ème} producteur d'huile d'olive à l'échelle mondiale [234].

Malheureusement, l'olivier est soumis à plusieurs stress biotiques, influençant la santé, le rendement et la qualité de l'huile et des fruits [235,236] (**Tableau 4** et **Figure I. 26**). Parmi les principaux agents pathogènes de l'olivier :

- *Pseudomonas savastanoi* pv. *Savastanoi* : c'est la bactérie la plus étudiée en oléiculture, c'est l'agent causal de la tuberculose de l'olivier, signalée pour la première fois au Maroc en 1960 [237]. Elle est actuellement répandue dans toutes les cultures d'olivier, affectant la productivité et la qualité de l'huile d'olive [238].
- *Xylella fastidiosa* : c'est une bactérie qui se développe dans le xylème des plantes, originaire de l'Amérique. Suite à sa propagation dans l'Union européenne (EU) à travers l'Italie en 2013, *X. fastidiosa* a provoqué le syndrome de déclin rapide de l'olivier (OQDS) aux oliveraies de la zone méditerranéenne [239]. Actuellement, aucun moyen

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curatif pour lutter contre cette bactérie n'est disponible. Le contrôle des insectes vecteurs et la destruction des plantes contaminées sont parmi les mesures préconisées pour limiter la propagation bactérienne [240].

- ***Colletotrichum spp.*** : est un genre de champignon qui attaque la partie aérienne et qui est responsable de l'antracnose de l'olivier [241]. Elle affecte principalement les fruits et est considérée comme l'une des maladies de l'olivier les plus destructrices et les plus répandues dans le monde [241]. D'autres pathogènes fongiques affectant la partie aérienne de l'olivier ont également été identifiés tels que *Venturia oleaginea*, *Spilocaea oleaginea* (œil du paon) [240], *Pseudocercospora cladosporioides* (maladie du plomb de l'olivier) [242], *Fomitiporia mediterranea* (pourriture du bois) [243], *Alternaria alternata* (Alternariose) [244] et *Botryosphaeria dothidea* [245].
- ***Verticillium dahliae*** : l'agent causal de la verticilliose de l'olivier (VVO) est considéré comme le champignon vasculaire le plus limitant de la production d'olives dans le monde [246]. D'autres champignons telluriques tels que *Fusarium spp.*, *Cylindrocarpon destructans* et *Rhizoctonia solani* sont également capable de causer des dégâts dans les cultures de l'olivier [247].
- ***Phytophthora spp.*** : les oliviers sont également affectés par les oomycètes du genre du *Phytophthora* tels que *P. oleae*, *P. megasperma*, *P. inundata* ou *P. palmivora* [248]. D'autre part, différents virus, insectes et nématodes causent de sérieux dégâts, engendrant des problèmes économiques importants [240,249] (**Tableau 4**).

Tableau 4. Liste des différents agents pathogènes de l'olivier.

Types de pathogènes	Noms des pathogènes	Références
Champignons	<i>Spilocaea oleagina</i> , <i>Verticillium dahliae</i> , <i>Colletotrichum spp.</i> , <i>Venturia oleaginea</i> , <i>Pseudocercospora cladosporioides</i> , <i>Fomitiporia mediterranea</i> , <i>Alternaria alternata</i> , <i>Botryosphaeria dothidea</i> , <i>Fusarium spp.</i> , <i>Cylindrocarpon destructans</i> , <i>Rhizoctonia solani</i>	[240-244,247,250]
Oomycètes	<i>Phytophthora oleae</i> , <i>P. megasperma</i> , <i>P. inundata</i> ou <i>P. palmivora</i>	[248]
Bactéries	<i>Xylella fastidiosa</i> , <i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i>	[238,240,251]

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Virus	Olive latent ringspot virus (OLRV), Olive leaf yellowing-associated virus (OLYaV), Olive latent virus 3 (OLV-3), Olive latent virus 1 (OLV-1), Olive latent virus 2 (OLV-2), Olive mild mosaic virus (OMMV), Olive vein yellowing associated virus (OVYaV), Olive yellow mottle and decline-associated virus (OYMDaV), Olive semilatif virus (OSLV), Arabis mosaic virus (ArMV), Strawberry latent ringspot virus (SLRSV), Cherry leafroll virus (CLRV)	[240]
Nématodes	<i>Meloidogyne</i> spp., <i>Xiphinema</i> spp.	[249,252,253]
Insectes	<i>Bactrocera oleae</i>	[240]

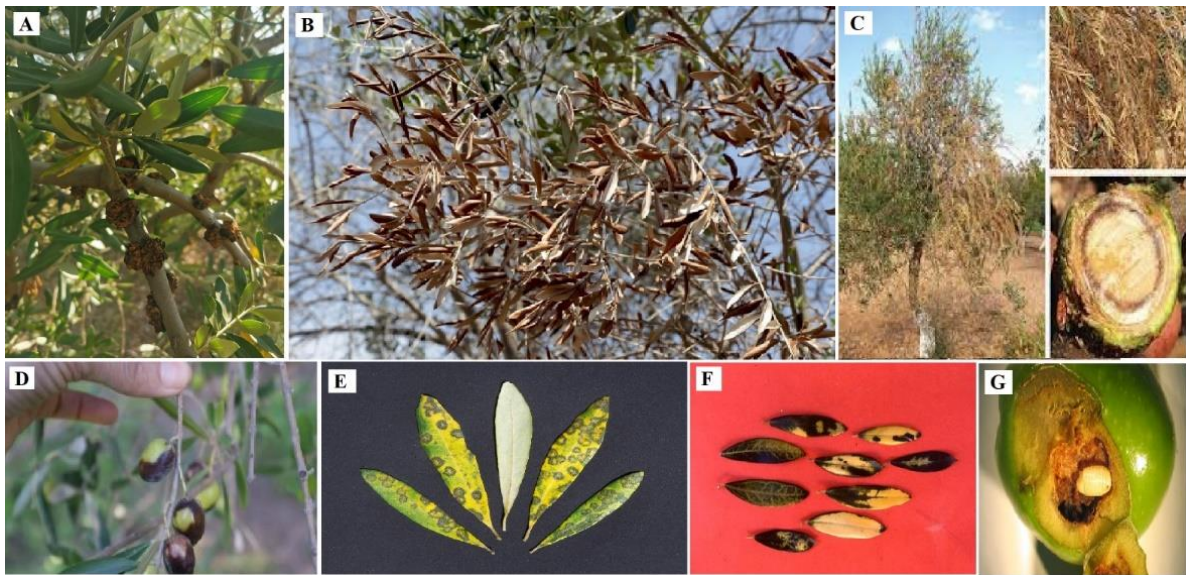


Figure I. 26. Symptômes des principales maladies de l'olivier. Tuberculose de l'olivier causée par *Pseudomonas savastanoi* pv. *savastanoi* (A) ; Syndrome de déclin rapide de l'olivier (OQDS) causé par *Xylella fastidiosa* (B) ; Verticilliose de l'olivier (VVO) causée par *Verticillium dahliae* (C) ; Anthracnose de l'olivier causée par *Colletotrichum* spp. (D) ; Cycloconium ou "œil de paon" causée par *Spilocaea oleaginea* (E) ; Jaunissement des feuilles de l'olivier causé par le virus jaune de la veine olive (OVYV) (F) ; Olive affectée par la *Bactrocera oleae* (la mouche de l'olive) (G).

En raison de la grande diversité des agents pathogènes qui affectent la culture de l'olivier, aujourd'hui, de nombreux agriculteurs utilisent les intrants chimiques pour la protection de cette culture. En revanche, de nombreux groupes de recherche ont concentré leurs efforts sur l'utilisation des BCAs tels que les AMFs, les rhizobactéries favorisant la croissance des plantes (PGPR), les champignons favorisant la croissance des plantes (PGPF) et les endophytes comme alternatives écologiques [232]. La production des cultivars résistants est également une mesure efficace et respectueuse de l'environnement pour la gestion des maladies de l'olivier, mais elle

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reste limitée par le manque d'informations génomiques adéquates [240,254]. La stimulation des défenses naturelles par des biomolécules extraites de ressources naturelles et abondantes telles que les algues marines pourrait être une bonne alternative pour prévenir le développement des maladies chez l'olivier et réduire la dépendance de sa culture aux produits chimiques toxiques pour l'environnement et la santé humaine.

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CHAPITRE II

EVALUATION DE L'EFFET ÉLICITEUR DES ALGINATES (POLYSACCHARIDES NON SULFATÉS) EXTRAITS D'ALGUES BRUNES MAROCAINES, ET LEURS DÉRIVÉS SUR LES PLANTS DE TOMATE



Article 1 : Accepté en 2020

Meriem Aitouguinane, Soukaina Bouissil, Anouar Mouhoub, Halima Rchid, Imen Fendri, Slim Abdelkafi, Mohamed Didi Ould El-Hadj, Zakaria Boual, Pascal Dubessay, Christine Gardarin, Philippe Michaud, Zainab El Alaoui-Talibi, Cherkaoui El Modafar, Guillaume Pierre and Cédric Delattre. Induction of natural defenses in tomato seedlings by using alginate and oligoalginates derivatives extracted from Moroccan brown algae. **Mar. Drugs** **2020**, *18*, 521, doi: 10.3390/md18100521. **IF (6.085)**.

Article 2 : Prochainement soumis

Meriem Aitouguinane, Zainab El Alaoui-Talibi, Halima Rchid, Imen Fendri, Slim Abdelkafi, Mohamed Didi Ould El-Hadj, Zakaria Boual, Pascal Dubessay, Philippe Michaud, Guillaume Pierre, Cédric Delattre, Cherkaoui El Modafar. Elicitor activity of low molecular weight alginates obtained by oxidative degradation of alginates extracted from *Sargassum muticum* and *Cystoseira myriophylloides*.

Contexte scientifique

Les macroalgues sont des ressources marines qui représentent une importance écologique capitale, contribuant à une grande partie de la biodiversité mondiale. En raison de leur abondance et leur potentiel élevé de prolifération et de production rapide, de nombreuses recherches sont actuellement axées sur la valorisation de cette biomasse marine dans différents domaines d'application. En 2018, la production mondiale de macroalgues représentait 32 millions de tonnes/an avec 1,1 million de tonnes/an provenant de la récolte sauvage, atteignant des revenus annuels de 13,3 milliards USD [1]. Actuellement, environ 250 espèces d'algues sont exploitées commercialement dans le monde, y compris quelques genres intensivement cultivés est principalement destinés à l'alimentation humaine [1,2]. Parmi les trois grands groupes d'algues, les algues brunes. Elles représentent le groupe le plus important économiquement en termes de biomasse produite et de composition en polysaccharides [3]. Leurs parois cellulaires sont constituées essentiellement de cellulose, d'hémicellulose, d'alginate, de fucoïdanes et de la laminarine [4]. L'alginate est le polysaccharide fondamental des algues brunes, représentant plus de 40 % de leur poids sec. C'est l'un des biopolymères les plus abondants [5]. Il est estimé que 23000 tonnes d'alginate sont produites à partir de 85000 tonnes d'algues brunes par an [6]. En outre, le marché de ces polysaccharides devrait connaître un taux de croissance important d'ici 2025 atteignant une valeur de 923,8 millions USD [7]. Les genres d'algues brunes tels que *Ascophyllum*, *Durvillaea*, *Ecklonia*, *Laminaria*, *Lessonia*, *Macrocystis* et *Sargassum* sont reconnus comme les principales sources commerciales d'alginate [7]. Les propriétés physicochimiques uniques, la biocompatibilité environnementale, la biodégradabilité, la non-toxicité et la longue durée de conservation, font de l'alginate un biopolymère idéal qui pourrait être utilisé dans un large éventail d'applications, dont l'agriculture. Comme discuté dans le chapitre précédent, ce polysaccharide a été largement utilisé pour stimuler la croissance et le développement des végétaux [8,9]. Cependant, peu d'études ont rapporté sa capacité à induire les mécanismes de résistance des plantes.

L'application des alginate en tant qu'éliciteur peut parfois être limitée par sa faible solubilité dans l'eau et sa viscosité lorsque des concentrations élevées sont nécessaires pour conférer des effets spécifiques sur les végétaux. Pour cette raison, des alginate à faible poids moléculaire (LMW : Low Molecular Weight) ou des oligoalginate peuvent être produits pour obtenir des nouveaux dérivés fonctionnels avec une meilleure solubilité et biodisponibilité tout en concevant de nombreuses propriétés physicochimiques du polymère natif. Ceci permet en outre

Chapitre II

de préparer des concentrations élevées sans augmentation significative de la viscosité. De nombreuses techniques physico-chimiques ont été décrites pour obtenir des oligoalginates. Cependant, l'hydrolyse radicalaire par l' H_2O_2 est considérée comme la méthode la plus écologique, la plus facile, la moins coûteuse et la plus reproductible d'un point de vue industriel. Pour cela, notre choix s'est porté sur cette technique pour dépolymériser les alginates extraits des différentes algues marines étudiées.

Dans ce chapitre, nous traitons l'évaluation du potentiel SDP des alginates extraits des algues brunes de poids moléculaires différents ainsi que l'impact des structures de ces molécules sur l'efficacité de cette fonction.

Pour ce faire, des alginates ont été extraits à partir de trois algues brunes, *Bifurcaria bifurcata*, *Sargassum muticum* et *Cystoseira myriophylloides* et leurs dérivés saccharidiques ont été préparés par hydrolyse radicalaire.

Dans les côtes marocaines, l'accumulation de ces trois espèces et surtout *S. muticum*, cause des problèmes environnementaux et économiques importants. Cependant, leur élimination par le gouvernement et les entreprises s'avère coûteuse. De ce fait, la valorisation de cette biomasse abondante dans le domaine agricole peut résoudre les problèmes associés. Elle peut également faire de ces espèces algales une opportunité économique en vue de leur richesse en molécules fonctionnelles, notamment les polysaccharides. Ceci pourrait en outre contribuer à développer une agriculture plus durable en réduisant le recours aux produits chimiques.

Les résultats de ce travail ont fait l'objet de deux publications. La première est parue en **octobre 2020** dans le journal « **Marine Drugs** » (**Impact factor : 6.085**). Elle porte sur l'induction des défenses naturelles des plants de tomate en réponse aux alginates extraits de *B. bifurcata* et leurs oligomères. L'élicitation a été évaluée en utilisant l'injection des solutions saccharidiques au milieu des tiges, comme mode d'application. Le suivi de l'activité de la phénylalanine ammonia-lyase (PAL) ainsi que la teneur en polyphénols a été effectué au niveau des feuilles des plants de tomate. Cette étude qui est également la continuité des travaux antérieurs réalisés sur le palmier dattier [10,11], nous a permis d'élargir le spectre d'action des poly/oligoalginates en prouvant leur efficacité en tant que SDP sur un autre modèle expérimental : la tomate (Plante herbacée annuelle). Ainsi, deux types d'alginates ont été extraits de *S. muticum* et *C. myriophylloides*, et leurs dérivés oligosaccharidiques ont été obtenus par hydrolyse radicalaire. Ces molécules ont été appliquées par pulvérisation, et l'élicitation a été évaluée par le suivi de la PAL, la lignine et le contenu en composés phénoliques au niveau des racines et des feuilles

Chapitre II

afin d'examiner l'aspect systémique des réponses déclenchées. Les résultats de ce travail sont présentés sous forme d'une **seconde publication**, qui sera **prochainement soumise**.

Chapitre II

Références

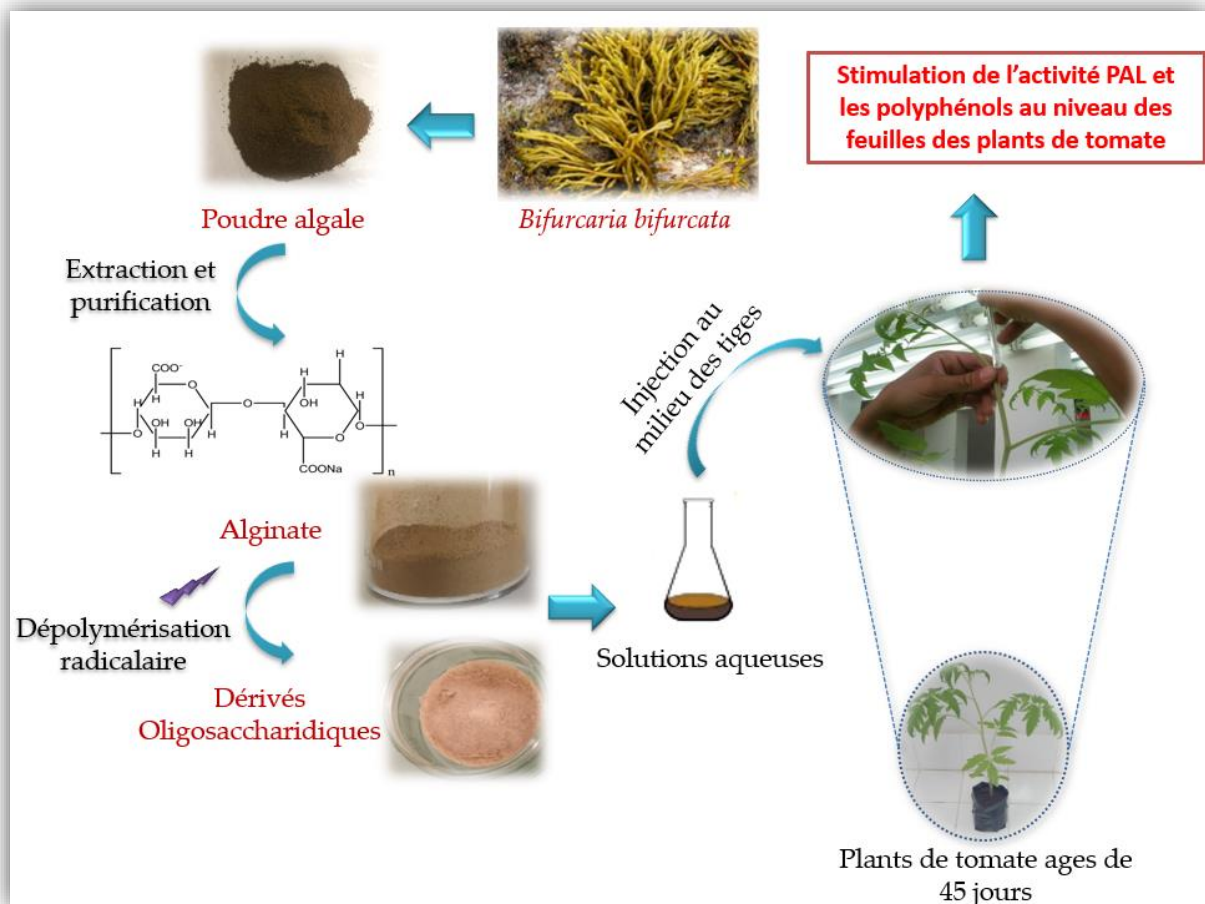
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Article 1

Induction of natural defenses in tomato seedlings by using alginate and oligoalginates derivatives extracted from Moroccan brown algae

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Résumé graphique :



Induction of natural defenses in tomato seedlings by using alginate and oligoalginates derivatives extracted from Moroccan brown algae

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Abstract

Polysaccharides extracted from marine algae have attracted much attention due to their biotechnological applications, including therapeutics, cosmetics, and mainly in agriculture and horticulture as biostimulants, biofertilizers, and stimulators of the natural defenses of plants. This study aimed to evaluate the ability of alginate isolated from *Bifurcaria bifurcata* from the Moroccan coast and oligoalginates derivatives to stimulate the natural defenses of tomato seedlings. Elicitation was carried out by the internodal injection of bioelicitor solutions. The elicitor capacities were evaluated by monitoring the activity of phenylalanine ammonia-lyase (PAL) as well as polyphenols content in the leaves located above the elicitation site for 5 days. Alginate and oligoalginates treatments triggered plant defense responses, which showed their

capacity to significantly induce the PAL activity and phenolic compounds accumulation in the leaves of tomato seedlings. Elicitation by alginates and oligoalginates showed an intensive induction of PAL activity, increasing from 12 h of treatment and remaining at high levels throughout the period of treatment. The amount of polyphenols in the leaves was increased rapidly and strongly from 12 h of elicitation by both saccharide solutions, representing peaks value after 24 h of application. Oligoalginates exhibited an effective elicitor capacity in polyphenols accumulation compared to alginate polymers. The alginate and oligosaccharides derivatives revealed a similar elicitor capacity in PAL activity whereas the accumulation of phenolic compounds showed a differential effect. Polysaccharides extracted from the brown seaweed *B. bifurcata* and oligosaccharides derivatives induced significantly the phenylpropanoid metabolism in tomato seedlings. These results contribute to the valorization of marine biomass as a potential bioresource for plant protection against phytopathogens in the context of eco-sustainable green technology.

Keywords: Marine seaweed; Alginate; Oligoalginates; Elicitation; PAL activity; Phenolic compounds; Tomato seedling

1. Introduction

Today, pesticides are among the most widely used substances in agriculture worldwide to control losses caused by phytopathogens. The use of these chemical compounds in crop protection has major impacts on the environment, and public health [1]. The increasing thrust toward the development of sustainable agriculture and green revolution technologies has led to the emergence of new molecules derived from natural sources, biopesticides. Despite their non-toxic mechanisms and eco-friendly manner, their usefulness is increasingly restricted because of their expensive cost of production, the broad spectrum, which might lead to negative effects on non-target organisms, and low reliability due to low stability [2]. In this context, new promising alternative approaches were developed by researchers, one of them, the stimulation of the natural plant defenses using natural elicitors that does not exert selective effects on the pathogen. This strategy aims to induce plant resistance against a broad spectrum of microorganisms [3].

Seaweed offers an abundant and valuable source of bioactive constituents, they have been recognized with their application in human food, nutraceutical, cosmetic, and pharmaceutical

industries as well as agri-horticultural sectors, as fertilizers and soil conditioners [4]. Recently, oligo/polysaccharides, derived from green, red, and brown macroalgae have gained much attention among researchers due to their elicitor activities leading to stimulate plant defense mechanisms and protect against a wide range of phytopathogens [5]. Ulvan from green seaweed, laminarin isolated from brown algae as well as carrageenan from red ones, and their oligomers derivatives, are extensively studied due to their capacity to enhance various plants defenses [5-10]. In the case of alginate purified from brown seaweed and oligoalginates, only few studies have been conducted.

This study aimed to investigate the ability of alginate isolated from the brown seaweed *Bifurcaria bifurcata* and oligosaccharides derivatives obtained after radical depolymerisation, to induce the phenylpropanoid defense pathway, in particular, phenylalanine ammonia-lyase (PAL) activity and phenolic compounds accumulation in the leaves of tomato seedlings. This is the first report on these saccharides as elicitors of tomato plant defenses.

2. Materials and Methods

2.1. Collection of *Bifurcaria bifurcata* seaweed samples

The brown seaweed *Bifurcaria bifurcata* was collected from the intertidal zone at low tide, from the Moroccan Atlantic coast in the south of El Jadida city (Latitude 32°15' to 33°15'; longitude 7°55' to 9°15'), during December 2017. After harvesting, the samples were washed extensively with seawater, then transported to the laboratory in plastic bags and rinsed again with distilled water in order to discard salt, sand, and any foreign particles that could affect the biological activities. The seaweed biomass was air-dried in the dark for 15 days, oven-dried for 6 h at 50 °C, and then blended into a fine powder using an electric mill (Moulinex (a subsidiary of Groupe SEB, Lyon, France)).

2.2. Preparation of sodium alginate and oligoalginates derivatives extracts

The polysaccharides and oligosaccharides used in this study were provided in the Institut Pascal, University Clermont Auvergne in France. The extraction and purification of alginate and oligoalginates derivatives, from the brown algae *Bifurcaria bifurcata*, were previously described in detail [11,12], and the Chemical Analysis, High-Performance Steric Exclusion Chromatography (HPSEC) (Agilent Technologies, Santa Clara, CA, USA) and NMR

Spectroscopy Analysis (Bruker Scientific Instruments, Billerica, MA, USA), were established according to the published protocol.

2.3. Plant cultivation and elicitor applications

Sterile Campbell 33 tomato seeds were used. The seeds were sown in individual 7 cm pots containing peat substrate for germination. Two weeks after growing, the seedlings were transplanted into plastic poly grow bags (22 × 10 × 8 cm) containing a mixture of soil and peat (v/v). The plants were grown in a culture room at 26 °C and a cycle of 16 h/8 h (light/dark). When the seedlings were 45 days old, the elicitation was carried out by syringe infiltration of 20 μL of each saccharide solution (3 g/L in distilled water, pH 6) into the internodal middle of the tomato seedlings. The control plants were injected with distilled water. The shoot system lengths of all plants used were 50 cm, at the eight-leaf stage. Eight tomato seedlings were collected after 0, 12, 24, 72, and 120 h in each treatment group. The two apical leaf samples from two plants were pooled, frozen in liquid nitrogen, and ground in a mortar and then stored at −20 °C for subsequent analysis. All experiments were conducted thrice under identical conditions. **Figure II. 1. 1** summarizes the elicitor application method.

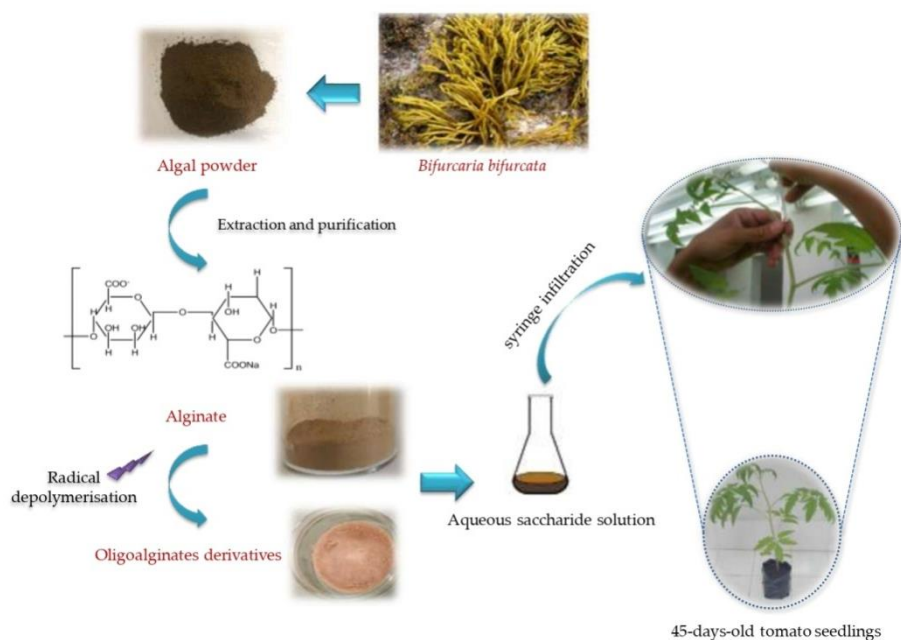


Figure II. 1. 1. Overview of the elicitor application method.

2.4. Extraction and measurement of phenylalanine ammonia-lyase (PAL) activity

Tomato leaf powders were weighed and macerated in 2 mL of extraction buffer (100 mM Tris-HCl, pH: 7, 14 mM 2-mercaptoethanol, and 3% Polyvinylpolypyrrolidone (PVPP)). The homogenate was centrifuged (UNIVERSAL 320 R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 10,000× g for 30 min at 4 °C and the supernatant was collected. The enzyme reaction mixture contained 1 mL of 100 mM Tris-HCl, pH: 6, 200 µL of 20 mM L-phenylalanine, and 200 µL of enzyme extract. The reaction was carried out at 30 °C for 30 min and stopped by addition of 100 µL of 6 N HCl [6]. The PAL activity was estimated by measuring *trans*-cinnamic acid synthesized at 290 nm using an extinction coefficient of 16.8901 L/mol/cm [13]. The PAL specific activity was expressed in µmole of *trans*-cinnamic acid/mg of protein/h/mg of fresh weight (FW).

2.5. Extraction and quantification of total phenolics contents

The leaves powder was weighed and extracted in 2 mL of acetone 80%. Samples were incubated in an ultrasonic bath for 30 min at 4 °C. The extracts were then centrifuged at 10,000× g for 15 min at 4 °C. Two hundred microliters of the acetonic extract was added to 250 µL of the Folin–Ciocalteu reagent (1/3) and 500 µL of sodium carbonate (20%). The samples were kept at 40 °C for 30 min [14]. The Folin–Ciocalteu (FC) assay shows interference from many oxidizable compounds such as tyrosine, tryptophan, ascorbic acid, and urea, and even diethyl ether [15]. To avoid the interaction of possible non-phenolic substances in the calorimetric estimation, another assay was performed under the same conditions by adding 3% of PVPP to the phenolic extract. After stirring and centrifugation, the obtained supernatant was treated according to the same procedure described above. The absorbance was measured at 760 nm (Jenway 6305 Spectrophotometer, Jenway, Dunmow, Essex, UK). The difference between the first (without PVPP) and the second measurement (with PVPP) was calculated to determine the amount of total phenolic compounds (gallic acid equivalent µg/g of FW).

2.6. Protein activity assays

Total soluble protein was measured by the Bradford method, using bovine serum albumin (BSA) as the standard [16]. One hundred microliters of each sample were added to 2 mL of diluted Bio-Rad protein assay reagent (1/5). After 10 min incubation in the dark at room temperature min, the absorbance was measured at 595 nm.

2.7. Data analysis

The data for the comparison of the PAL activity and the accumulation of phenolic compounds in the leaves of tomato seedlings between treated and control plants in each period of elicitation were analyzed using ANOVA test in SPSS version 25.0 software (IBM SPSS Statistics for Windows, Armonk, NY). The results were presented as mean value \pm standard error (SE) of four replicates with two tomato seedlings per replicate. Statistical differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Structural and chemical characterization of the alginate extracted from *Bifurcaria bifurcata* and their oligomers derivatives

Alginate was extracted from the brown seaweed *Bifurcaria bifurcata* from the Moroccan coast. The polysaccharides were extracted through a series of steps involving acid and alkali heat treatment and purified by rinsing several times with ethanol, and then analyzed chemically and structurally [11,12]. Oligoalginates were obtained after radical depolymerization of the alginate by aqueous hydrogen peroxide (H_2O_2) solution for 6 h at 70 °C. The hydrolysis process was described in detail in the previous study [11]. Extraction and purification techniques were potentially optimized to achieve higher yield of target compound. As described in the previous study [12], the yield of alginate was about 24% (w/w), and the chemical composition was revealed as 48.6% of total sugars, with the abundance of uronic acids (58.4%), and the presence of minor impurities (phenolic compounds, proteins, and sulfate groups). Total carbohydrates were determined by the Dubois method [17]. In general, two colorimetric methods can be used, the Dubois and the anthrone assays [18]. Both methods give different results depending on the standard sugars used and the composition of carbohydrates in the biological samples. These colorimetric assays remain problematic and could involve an over or underestimation of carbohydrate content, because of the presence of various biochemical compounds that can have a possible interaction in the quantification [19]. Extracted alginate showed substantial quantitative yields as well as an appropriate composition, to make it a good product for biological activities. Widely, the alginate yield of studied brown seaweed genus, such as *Sargassum*, *Turbinaria* [20], *Cystoseira* [21,22], *Laminaria* [23] is largely in the range of 20–50% (w/w). The reported alginate yield and chemical properties depend greatly on the algal species and its polysaccharide content, harvesting conditions, and extraction methods [24].

Naturally, algae live in a very changing environment, which leads to morphological, content, and composition diversity. Several studies have already shown temporal and spatial variability in the content and composition of algae compounds when exposed to various environmental factors, such as nutrients, temperature, salinity, or light [25]. Furthermore, the season of seaweed cultivation also plays an important role in their structural and biochemical composition [26].

In order to predict the different mannuronic and guluronic acid residues ratio (M/G ratio) of intact alginate powder and to determine the block structure, ¹H-NMR spectroscopy was performed. The molecular weight was determined by the High-Performance Steric Exclusion Chromatography HPSEC. Alginate from brown seaweed *Bifurcaria bifurcata* was characterized by a weight average molecular weight (M_w) of 22.5×10^4 g/mol, and 5500 g/mol for the produced oligoalginates. The estimated M/G ratio from ¹H-NMR spectra shows higher values of guluronic acid (68%) than mannuronic acid blocks (32%, M/G < 1), indicating that the samples are rich in guluronic acid. All structural and chemical analyses of alginate were presented in the previous study with a detailed description [12]. The determination of the composition of alginates and their physical properties is an important criterion for knowing the gelling properties in order to select the appropriate concentrations for biological tests. In general, alginates with an M/G ratio (<1) form strong and brittle gels, making them suitable for biomedical or environmental applications, whereas alginates with M/G ratio (>1) produce an elastic gel that can be used for many industrial applications [27]. In this work, the concentration used for elicitation was 0.3% (w/v), which means that the flow behavior of the sodium alginate has a Newtonian effect. This avoids several irregular modifications of the functions and cellular mechanisms due to the high viscosity of the solution. Previous studies have emphasized that lower concentrations (<2% w/v) of alginates in aqueous solution represent Newtonian flow while, higher concentrations (>2% w/v) improve viscosity and shear thinning behavior [28-31]. The bioelicitor concentration of 3 g/L was chosen according to the previous study, which was carried out under the same conditions on tomato seedlings [6].

3.2. Polysaccharides from *Bifurcaria bifurcata* and derived oligosaccharides induce defense responses in tomato seedlings

3.2.1. Effect of alginate extracted from *Bifurcaria bifurcata* and oligoalginates derivatives on the PAL activity

The induction of the phenylpropanoid defense pathway in the leaves located above the elicitation area of the tomato seedlings in response to alginates, and oligoalginates treatments was evaluated by monitoring the PAL activity and the phenolic compounds biosynthesis over 5 days. The phenylpropanoid mechanisms play an important role in the signaling pathways of plant defense in response to phytopathogens attack as well as bioelicitor application [32]. This pathway is activated by the formation of cinnamic acid via the action of phenylalanine ammonia-lyase, the key enzyme in the defense system that initiates both primary (shikimate pathway) and secondary (phenylpropanoid) metabolisms [33].

The application of alginate and oligoalginates derivatives (3 g/L), by internodal infiltration in the tomato seedlings induced significantly the PAL activity in the leaves ($p < 0.05$). The elicitation response depended on the treatment duration (**Figure II. 1. 2**). The PAL activity increased substantially in the leaves for each of the alginate and their oligomers ($p < 0.05$) and remained at high levels throughout the incubation period. In the response to alginate treatment, levels rose steadily from 12 h of elicitation, reaching a peak value 2-fold higher compared to control (water) after 72 h of treatment, to be decreased slightly in remaining days. Besides, the elicitation by oligoalginates derivatives showed a similar pattern. As illustrated in **Figure II. 1. 2**, both alginate and oligoalginates exhibited similar effectiveness and efficiency to enhance PAL activity in the leaves of tomato seedlings.

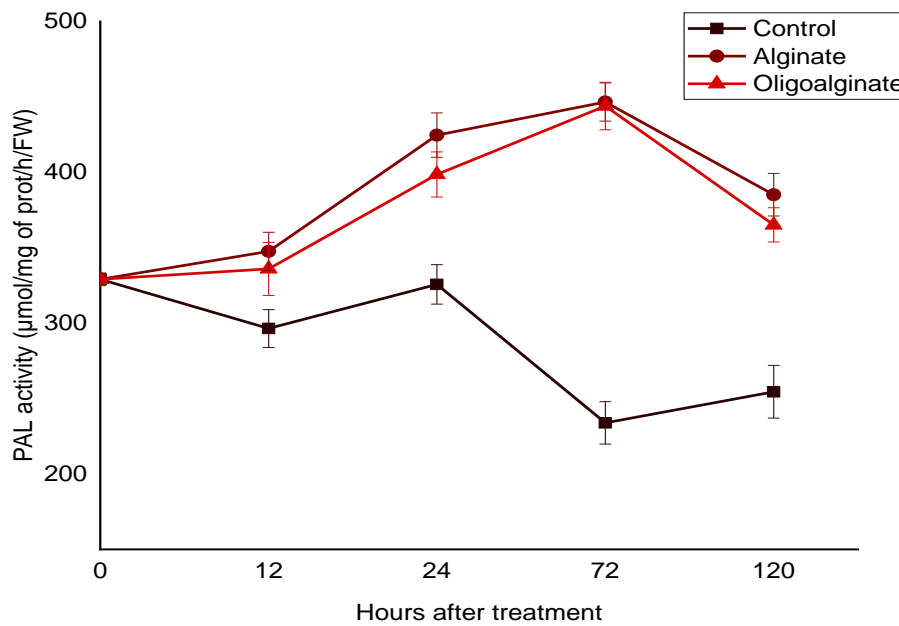


Figure II. 1. 2. Effects of alginate and oligoalginates derivatives on PAL activity in the leaves located above the elicitation site of tomato seedlings for 5 days. Each value is the mean of four repetitions \pm SE.

3.2.2. Effect of alginate extracted from *Bifurcaria bifurcata* and oligoalginates derivatives on the phenolic compounds content

The phenolic compounds synthesis in the leaves of tomato seedlings was significantly promoted ($p < 0.05$), in response to treatment with alginate and oligoalginates derivatives (3 g/L). As illustrated in **Figure II. 1. 3**, the polyphenol content was enhanced by both bioelicitor applications compared to the control (water). The elicitation response depended on the saccharide elicitor and the duration of treatment. For each of alginate and their oligosaccharides derivatives, the accumulation of these metabolites in the leaves showed similar trends, it initially increased rapidly and strongly from 12 h of elicitation, representing peaks value after 24 h of application. This maximal level was 2.6 and 4 times higher as compared to untreated plants (control), for alginate and oligoalginates, respectively. Thereafter, levels suddenly decreased attaining a control value after 72 h of treatment to increase significantly in the remaining days. Compared to alginate, oligoalginates were more effective, they expressed an intense elicitor capacity in polyphenols synthesis, that was almost 1.5 times more important than that observed in response to alginate polymer after 24 h of treatment (**Figure II. 1. 3**).

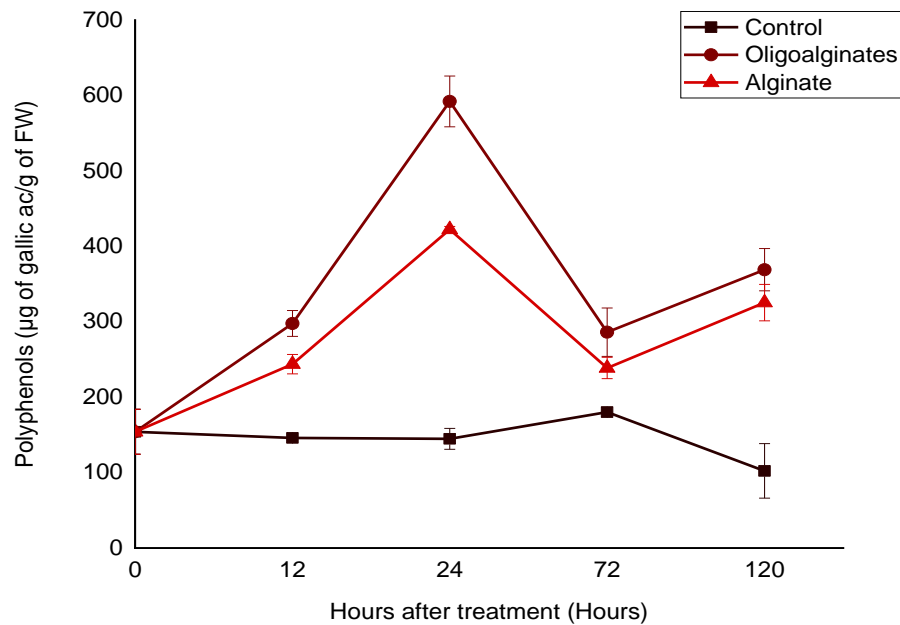


Figure II. 1. 3. Effects of alginate and oligoalginates derivatives on phenolic contents in the leaves located above the elicitation site of tomato seedlings for 5 days. Each value is the mean of four repetitions \pm SE.

These studies revealed clearly, the capacity of alginates, extracted from the brown seaweed *Bifurcaria bifurcata*, and oligoalginates derivatives to induce PAL activity (**Figure II. 1. 2**) and polyphenols biosynthesis (**Figure II. 1. 3**) in the leaves of tomato seedlings. Based on these results, and on previous study realized on date palms using the same alginates [12], we suggest that polysaccharides isolated from *Bifurcaria bifurcata* and their oligomers could be important elicitors to trigger the natural defenses of different plants enhancing protection against a wide range of phytopathogens. Additional studies are therefore required for exploring the protective effects of these molecules. As far as we know, very limited research has examined the stimulator activities of alginate and oligoalginates purified from brown algae. However, other polysaccharides extracted from green and red algae and their oligomers have been extensively studied for their potential to enhance a wide range of defense reactions in different plants, such as ulvan, the sulphated polysaccharide from green seaweeds [6,7,34,35], carrageenan from red seaweed [9,36-38], laminarin and fucoidan from brown seaweed [39-43].

Alginate and oligoalginates derivatives expressed an important and persistent elicitor effect of PAL activity (**Figure II. 1. 2**). This was supplemented by a strong accumulation of phenolic compounds in the leaves of tomato seedlings, in the response of both saccharide bioelicitors. However, oligosaccharides presented an effective elicitor capacity compared to alginate



polymers (**Figure II. 1. 3**). Similar contributions were observed in a recent study, carried out on tomato plants after application of ulvan, oligoulvans, glucuronan, and oligoglucuronans from green seaweed [6]. Treatment with these saccharide elicitors increased significantly the PAL activity as well as the content of phenolic compound in the leaves above and below the elicitation site, this response was systemic and seemed to be related to the salicylic acid pathway [6]. The application of these saccharide molecules reduced significantly wilt development caused by *Fusarium oxysporum* f. sp. *lycopersici*, particularly the oligoulvans which strongly reduced wilting and mortality of tomato plants, by 44% and 54%, respectively, compared to the control plants [6]. The results presented by Abouraïcha et al. reported that the PAL activity and phenolics content have been greatly enhanced in the apple fruit after elicitation by ulvan and oligoulvans, indicating the effectiveness of these saccharides in protection against blue and grey molds [7]. The implication of the PAL activity and polyphenols on plant resistance after seaweed polysaccharides treatment have been largely reported [7,8,44,45].

On a worldwide scale, tomato constitutes an essential vegetable fresh crop. In spite of its economic importance, bacterial and fungal diseases and pests became the major common constraints accounting for poor tomato production [46]. Bacterial disease caused by *Xanthomonas campestris* pv. *vesicatoria*, early blight (*Alternaria linariae*), late blight (*Phytophthora infestans*), and powdery mildew (*Golovinomyces lycopersici*) are considered the most disastrous problems of tomato [47,48]. For instance, several reports have shown that the application of crude or purified algal preparations protect against a large range of pathogens in tomato seedlings [46,49,50].

The results revealed a similar elicitor capacity in PAL activity whereas the accumulation of phenolic compounds showed a differential effect in the response of alginate and oligosaccharides derivatives. PAL is one of the most thoroughly studied enzymes, being the first committed enzyme that controls the interface between phenylalanine and the secondary phenylpropanoid metabolism in response to biotic and abiotic stress in plants [51]. After the first reaction, which consists of catalyzing the phenylalanine in the presence of PAL enzymes, polyphenols are produced by three different biogenetic routes, the shikimate, malonate, and mevalonate pathways. The shikimate pathway is the most important in the biosynthesis of the majority of plant phenolics which comprise a diverse group of molecules including flavonoids (flavones, anthocyanidins), stilbenes, tannins, lignans, and lignin [51]. These compounds play an important role in structural and defense-related functions. A recent study has revealed that



high levels of these metabolites at the site of pathogen invasion can restrict or slow the growth of the pathogen [7]. The shikimic acid pathway consists of many sequential enzymatic steps, each reaction produces different metabolites that play an important role as branch point compounds serving as factors that induce the synthesis of various phenolic compounds in the plant [52]. Seven enzymes could intervene in this pathway for the development of the majority of secondary metabolites depending on the elicitor molecules [51-53]. From this, it can be suggested that the difference in the expression of phenolic compounds in the leaves of tomato seedlings between alginates and oligoalginates could be explained by the distinct target of the enzymatic reactions stimulated by each elicitor in the shikimate pathway. The presence of phenolic compounds synthesized with polyphenol oxidase and peroxidase enzymes in plants may also influence the content of these metabolites.

Additionally, the differential elicitor responses between alginates and their oligomers might be associated to the saccharide chain length as well as the structure of both oligosaccharides and polysaccharides, which leads to multiple variations in cell surface interaction which means widely varying receptors that are important for recognition, regulation, and signal transduction. In this work, the oligoalginates derivatives were characterized by $2 \leq DP$ (degree of polymerization) ≤ 24 . A series of recent studies have indicated that seaweed-derived oligosaccharides with a low degree of polymerization (DP) strongly stimulate plant defense mechanisms compared to polysaccharides. Tomato seedlings and apple fruit treated with oligoulvans and oligoglucuronans, obtained after enzymatic depolymerization from ulvan and glucuronan, respectively, revealed a strong induction of PAL activity and other defense-related enzymes and metabolites, that were more effective than polysaccharides [6,7,44]. In previous studies, it was suggested, that oligoalginates enriched polymannuronic acid (Poly-M) fraction, obtained after chemical depolymerization, induced a sustained increase in PAL activity in wheat and tobacco leaves, while, polyguluronic acid (Poly-G) showed low elicitor activity, mentioning that only Poly-M activate defense responses in plants probably through the salicylic acid (SA) signaling pathway [54,55]. However, other studies reported that some metabolites could be induced by high DP instead of low DP [39], although all of them could act as elicitors. It has been demonstrated, in tobacco, that although two different oligosaccharides have similar DP, defense responses varied from each other [56]. Compared with curdlan oligosaccharides with low DP, laminarin showed a durable effect on plant defense mechanisms, whereas oligomers took an active part in early defense reactions [56]. Chitin is a polymer of *N*-acetyl-



d-glucosamine, found in the cell walls of fungi. Barber et al. reported that the monomer and dimer of this molecule were inactive, while the tetramer, pentamer, and hexamer significantly induced lignin synthesis in wheat leaves [57]. On the other hand, the smaller chitooligosaccharides (COSs) with DP between 3 and 6 showed lower elicitor activity. In contrast, pentameric and hexameric COSs ($DP \geq 20$) effectively stimulated cellular responses in rice [58]. In addition, the production methods of oligosaccharides from native polymers, including chemical hydrolysis or enzymatic hydrolysis, could influence the elicitor behavior of these molecules. A previous study conducted on animal cells showed that enzymatically depolymerized alginate oligomers (unsaturated guluronate and mannuronate oligomers) were highly active triggers for cytokine production, while saturated guluronate and mannuronate oligomers produced by acid hydrolysis were less effective. This suggests that the unsaturated terminal structure with double bond is important for the bioactivity of alginate oligomers, regardless of molecular size or structure [59]. This hypothesis has yet to be proven on plant cells.

The difference between elicitors effectiveness could also depend on the specificity of the receptors in the cell wall towards these molecules, which activate the defense signaling pathways in different ways. Plants have a perceptual system that is responsible for regulating the defense against foreign molecules, called elicitors, which can be from pathogens (fungi, bacteria, viruses, insects, etc.), animals, plants, or seaweeds [60]. These general elicitors are designated as PAMPs (pathogen-associated molecular patterns) when isolated from pathogenic agents, MAMPs (Microbe-Associated Molecular Patterns) from non-pathogenic microorganisms and chemical compounds or DAMPs (Damage-Associated Molecular Patterns) corresponding to endogenous signals derived from the plant host resulting from the action of pathogens [61]. They are recognized by surface-based receptors called PRRs (pattern recognition receptors) or by specific intracellular receptors that recognize the specific pathogenic effector molecules [62,63]. PRR surface receptors are known as plasma membrane resident proteins, including receptor-like kinases (RLK), receptor-like proteins (RLP), and extracellular binding proteins. In contrast, intracellular receptors, known as NB-LRR, constitute the nucleotide-binding site (NB) and leucine-rich repeat (LRR) [64].

Regarding the recognition of poly-oligosaccharides by plants, it has been shown that ^{14}C -labeled mycolaminarin isolated from *Phytophthora* sp. presents a high-affinity binding site in membrane preparations from soybean roots [65]. Other studies have demonstrated the presence

of a single class of specific binding receptors with high affinity for β -glucan oligosaccharide elicitor in the cell membrane preparations from the root and other parts of the soybean cotyledon [66]. A radio-labeled chitin oligosaccharide elicitor also showed a high-affinity binding site in the plasma membrane of rice [67], tomato cells [68], and soybean root [69]. Lectins and Lectin Receptor-Like Kinases seem to have an important role in signaling and perception cascades activating defense mechanisms in response to saccharide elicitors. It has been identified as carbohydrate-binding proteins [70]. Therefore, it is essential to understand the interaction between these elicitors and the host to develop durable resistance in plants and to find new approaches for disease control. For this, more specific studies are planned to investigate the mechanisms underlying the perception of polysaccharides and oligosaccharides derived from marine seaweed.

4. Conclusions

In this study, it appears likely that the alginates isolated from the brown seaweed *Bifurcaria bifurcata* and oligosaccharides derivatives possessed elicitor activities. They showed the capacity to induce phenylpropanoid metabolism by enhancing the phenylalanine ammonia-lyase (PAL) activity and phenolic compounds accumulation in the leaves of tomato seedlings. The oligosalginates presented an effective elicitor capacity compared to alginate polymers. Additional studies are planned to understand more completely this difference in the mechanisms of interaction between polysaccharides and oligosaccharides derived from marine seaweed and the plants upon the expression of marker genes and transcriptomic analysis. Further studies are also needed to ensure the protective effects of these molecules against phytopathogens. Therefore, these results obtained highlight molecules of marine algae likely to contribute to the resistance of plants to pathogens by reducing/replacing the use of synthetic pesticides.

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M.D.O.E.-H.; I.F.; S.A.; G.P. All authors have read and agreed to the published version of the manuscript.

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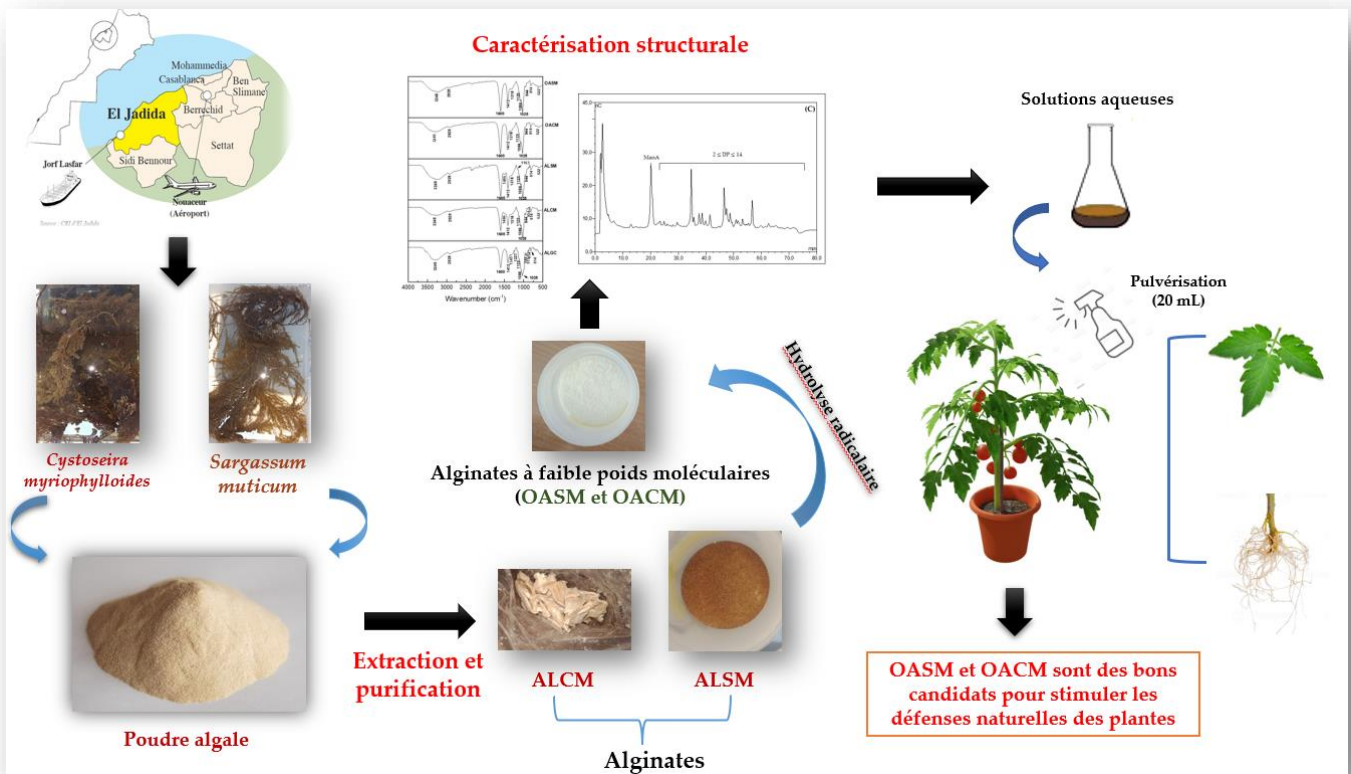
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Article 2 : Prochainement soumis

Elicitor activity of low molecular weight alginates obtained by oxidative degradation of alginates extracted from *Sargassum muticum* and *Cystoseira myriophylloides*

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Résumé graphique :



Elicitor activity of low molecular weight alginates obtained by oxidative degradation of alginates extracted from *Sargassum muticum* and *Cystoseira myriophylloides*

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Abstract

Alginates extracted from Moroccan brown seaweed and their derivatives were investigated for their ability to induce phenolic metabolism in the roots and leaves of tomato seedlings. Sodium alginates (ALSM and ALCM) were extracted from the brown seaweed *Sargassum muticum* and *Cystoseira myriophylloides*, respectively. The OASM and OACM fractions were obtained after radical hydrolysis of the alginates. Elicitation was carried out by foliar spray of 20 ml of aqueous solutions (1 g/L) to 45-day-old tomato seedlings. The elicitor capacities were evaluated by monitoring the phenylalanine ammonia-lyase (PAL) activity, polyphenols accumulation, and lignin content in the roots and the leaves, after 0, 12, 24, 48, and 72 h of treatment. The molecular weight (M_w) of different fractions was 202 kDa for ALSM, 76 kDa for ALCM, 19

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kDa for OACM, and 3 kDa for OASM. FTIR analysis revealed that the structure of OACM and OASM did not change after the oxidative degradation of native alginates. These molecules showed their differential capacity to induce the PAL activity, polyphenol accumulation, and lignin content in the leaves and roots of tomato seedlings. OASM and OACM fractions exhibited an effective induction of PAL activity compared to native alginate. These results suggest that the low molecular weight alginates could be considered good candidates to stimulate the natural defenses of plants.

Keywords: Alginates; Low M_w alginates; Elicitor; Seaweed; Tomato seedlings.

1. Introduction

Alginate is an anionic polymer isolated from the cell wall of brown algae. It is mainly composed of two conformational isomer residues, D-mannuronic acid (M) and L-guluronic acid (G), constituting homopolymeric (MM, GG) and heteropolymeric (MG, GM) sequential block structures [1]. Alginate has been widely used in food [2], pharmaceutical, biomedical [3], and industrial fields [4]. Recently, low molecular weight alginates, including oligomers, have gained much attention among researchers for their immunomodulatory [5], antimicrobial [6,7], antitumor [8], antioxidant [9,10], prebiotic [11], and antidiabetic activities [12]. LMW alginates are obtained by hydrolyzing alginate polymers using several methods, including physical, chemical, and enzymatic degradation [13]. Today, chemical methods such as acid, alkaline, and oxidative hydrolysis are the simplest, most effective, least expensive, and most reproducible on an industrial scale. Compared to acid degradation, radical depolymerization using H_2O_2 as an oxidizing agent is a green and eco-friendly processor producing high recovery low M_w alginates without generating toxic impurities as the only by-molecule is H_2O .

In agriculture, alginate and low M_w alginates have been recognized as fertilizers, soil conditioners, as well as inducers of natural defenses of plants [14-18]. The use of elicitors in crop protection is a new approach that could contribute to greater agricultural sustainability by minimizing chemical product applications and optimizing biocontrol strategies. Several studies have investigated some functional mechanisms of oligoalginates in plants [19,20]. Oligosaccharides involved in plant-pathogen interactions are originally produced by the degradation of cell wall polysaccharides of microorganisms or plants. The generated oligomers are then recognized as PAMPs (pathogen-associated molecular patterns), MAMPs (Microbe-Associated Molecular Patterns), or DAMPs (Damage-Associated Molecular Patterns) [21,22]. After signal transduction, a cascade of defense responses is activated. Systemic acquired

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resistance (SAR) is a distinct signal transduction pathway that plays an important role in plant resistance, activating enzymes responsible for secondary metabolite synthesis, leading to resistance to a wide range of phytopathogens [23].

In the present study, alginates isolated from *Sargassum muticum* and *Cystoseira myriophylloides* and low M_w alginates derivatives (OACM and OASM) were investigated for their ability to induce the phenolic metabolism in the leaves and roots of tomato seedlings. OASM and OACM were obtained after radical hydrolysis of alginate polymers.

2. Materials and Methods

2.1. Biological materials

Tomato Campbell 33 seeds were obtained from Technisem (Longué-Jumelles, France). Under greenhouse conditions, sterile seeds were germinated in peat substrate for 20 days and then transplanted into pots containing soil and peat (v/v) for 45 days.

The two brown algae, *S. muticum* and *C. myriophylloides* were collected from the intertidal zone of the station Sidi Bouzid, El Jadida, Morocco, in July 2019. The samples were washed to eliminate salts and impurities, oven-dried, and then milled to a fine powder.

2.2. Preparation and characterization of sodium alginates and their derivatives

As described previously, alginates (ALCM and ALSM) were extracted according to the Mazumber et al. method [24] with slight modification [25]. Briefly, the algal powder was treated with formaldehyde (2%), and then an aqueous solution of HCl (pH: 2-3; 60 °C; 3 h; 500 rpm) was added to the residue. The conversion of alginic acid to sodium alginate was carried out by an alkaline treatment (Na_2CO_3 ; 2-3%; 80 °C; 3 h; 500 rpm). The ALCM and ALSM were precipitated with ethanol (96°), purified using the Vivaflow 50R200 system, and then lyophilized.

The alginate polymers were hydrolyzed using the method previously described [25]. This method is based on the oxidation of ALCM and ALSM using hydrogen peroxide. Briefly, aqueous solutions of sodium alginates (1% w/v) were treated with H_2O_2 in a 1:1 (w/w) ratio (70 °C; 6 h; 300 rpm). After adding ethanol 96° (1/10 v/v), the samples were purified and freeze-dried to obtain the OACM and OASM fractions.

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The extracts were previously characterized by High-Performance Size-Exclusion Chromatography coupled with on-line Multi-angle Laser Light Scattering (HPSEC-MALLS), Gas Chromatography-Mass Spectrometry (GC-MS), and ^1H NMR [25]. Biochemical analyses were performed as well, to determine the total and neutral sugars, uronic acid, sulfate, protein, and polyphenol contents.

The distribution of DPs analysis was carried out by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). OACM and OASM were prepared at 10 g/l in Milli-Q water to be injected into the Dionex ICS-3000 system using a CarboPac PA-200 column (4 mm x 250 mm) fixed to a CarboPac PA-200 guard column (Dionex 4 mm x 50 mm). Mannuronic acid was used as a standard.

The FTIR analysis was carried out using a VERTEX 70 FTIR instrument. After 50 scans at room temperature, the spectra were collected in the range of 500-4000 cm^{-1} .

2.3. Elicitor treatment and biochemical analysis

Saccharide fractions were dissolved in distilled water at a final concentration of 1g/L, and the pH was adjusted to 6. The aqueous solutions were then applied by foliar spray of 20 mL to 45-day-old tomato seedlings. Control seedlings were treated with distilled water. After 0, 12, 24, 48, and 72 h, the upper leaves (n and n-1) and roots of four plants were collected and ground in liquid nitrogen for subsequent analysis.

Phenylalanine ammonia-lyase (PAL), phenolic compound, and lignin content were determined as described in the previous work [26]. PAL activity was estimated by measuring the absorbance of *trans*-cinnamic acid at 280 nm after 1 h of enzymatic reaction. Results were expressed in μmol of *trans*-cinnamic acid/mg protein/h/mg of dry weight (DW).

Proteins were estimated based on the Bradford assay, using bovine serum albumin (BSA) as a standard [27].

Total polyphenols were extracted, purified, and assayed using the Folin-Ciocalteu method. Findings were represented as μg of gallic acid/g of dry weight (DW).

Lignin contents were extracted and quantified by monitoring the absorbance of the formed thioglycolic acid-lignin complex at 280 nm. Results were given in A_{280} /g of dry weight (DW).

2.4. Statistical analysis

The data were analyzed using the ANOVA test in SPSS version 25.0 software. The findings were represented as a mean value \pm standard error (SE) of four replicates. At a p -value of less than 0.05, the differences were considered statistically significant.

3. Results and discussion

3.1. Structural analysis of the alginate extracted from *S. muticum* and *C. myriophylloides* and their derivatives

Alginates (ALCM and ALSM) were extracted and purified from the Moroccan brown seaweed (*S. muticum* and *C. myriophylloides*). The fractions of OACM and OASM were obtained after the oxidation of ALCM and OASM with H₂O₂, respectively, to prepare low molecular weight alginate. As described previously [25], the average molecular weight (M_w) of ALCM and ALSM was 76 kDa and 202 kDa, respectively. The OACM derivative was characterized by a M_w of 19 kDa, while OASM showed a low M_w of 3 kDa. The HPAEC-PAD analysis of both fractions confirmed that ALSM was depolymerized to oligoalginates (OASM) with a $2 \leq DP \leq 14$ (**Figure II. 2. 1C**), while ALCM was partially hydrolyzed, generating low molecular weight alginate (OACM) with a DP= 88 (**Figure II. 2. 1B**). The DPs were calculated based on the M_w of generated fractions and the M_w of repeated units of guluronic acid or mannuronic acid (216 g.mol⁻¹). To obtain low molecular weight alginate, various methods have been used, such as enzymatic depolymerization [10], acid hydrolysis, and the oxidation method [28]. During the enzymatic degradation of alginate by alginate lyases, unsaturated, non-reducing terminal residues are formed, which are not always desirable [10]. Besides, the main issue with this process is the high cost and long reaction time requirements. Acid hydrolysis using H₂SO₄ [29], and HCl [28] is a simple and easy method, but it involves acid impurities in final products, that are difficult to remove. Moreover, it generates a low yield of produced derivatives [28]. H₂O₂ oxidation became an alternative eco-friendly method for alginate degradation with a recovery rate achieving 87 % [28]. In this study, the yield of OASM and OACM fractions was about 82% and 81.26%, respectively. The rate and the M_w of derived fractions depended on alginate structure, reaction time, temperature, solution pH, and H₂O₂ concentration [30]. The hydrogen peroxide hydrolysis consists of a detachment of hydrogen atoms at all ring C-H bonds of polysaccharides. Hydroxyl radicals attack leads to the formation of carboxyl groups via 2 ways. The first is the oxidation of -OH at C6 to carbonyl groups and then to COOH, and the second is the breakdown of glycosidic bonds by the action of generated radicals at the C1 position,

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forming terminal carboxyl [31-33]. Several studies have used H₂O₂ to oxidize polysaccharides, including alginate [28,31,34], amylose[35], κ-carrageenan[36], chitosan [37], gellan gum [33], and cellulose [38]. In this study, despite its high M_w (202 kDa), ALSM was hydrolyzed faster than ALCM (76 kDa) under the same conditions. This could be explained by resistance to hydroxyl radical attack due to the presence of sulfates (7,29%) in ALCM (fucoidan contamination) [25]. Moseley et al. have reported that sulfated glycosaminoglycans (GAG) were less susceptible to degradation and chemical modification compared to non-sulfated GAG, suggesting that the presence of sulfate could protect molecules against ROS hydrolysis [39]. It has been reported that sulfate contents had an effect on the scavenging hydroxyl radical generated [40]. Further studies are needed to develop and confirm this hypothesis.

In order to analyze the changes in composition and structure of oxidized alginate, FTIR analysis was performed. The spectra were compared to unmodified alginates, including commercial alginate (ALGC), as shown in **Figure II. 2. 2**. The native alginates and their derivatives present characteristic bands around 1600, 1412, 1086, and 1025 cm⁻¹. Peaks at 3245 and 2929 cm⁻¹ are attributed to -OH and -CH stretching bonds, respectively. The two intense bands at 1600 and 1412 cm⁻¹ corresponded to asymmetric and symmetric stretching vibrations of carboxyl groups (COOH) of alginate, respectively [41]. Taking the peak at 1600 cm⁻¹ as the reference, the COOH groups did not change after the oxidation of native alginates (ALCM and ALSM) (**Figure II. 2. 2**). The weak bands at 1086 cm⁻¹ and strong peaks at 1025 cm⁻¹ may be assigned to C=O and C-C of guluronic and mannuronic units, respectively [41,42]. Signals at 944 cm⁻¹ are assigned to the C=O stretching vibration of uronic acids [42]. The characteristic bands of β-mannuronic acid residues are located at 814 cm⁻¹ [41]. When compared to ALGC, the positions of the characteristic bands of ALCM and ALSM are similar. No obvious differences were reported in the FTIR spectrum of alginates and their derivatives (OACM and OASM). These results indicate that the depolymerization of H₂O₂ with a ratio of 1:1 (w/w) under the conditions described above allows the breakdown of glycosidic bonds of alginate, keeping their main chain and structure unchanged. It has been reported that H₂O₂ treatment under extreme conditions changes the structure, with the appearance of a new band at 1730 cm⁻¹ indicating the introduction of carboxyl and carbonyl groups to the structure of polysaccharides. The peak became more intense with the increase in oxidation degree [31,33,36].

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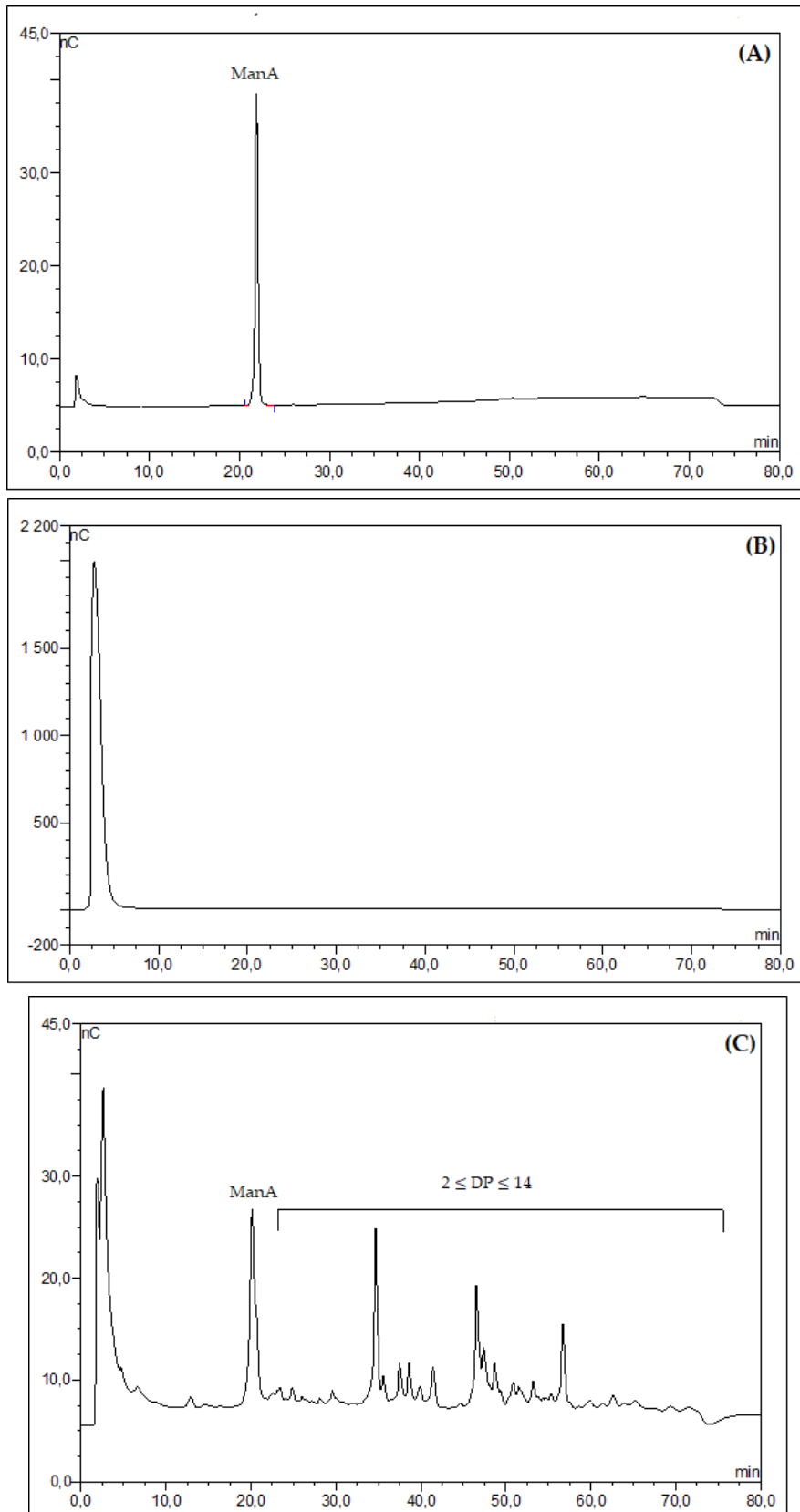


Figure II. 2. 1. HPAEC-PAD analysis of OACM (A) and OASM (B) fractions obtained after 6 h of radical hydrolysis of alginates (ALSM and ALCM) using H₂O₂; (C) HPAEC-PAD profile of mannuronic acid.

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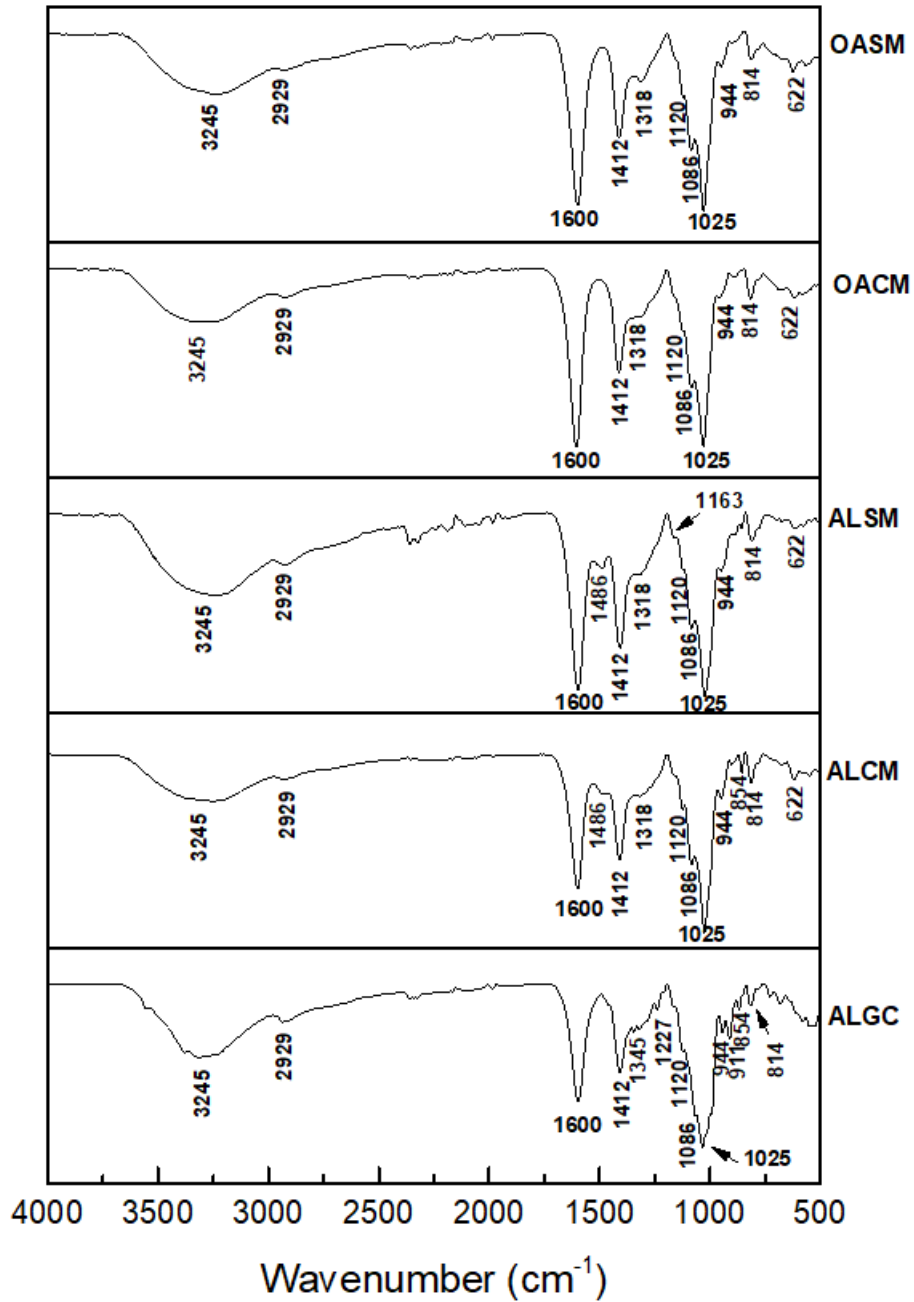


Figure II. 2. 2. FTIR spectrum of native alginates extracted from *C. myriophylloides* (ALCM) and *S. muticum* (ALSM), commercial alginate (ALGC), and oxidized fractions (OACM and OASM).

3.2. Effect of alginates and their derivatives on the phenolic metabolism in tomato seedlings

Alginates extracted from the brown seaweed *C. myriophylloides* (ALCM) and *S. muticum* (ALSM), and their derivatives were applied by foliar spray of 45-day-old tomato seedlings to evaluate their elicitor effects. The systemic responses were investigated by measuring the PAL

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activity, the phenolic compounds, and the lignin content in the roots and the upper leaves (n and n-1) after 0, 12, 24, 48, and 72 h. Control plants were treated with distilled water.

3.2.1. PAL activity

PAL activity was significantly induced in tomato roots and leaves after the treatment by alginates and their derivatives, depending on the incubation time ($p < 0.05$).

In comparison to native polymers (ALCM and ALSM), foliar spray application of hydrolyzed alginates (OACM and OASM) showed an earlier onset of PAL activity. Plants stimulated by OACM manifested an increase in PAL levels as early as 12 h in the leaves and 24 h in the roots and remained high throughout the incubation period (**Figure II. 2. 3**). In response to OASM, levels rose steadily from 12 h in the roots, reaching a peak value after 72 of elicitation. Additionally, the fall of PAL levels in roots was accompanied by their increase in leaves after 48 h (**Figure II. 2. 3**). The responses were expressed from 24 h in the roots of seedlings treated by ALSM, and from 48 h after the application of ALCM (**Figure II. 2. 3B**). Levels increased in leaves after 48 h of ALSM elicitation. PAL activity in leaves was not appeared to be affected by ALCM treatment (**Figure II. 2. 3A**).

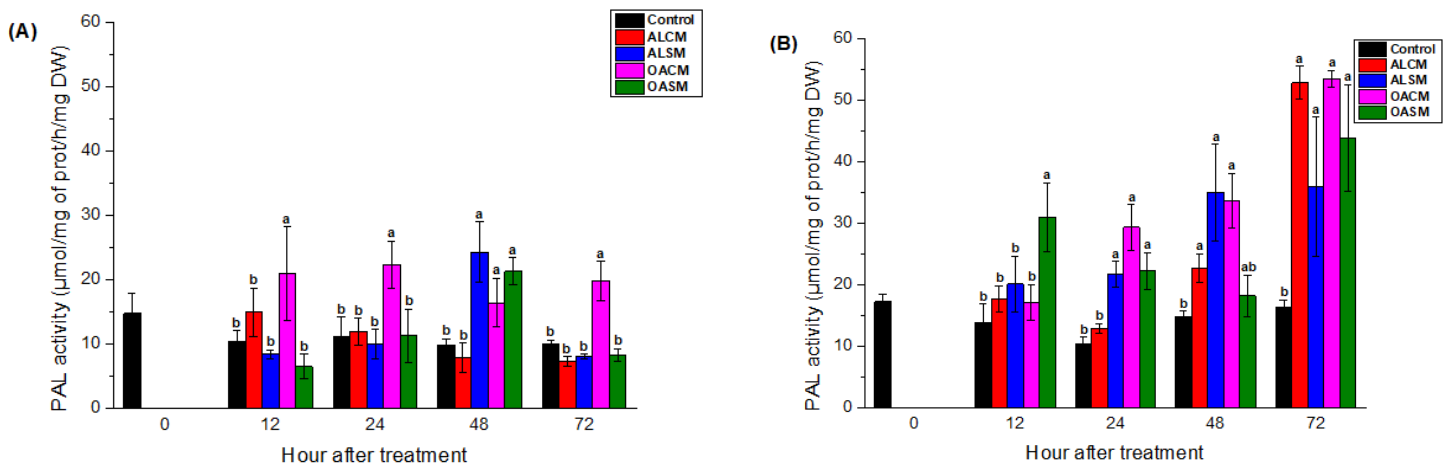


Figure II. 2. 3. Effect of alginates (ALCM and ALSM) and their derivatives (OACM and OASM) on the PAL activity in upper leaves (A) and roots (B) of tomato seedlings. Each value is the mean of four repetitions \pm SE. The letters (a, b) indicate significant differences between treatments at each point time.

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3.2.2. Phenolic compounds

The effect of alginates and their derivatives on the accumulation of phenolic compounds is presented in **Figure II. 2. 4**. The responses were dependent on the duration after elicitation ($p < 0.05$).

The foliar spray of ALSM, OACM, and OASM significantly induced polyphenol production in the leaves after 12 h (**Figure II. 2. 4A**). Levels declined sharply in the following days, except for plants treated with OACM, which showed again an upward trend after 72 h (**Figure II. 2. 4A**). In root tissues, ALSM and their oligomers followed similar patterns. Initially, levels rose from 24 h, then declined sharply after 48h, reaching control values. After 72 h, polyphenol content significantly increased to rise towards its peak (**Figure II. 2. 4B**). The effects of ALCM on the synthesis of these compounds in the leaves were not observed. However, they manifested elicitor activity after 12, 48, and 72 h in roots. The phenolic contents were promoted in the roots of tomato plants after 48 and 72 h of elicitation by OACM (**Figure II. 2. 4B**).

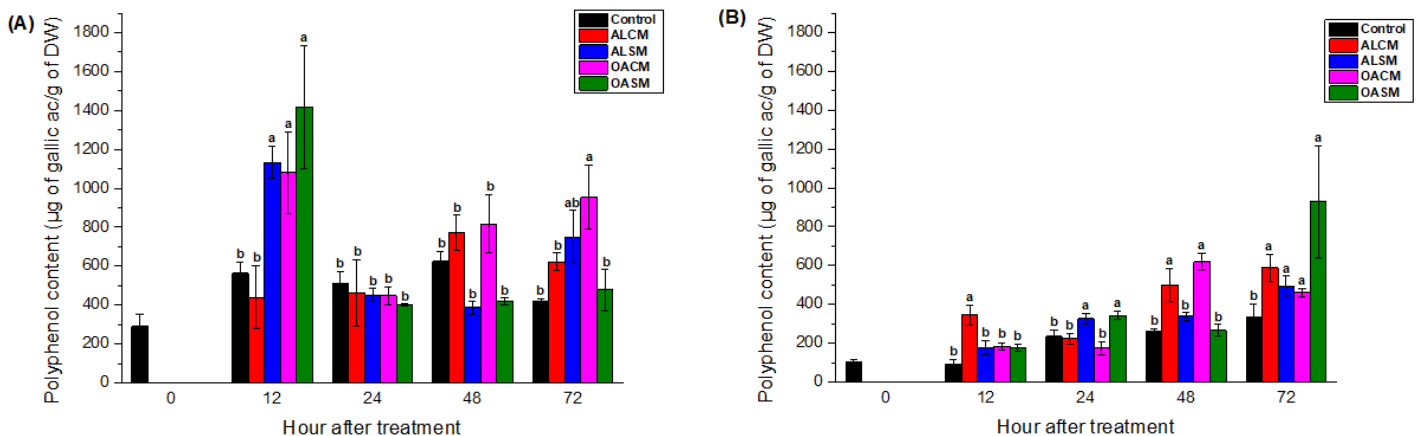


Figure II. 2. 4. Effect of alginates (ALCM and ALSM) and their derivatives (OACM and OASM) on the accumulation of polyphenols in upper leaves (A) and roots (B) of tomato seedlings. Each value is the mean of four repetitions \pm SE. The letters (a, b, c) indicate significant differences between treatments at each point time.

3.2.3. Lignin content

As illustrated in **Figure II. 2. 5**, all fractions showed their ability to enhance the synthesis of lignin content in root and leaf tissues of tomato seedlings, depending on the duration time ($p < 0.05$). The lignin contents were increased in the leaves 48 h after foliar treatment. This was

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accompanied by a decrease in the polyphenol contents (**Figure II. 2. 4A**), meaning that probably the phenolic compounds were used as precursors to lignin biosynthesis. It is known that lignin is formed via oxidative polymerization of monolignols (sinapyl alcohol, coniferyl alcohol, and *p*-coumaryl) coming from the phenylpropanoid pathway [43]. Other phenolic monomer such as hydroxycinnamaldehydes, Tricin, hydroxystilbenes have also been reported to form lignin subunits [44-46].

When compared to their native polymers, the alginate derivatives (OACM and OASM) exhibited an important production of lignin content in the roots and the leaves after 72 h (**Figure II. 2. 5**). After 12, 24, 48, and 72 h, respectively, the first elicitor activity of ALCM, ALSM, OACM, and OASM was detected in the roots (**Figure II. 2. 5B**). When compared to their derivatives, the native alginates showed an earlier production of lignin (**Figure II. 2. 5B**).

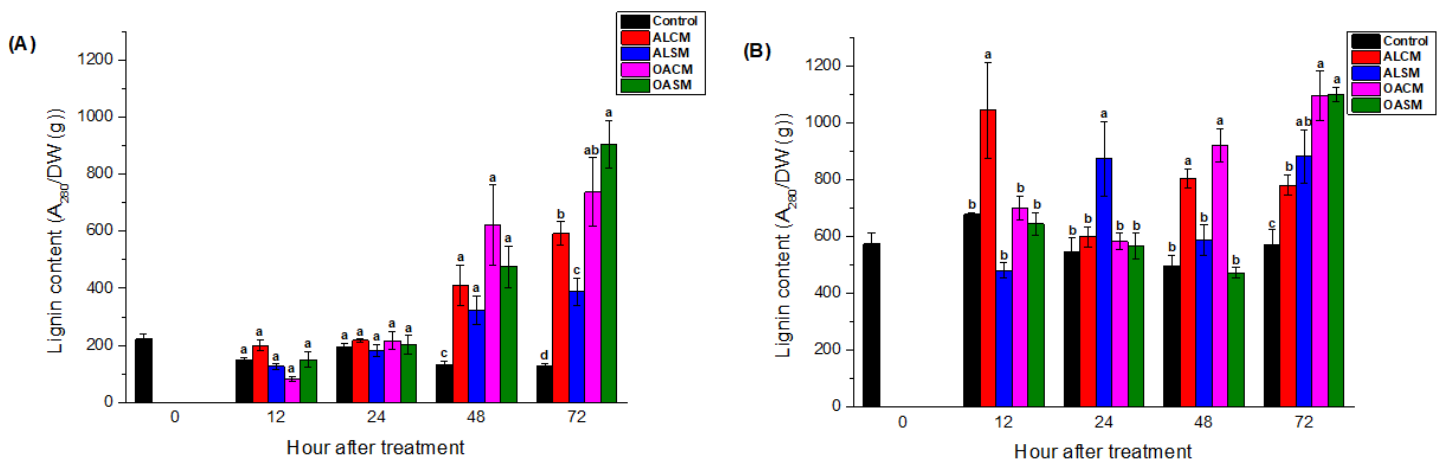


Figure II. 2. 5. Effect of alginates (ALCM and ALSM) and their derivatives (OACM and OASM) on the lignin content in upper leaves (**A**) and roots (**B**) of tomato seedlings. Each value is the mean of four repetitions \pm SE. The letters (a, b, c, d) indicate significant differences between treatments at each point time.

In this study, alginates and their derivatives triggered the PAL activity, phenolic compounds, and lignin production in the roots and leaves of tomato seedlings depending on the incubation time. The responses were systemic. PAL is one of the enzymes the most studied in plant defense responses. It initiates the primary (shikimate pathway) and secondary (phenolic pathway) metabolisms by catalyzing the conversion of L-phenylalanine to *trans*-cinnamic acid. This leads to the formation of the majority of phenylpropanoid compounds, including flavonoids, stilbenes, tannins, and complex polymers (lignin and suberin) via a series of methylation,

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dehydration, polymerization, and hydroxylation reactions [47,48]. These metabolites are then degraded by several oxidative enzymes such as polyphenol-oxidases (PPO), POD, LAC, and lipoxygenases (LOX) [48]. A significant increase in PAL, total phenolics, and lignin was observed in tomato seedlings treated with the lactic acid bacterium (LAB) isolated from rhizosphere soil, *Fusarium* mycelium extract, chitosan, *Fusarium* culture filtrate, *Trichoderma* mycelium extract, and salicylic acid (SA) [49-51]. Polysaccharides from algae have also been reported as inducers of natural defenses of apple fruits, date palm, tomato, olive leaves, and twigs [26,52-55]. Alginates derived from *S. muticum* and *C. myriophylloides* as well as their derivatives were found to be effective in inducing phenolic metabolism in tomato seedlings and olive leaves [25], indicating their broad-spectrum activity. A previous study has reported the ability of alginate isolated from the brown seaweed *Bifurcaria bifurcata* and *Fucus spiralis* to induce PAL and polyphenol production in the leaves of tomato plants and the roots of date palm [14,15]. Low molecular weight alginates have been widely used as antimicrobial, antioxidant, immunomodulatory, prebiotic, antihypertensive, antidiabetic, anticoagulant, and antitumor agents [56]. But their applications in agriculture as plant defense stimulators remain limited. In the present work, the oligomers OASM (3 kDa) and the low molecular weight alginate (OACM) were found to be more effective elicitors in inducing PAL activity than native polymers. Similar conclusions were reached after the treatment of tomato plants by oligoalginates with a M_w of 5 kDa [14]. OASM fractions have also shown their effectiveness to enhance PAL and TAL activities in olive leaves compared to alginate polymers [25]. Previous studies have reported the ability of oligoalginates to stimulate defenses against biotic and abiotic stresses, and growth in plants [57]. Oligoalginates produced by enzymatic degradation have reduced the effects of salt stress in *Brassica campestris* L. [17]. Wheat seedlings treated with exogenous alginate oligosaccharides (AOS) have improved resistance to drought stress during the growth period, by enhancing biomass, the content of chlorophyll, proline, and antioxidant enzyme activities [20]. The application of alginoligosaccharides obtained using alginate lyase from *Flavobacterium* sp. has shown the induction of PAL activity and an accumulation of phytoalexin in soybean cotyledon [58]. Furthermore, these oligosaccharides have shown their protective effects against bacteria, fungi, and viruses by activating the SA pathway and inducing the synthesis of secondary metabolites in various plants [57,59,60]. In addition, many other oligosaccharides were reported to induce plant defenses. Chitosan and carrageenan-derived oligosaccharides have revealed their effects in stimulating plant defenses against a wide range of pathogens including *Fusarium solani*, *Botrytis cinerea*, Tobacco Necrosis Virus (TNV), *Alternaria kikuchiana*, *Physalospora piricola*, *Sclerotinia sclerotiorum*, *Lophodermium* sp.,

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Pseudomonas syringae DC3000, Tobacco Mosaic Virus (TMV), and *Peptobacterium carotovorum* [57]. oligo-galacturonides (OGAs) and oligo-xyloglucans (OXGLs) derived from plant cell walls have shown to be able to elicit defense responses mainly the accumulation of ROS, the activation of mitogen-activated protein kinases (MAPKs), and pathogenesis-related proteins (PR) as well as the callose deposition leading to protect plants against pathogen infections [61,62]. The elicitor activities are depended on several factors including the degree of polymerization (DP), conformation, branching characteristics, features of glycosidic linkages, monosaccharide composition, functional groups, and the specificity of receptors [63,64]. This led to activating plant defense mechanism in different ways. Plants surface receptors (PRRs; Pattern Recognition Receptor) are able to recognize carbohydrates (poly/oligosaccharides) as MAMPs or PAMPs, such as chitin, peptidoglycan (PGN), glucans, and trigger an immune defense called PAMP-triggered immunity (PTI) [62,63]. Important biological activities are mediated by the interaction of saccharide elicitors and their associated protein. The specific type of proteins binding with specific carbohydrates are classified as lectins which play an important role as PRRs [63]. Plant PRRs are receptor-like kinases (RLKs), or receptor-like proteins (RLPs) associated with a ligand binding ectodomain (ECD) and a single-pass transmembrane domain. The ECDs contain leucine-rich repeats, lysine motifs (LysMs), lectin motifs, or epidermal growth factor (EGF)-like domains. LysM-RLPs/RLKs are also known to recognize the carbohydrate ligands. For example, chitin elicitor receptor kinase 1 (CERK1) is reported as a co-receptor induced by several carbohydrate elicitors [63]. On the other side, the wall-associated kinases, or WAKs, are receptors for pectin and OGAs, activated during pathogen responses [65]. However, WAKs have been recently reported to bind with alginates in the presence of calcium and ionic conditions for the formation of Ca^{2+} bridges between oligo-and polymers [66]. This conformation known as “egg boxes” is necessary for the perception system of plants [67]. Nevertheless, the perception of polysaccharides and oligosaccharides by plants is extremely complex, and more research is required.

A complex of oligochitosans and oligopectates has successfully been released into the market for crop protection under the brand FytoSave[®]. This product has shown protective effects against downy mildew of grapes and cucumbers by reducing the severity of the disease [68]. Stemicol[®] is another commercial phytovaccine composed of chito-oligosaccharide as the active substance. It is characterized by a M_w of less than 3 kDa and a DP of 5 to 18 monomers [69]. As a result, oligoalginates and low molecular weight alginates could be an efficient defense-

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inducing agents that are readily recognized by plant receptors, leading to rapid signal transduction.

4. Conclusion

In this study, alginates were extracted from two Moroccan brown seaweed *S. muticum*, and *C. myriophylloides*. Low M_w alginates were obtained by oxidative hydrolysis. The M_w of different fractions ranged from 202 to 3 kDa. The FTIR analysis confirmed that the structure of oxidized alginates remained unchanged, indicating that the depolymerization using H_2O_2 with a ratio of 1:1 (w/w) seems to be an adequate method for alginate modification. The application of the alginates and their derivatives showed their capacity to induce PAL activity, phenolic compounds, and lignin content in the leaves and roots of tomato seedlings. Low M_w alginates exhibited an effective induction of PAL activity compared to their native polymers, supplemented by a strong accumulation of secondary metabolites in both roots and leaves. In light of the foregoing, these findings underline the importance of oligoalginates and low M_w alginates as potential inducers of natural defenses of plants that may be used in crop protection for green and sustainable agriculture.

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CHAPITRE III

CARACTÉRISATION ET ÉVALUATION DE L'EFFET
ÉLICITEUR DES ARABINOGALACTANES
(POLYSACCHARIDES SULFATÉS) EXTRAITS D'ALGUE
VERTE MAROCAINE *CODIUM DECORTICATUM*, SUR LES
PLANTS DE TOMATE



Article 3 : Accepté en 2022

Meriem Aitouguinane, Zainab El Alaoui-Talibi, Halima Rchid, Imen Fendri, Slim Abdelkafi, Mohamed Didi Ould El-Hadj, Zakaria Boual, Pascal Dubessay, Philippe Michaud, Didier Le Cerf, Christophe Rihouey, Guillaume Pierre, Cédric Delattre, and Cherkaoui El Modafar. A novel sulfated glycoprotein elicitor extracted from the moroccan green seaweed *Codium decorticum* induces natural defenses in tomato. **Appl. Sci.** **2022**, 12(7):3643, doi: 10.3390/app12073643. **IF (2.838)**.

Contexte scientifique

En plus des polysaccharides anioniques, la paroi cellulaire des algues marines est riche en polysaccharides sulfatés (SPs). Ils sont trouvés principalement sous forme de fucoïdanes chez la famille des Phaeophyceae, de carraghénane chez la famille des Rhodophyceae et d'ulvane chez la famille des Chlorophyceae. Ces SPs sont en majorité solubles dans l'eau et possèdent plusieurs propriétés biologiques notamment, anticoagulantes, antioxydantes, antilipidémiantes, antithrombotiques, antivirales, et immuno-inflammatoires [1-3]. Récemment, les SPs ont attiré l'attention des chercheurs pour leur activité stimulatrice des défenses naturelles des plantes [4-6].

Parmi les trois grands groupes de macroalgues, les algues vertes représentent une source importante d'homo- et d'hétéropolysaccharides sulfatés, cependant, seuls quelques taxons ont été étudiés en détail. La classe des Ulvophyceae regroupent une large gamme d'espèces qui contiennent des SPs structurellement différents concernant la composition en monosaccharides et la linéarité de leurs squelettes [7]. Ces SPs sont classés selon leurs structures chimiques en deux grands groupes, les SPs riches en acide uronique présents chez les Ulvales, et les SPs pauvre ou dépourvus d'acide uronique, trouvés chez les Bryopsidales [7]. Les parois cellulaires des Ulvales, comprennent deux composants polysaccharidiques majeurs, les ulvanes et la cellulose, et deux composants mineurs, le xyloglucane et le glucuronane. L'ulvane est la famille des polysaccharides sulfatés des algues vertes la plus étudiée en agriculture en tant qu'inducteur des mécanismes de résistance des plantes [8-11]. Après les Ulvales, les Bryopsidales sont classés comme le deuxième groupe d'algues riches en SPs. Leur paroi cellulaire est composée principalement de mannanes sulfatés, d'arabinanes sulfatés, de galactanes sulfatés et des arabinogalactanes riche en protéines (AGPs) [7,12]. *Codium* sp. est le genre de Bryopsidales le plus étudié pour sa richesse en SPs [7]. Plusieurs études ont rapporté diverses activités biologiques de ces SPs pour des applications pharmaceutiques [13]. Néanmoins, aucune recherche antérieure n'a évalué ces composés en agriculture, notamment en tant que SDP.

Dans le chapitre précédent, des polysaccharides non sulfatés (alginates) extrait d'algues brunes ont été testés pour leur pouvoir d'induire les défenses naturelles des plants de tomates. Dans cette deuxième partie des résultats, des polysaccharides sulfatés (arabinogalactanes) extraits d'une algue verte ont été caractérisés et étudiés dans le but de répondre à trois grandes questions :

Chapitre III

- ✓ Etant donné que les arabinogalactanes sulfatés n'ont jamais été testé pour leur potentiel éliciteur, pourraient-ils être capable de mobiliser les mécanismes de défenses des plants de tomate ? Pour répondre à cette question des arabinogalactanes sulfatés extraits de l'algue verte marocaine *Codium decorticatum* ont été caractérisés et évalués pour leur pouvoir d'induire les défenses naturelles des plants de tomate. Ceci permet en outre de valoriser cette biomasse algale inexploitable au niveau des côtes marocaines, surtout que les espèces du genre *Codium* sont des algues envahissantes dans les côtes marocaines et mondiales. Elles forment des efflorescences nuisibles sur les récifs favorisant l'augmentation de l'eutrophisation dans les milieux marins [13,14]. Les résultats obtenus dans cette étude sont présentés sous forme d'une publication acceptée en **2022** dans le journal « **Applied Sciences** » (**Impact factor : 2.838**). L'article est intitulé « **A novel sulfated glycoprotein elicitor extracted from the moroccan green seaweed *Codium decorticatum* induces natural defenses in tomato** ».
- ✓ L'efficacité des réponses de défense est-elle influencée par la structure de l'éliciteur, et plus précisément la présence des groupements sulfatés et des protéines ?
- ✓ Un mélange de polysaccharides sulfatés et non sulfatés pourrait-il améliorer l'efficacité de la réaction en exerçant un effet synergique ?

Pour répondre à ces deux dernières questions des résultats complémentaires sont apportés à la fin de ce chapitre.

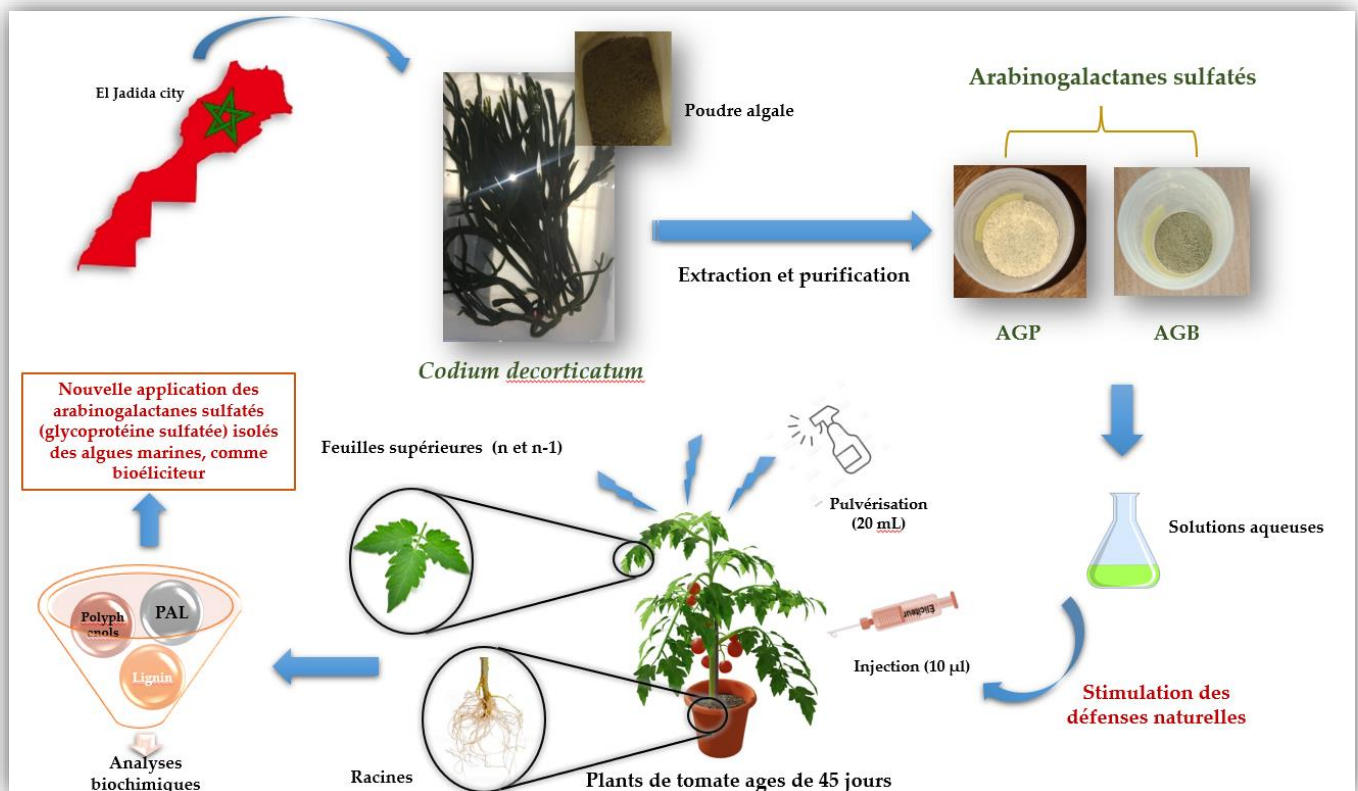
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A Novel Sulfated Glycoprotein Elicitor Extracted from the Moroccan Green Seaweed *Codium decorticum* Induces Natural Defenses in Tomato

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Résumé graphique :



A Novel Sulfated Glycoprotein Elicitor Extracted from the Moroccan Green Seaweed *Codium decorticum* Induces Natural Defenses in Tomato

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Abstract

Sulfated glycoproteins extracted for the first time from the Moroccan green seaweed *Codium decorticum* were investigated for their ability to induce a natural defense metabolism in the roots and the upper leaves of tomato seedlings. The crude (AGB) and the purified fractions (AGP) were characterized chemically (Colorimetric assays) and structurally (SEC-MALS, GC-EI/MS, ATR-FTIR). The elicitor aqueous solutions (1 g/L) were applied by foliar spray and syringe infiltration into the internodal middle of 45-day-old tomato seedlings. Phenylalanine

ammonia-lyase (PAL) activity, polyphenols, and lignin contents were measured in the roots and the leaves after 0 h, 12, 24, 48, and 72 h of treatment. The AGB and AGP extracts contained 37.67% and 48.38% of the total carbohydrates, respectively, and were mainly composed of galactose, glucose, arabinose, and a minor amount of xylose and rhamnose. They were characterized by an important molecular weight (M_w) > of $2000 \times 10^3 \text{ g} \cdot \text{mol}^{-1}$ and a high degree of sulfation and protein (12–23% (w/w)), indicating that the extracted polysaccharides could be an arabinogalactan-rich protein present in the cell wall of the green seaweed *C. decorticum*. Both crude and purified fractions exhibited an elicitor effect by inducing the PAL activity, the accumulation of phenolic compounds and lignin contents in the roots and the leaves of tomato seedlings. These responses were systemic in both the methods used (injection and foliar spray) and were mobilized throughout tissues that are not directly treated (roots and/or leaves). Regarding the elicitor activities, AGB and AGP presented globally similar patterns, which revealed the importance of crude extracts in the stimulation of plant immunity. These results suggest the new application of sulfated glycoprotein isolated from green seaweed in agriculture as inducers of natural defenses of plants.

Keywords: Sulfated arabinogalactan-rich protein; Green seaweed; PAL activity; Polyphenols; Lignin; Elicitor; Tomato seedlings

1. Introduction

Macroalgae constitute an interesting marine biomass rich in diverse bioactive compounds, such as proteins (1–50% dry weight), lipids (0.3–5% dry weight), and carbohydrates (15–76% dry weight) [1]. More recently, marine polysaccharides present in the cell wall and the extracellular matrix of seaweed (alginate, agar, carrageenans, fucoidan, and laminaran) are the most extensively exploited components in cosmetics, nutraceutical, pharmacology, chemistry, and sustainable agriculture [2]. Polysaccharides derived from macroalgae are largely reported as bio-elicitors capable of inducing the natural defenses of plants to provide protection against a wide range of phytopathogens [3].

Sulfated carbohydrates (SPs) are most commonly used for diverse biological activities; for example, it has anticoagulant, antioxidant, antilipidemic, antithrombotic, antiviral, antihyperlipidemic, and immuno-inflammatory effects, as well as elicitor effects [4,5]. They are found predominantly as fucoidans, carrageenans, and ulvans in red, brown, and green algae [6]. The sulfated fucans, ulvans, and galactans are widely studied in crop protection [3,5,7,8].

The green algae contain a range of sulfated homo and heteropolysaccharides that are still rarely explored for their elicitor activities in agriculture. The ulvans (8 to 29% of the algal dry weight) are recognized by their high degree of sulfation [9]. These SPs are usually the most phycocolloid studied in various fields [9,10]. Recently, the SPs from the genus *Codium* were found to be an interesting bioactive compound, particularly used for medical purposes [11]. Currently, the *Codium* genus (125 species) is classified as the second group of algae rich in SPs, after the *Ulva* genus. *Codium fragile* ssp. *fragile* (formerly *Codium fragile* ssp. *Tomentosoides*) is the most invasive green algae in the world [12,13]. The cell walls of the *Codium* species are mainly composed of sulfated mannans, arabinans, galactans, and arabinogalactan-rich proteins (AGPs) [14-16].

AGPs, also known as arabinogalactan type II (AG type II), are cell wall glycoproteins belonging to the hydroxyproline-rich glycoproteins (HRGPs) family. They are found principally in plants, some microorganisms, and have recently been detected in the seagrass species *Zostera marina* L. [17-19]. Arabinogalactans from the green seaweed *Codium* seem to be almost identical to terrestrial plants [16]. They are structurally characterized by the presence of a β -(1,3) and/or β -(1,6)- linked D-galactopyranose chain attached to hydroxyproline (Hyp) residues [17,18]. The non-reducing ends often consist of a main chain of L-arabinofuranose, L-arabinopyranose, D-mannose, D-xylose, D-glucose, L-rhamnose, L-fucose, D-glucosamine, and D-glucuronic acid [17,19,20]. Concerning the *Codium* genus, the AGPs have been detected for the first time in *C. fragile*, which were composed mainly of linear (1 \rightarrow 4)- β -D-mannans, pyruvylated arabinogalactan sulfates (pAGS), and low amounts of HRGP [16]. A similar composition has been reported in the cell wall glycoproteins of *C. vermilara* [21]. Several studies have focused on the pharmaceutical applications of AGPs, but no previous research has investigated the use of these compounds in sustainable agriculture, especially as inducers of the natural defenses of plants. This approach is one of the new promising alternatives to chemical pesticides that focus on the use of natural products initiating the defense mechanisms leading to the stimulation of plant resistance against a wide range of phytopathogens.

Codium decorticutum (formerly *Codium elongatum*) is a dark green alga belonging to the family of Codiales, which grows on hard substrate in the lower mediolittoral zone. On the Moroccan coast, this species exhibits massive growth and large distribution. Despite their richness in bioactive compounds, they are not valorized locally [22]. As far as we know, the AGPs have never been isolated from *C. decorticutum* or used as a bio-elicitor of the natural

defenses of plants. Therefore, the main aim of this work is to determine the structure of the cell wall glycoprotein of the Moroccan green seaweed *C. decorticans* and to investigate, for the first time, their ability to induce natural defense mechanisms, through the measurement of the phenylalanine ammonia-lyase (PAL) activity, the accumulation of phenolic compounds, and the lignin contents in the roots and the leaves of tomato seedlings.

2. Materials and Methods

2.1. Collection of *C. decorticans* Samples

The seaweed samples were collected by hand, during July 2019, at low tide, from the intertidal zone of the Moroccan Atlantic coast in the south of El Jadida city (Sidi Bouzid station: Latitude 32° 15' to 33°15'; longitude 7°55' to 9°15') and placed into an adequate container before being transported to the laboratory. After being washed thoroughly with distilled water, the samples were oven-dried at 50 °C and then milled in fine powder.

2.2. Extraction and Purification of Sulfated Polysaccharides from *C. decorticans*

One hundred of the algal powder was initially treated with 96° ethanol at room temperature for 24 h under constant stirring to remove impurities (pigments, lipids, and terpenoids). The obtained residue was dried in the oven at 50 °C and soaked in 20 volumes (w/v) of distilled water for 48 h under stirring at room temperature. After centrifugation (7000 g, 20 min), the supernatant was concentrated and precipitated with 3 volumes of 96° ethanol and left overnight at −20 °C. The lyophilized fraction constitutes the crude polysaccharides (AGB). On the other side, a purified fraction (AGP) was obtained by ultrafiltration using Vivaflow 50R-200 with a membrane of 100 kDa MWCO. This system involves the desalting and removal of components with a molecular size of less than 100 kDa. The final solution was then freeze-dried for 72 h (**Figure III. 1**).

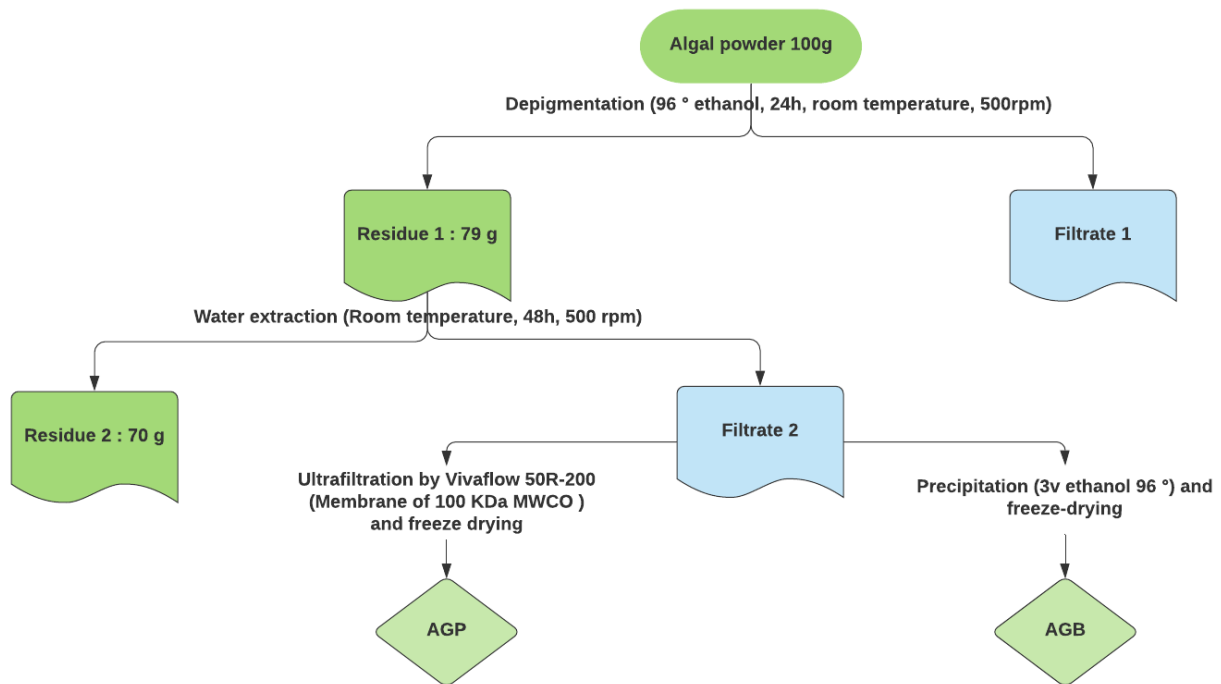


Figure III. 1. Process for extraction and purification of sulfated glycoproteins from *C. decorticum*.

2.3. Chemical Composition

Polysaccharide fractions were analyzed for their carbohydrates, proteins, sulfates, and polyphenol contents.

Total sugars were estimated by the Dubois assay using d-glucose as standard [23]. Neutral sugars were determined by the resorcinol sulfuric acid method and quantified according to the corrective formula described by Monsigny et al. [24]. The uronic acids were measured with m-hydroxydiphenyl (m-HDP) assay [25]. The D-galactose and D-glucuronic acid were used as standards for neutral and acid carbohydrates, respectively.

The sulfate content of the polysaccharides was determined by the BaCl₂/gelatin turbidimetric method after hydrolysis with HCl (2 N) at 100 °C for 2 h. The potassium sulfate was used as the standard [26].

Proteins were estimated according to the Lowry method using bovine serum albumin (BSA) as the standard [27]. The evaluation of polyphenol contents was carried out by the Folin-Ciocalteu assay, and the results were expressed as gallic acid equivalents [28].

2.4. HPSEC-MALLS Analysis

Samples were prepared (1 g/L) in 0.1 M LiNO₃ and then incubated at room temperature for 24 h under stirring. Solutions were filtrated through a 0.45 µm regenerated cellulose membrane filter and then analyzed for 120 min on a SEC-MALS-dRI-UV-280 nm system using an 804–806 column at 0.5 mL/min with an injection volume of 100 µL. The Intrinsic viscosity, the number average molecular weight (M_n), the weight average molecular weight (M_w), and the polydispersity index (\mathcal{D}) were estimated using $dn/dc = 0.150$ mL/g.

2.5. Monosaccharide Compositions Analysis by GC-EI/MS

Dry fractions AGP and AGB (10 g/L) were treated with 2 M trifluoroacetic acid (TFA) and heated at 120 °C for 90 min under agitation in a dry bath. The TFA was discarded under a nitrogen blowdown dry evaporator (60 °C), and then the hydrolysates were washed twice with methanol to remove excess acid. Dry samples were dissolved in a mixture of pyridine and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (v/v), mixed with a vortex, then incubated for 2 h under stirring at room temperature. After evaporation, the trimethylsilyl-O-glycosides were resuspended into dichloromethane to be injected into the GC-MS system. The standard monosaccharides were treated with the same conditions as before. Analysis was carried out on a GCMS-GP2020 NX instrument from Shimadzu, using an OPTIMA-1MS (30 m, 0.25 mm, 0.25 µm) column with a constant flow rate of 1.75 mL/min. The helium pressure was set at 7.8 psi, the injection volume at 1 µL, and the split ratio at 50: 1. The oven program was started at 100 °C for 3 min; afterward, the temperature was increased at 8 °C/min to 200 °C for 1 min and finally at 5 °C/min to 215 °C. The mass spectra were obtained by the electronic impact of 70 eV applying the following parameters: injector temperature 250 °C, ion source temperature 150 °C, and the target ion range m/z 40–800 [29]. The data analysis was performed using MestReNova 7.1. software (Mestrelab Research, Escondido, CA, USA), and the results were expressed in a molar ratio (%).

2.6. ATR-FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopies of the crude extract (AGB) and the purified fraction (AGP) of *C. decorticutum*, were analyzed with a VERTEX 70 FTIR instrument using an ATR A225 diamante. The IR spectra of dried polysaccharides were recorded after 50 scans at room temperature and referenced against the air in the wavenumber range of 500–4000 cm⁻¹. The data analysis was performed using OPUS 7.2 software (Bruker, Billerica, MA, USA).

2.7. Elicitor Applications

The sterile seeds of tomato (Var. Campbell 33) were germinated in individual pots containing peat substrate for three weeks in a growth room at 30 °C, with a photoperiod of 16/8 h (day/night). The young seedlings were then transplanted into pots containing a mixture of soil and peat (v/v) and grown under greenhouse conditions. The polysaccharide aqueous solutions (1 g/L, pH 6) were applied to 45-day-old tomato seedlings by foliar spray until run-off (20 mL) and syringe infiltration (10 µL) into the internodal middle of plants. The control plants were treated with distilled water. The roots and two apical leaves (n and n – 1) of four tomato seedlings were collected after 0, 12, 24, 48, and 72 h for each treatment group.

2.8. Phenylalanine Ammonia-Lyase (PAL) Activity and Protein Assays

The determination of PAL activity was carried out according to the Liu et al. method with slight modifications [30]. Briefly, the root and the leaf tissues of tomato seedlings previously ground were treated by a buffer solution containing 100 mM boric acid, pH 6, 1 mM of ethylenediaminetetraacetic acid (EDTA), and 1% of insoluble Polyvinylpyrrolidone (PVPP). After centrifugation (10,000 g, 30 min, 4 °C), 200 µL of enzyme extracts were added to 1 mL of boric acid (100 mM) and 200 µL of L-phenylalanine (20 mM). The solutions were incubated for 60 min at 30 °C and the reaction was stopped by adding HCl (6 N). The absorbance was read at 280 nm and the results were expressed in µmol of trans-cinnamic acid/mg proteins/h/mg of dry weight (DW) using an extinction coefficient of 9116 L·mol⁻¹ cm⁻¹ [31]. The protein assays were performed according to the Bradford method, using bovine serum albumin (BSA) as the standard [32].

2.9. Extraction and Purification of Total Phenolic Compounds

The polyphenols were extracted according to the method described by El Modafar et al. [33]. Briefly, samples were ground in liquid nitrogen in the presence of acetone 80°, the homogenates were then incubated in an ultrasonic bath for 30 min at 4 °C and centrifuged at 10,000 g for 15 min at 4 °C. The recovered supernatants were evaporated, then depigmented and delipidated by adding 1 mL of distilled water and ½ v of petroleum ether. The obtained aqueous phases were treated with 2% of metaphosphoric acid and ethyl acetate (v/v), and then the phenolic extracts were evaporated and dissolved in acetone 80°.

The estimation of total phenolic compounds was performed according to the Budini et al. method [34] with slight modifications. One hundred microliters of purified polyphenols were

added to Folin Ciocalteu reagent (1/3) and sodium carbonate (20%). Afterward, the solutions were incubated at 40 °C for 30 min. The absorbances were read at 760 nm, and the results were expressed in µg of gallic acid/g of dry weight (DW).

2.10. Extraction and Quantification of Lignin Content

Root and leaf tissues were macerated in the presence of acetone 80°, then the homogenates were centrifuged at 7000 g for 20 min. The recovered pellets were dried at room temperature and treated with HCl (2 M) and thioglycolic acid (1 M). The mixtures were heated at 100 °C for 8 h and centrifuged at 7000 g for 20 min at 4 °C. The supernatants were discarded, and the pellets were washed with distilled water to remove residual acids and then resuspended in NaOH (1 M). After incubation (20 °C, 18 h) and centrifugation (7000 g, 20 min, 4 °C), the supernatants were recuperated and treated with HCl (12 N). Afterward, the samples were left overnight at 4 °C in order to precipitate the thioglycolic acid-lignin complex and then dissolved in NaOH (1 M). The absorbances were measured at 280 nm, and the lignin contents were expressed in A_{280}/g of dry weight (DW) [35].

2.11. Statistical Analysis

The data analysis was performed using the ANOVA test in SPSS version 25.0 software, and the results were presented as mean value \pm Standard error (SE). Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. Extraction Yield and Chemical Composition of AGB and AGP Fractions

The AGB and AGP polysaccharides were extracted from the green seaweed *C. decorticans* from the Moroccan coast of El Jadida city. The extraction was carried out using water and then precipitated by ethanol to obtain the crude polysaccharides (AGB). For the purified fractions (AGP), ultrafiltration was performed using a cut-off membrane of 100 kDa in order to discard all particles with molecular weight < 100 kDa. As illustrated in **Table III. 1**, the extraction yield of AGB (11%) was higher than that obtained for AGP fractions (5%).

Table III. 1. Chemical analysis and extraction yields of AGB and AGP fractions from green seaweed *C. decorticutum*.

	AGB ²	AGP ³
Yields (% w/w)	11	5
Total sugars (% w/w)	37.67	48.38
Neutral sugars (% w/w)	37.56	45.68
Uronic acids (% w/w)	3.05	4.04
Sulfate groups (% w/w)	22.68	13.21
Proteins (% w/w)	20.03	11.89
Phenolic compounds (% w/w)	Trace ¹	Trace ¹

¹ Trace: % w/w < 1. ² AGB: sulfated glycoprotein (crude extract) from *C. decorticutum*. ³ AGP: sulfated glycoprotein (purified extract) from *C. decorticutum*.

The chemical compositions of crude and purified polysaccharides are summarized in **Table III. 1**. AGB and AGP fractions contained 37.67% and 48.38% of total carbohydrates, respectively—mainly neutral sugars—37.56% for AGB, and 45.68% for AGP, with a minor amount of uronic acids (<5%). The presence of a high degree of sulfation and proteins, in the range of 12–23%, indicated that the extracted polysaccharides belonged to sulfated glycoproteins in the cell wall of *C. decorticutum*.

3.2. Molecular Weight Analysis of AGB and AGP Polysaccharides by SEC-MALLS

The molecular mass distribution of extracted polysaccharides was determined by High-Performance Size-Exclusion Chromatography coupled with on-line Multi-angle Laser Light Scattering (HPSEC-MALLS). AGB and AGP were characterized by M_n of $711 \times 10^3 \text{ g}\cdot\text{mol}^{-1}$ and $1295 \times 10^3 \text{ g}\cdot\text{mol}^{-1}$, respectively, and M_w of $2173 \times 10^3 \text{ g}\cdot\text{mol}^{-1}$ for the crude fraction and $2042 \times 10^3 \text{ g}\cdot\text{mol}^{-1}$ for the purified polysaccharides (**Table III. 2**).

Table III. 2. Determination of molecular weight of sulfated glycoprotein extracted from *C. decorticutum*.

	AGB ⁴	AGP ⁵
M_n ¹ (g.mol⁻¹)	$711 \times 10^3 (\pm 1.4\%)$	$1\ 295 \times 10^3 (\pm 0.9\%)$
M_w ² (g.mol⁻¹)	$2\ 173 \times 10^3 (\pm 0.4\%)$	$2\ 042 \times 10^3 (\pm 0.7\%)$
\bar{D} ³	$3.1 (\pm 1.4\%)$	$1.6 (\pm 1.2\%)$
Intrinsic viscosity (mL.g⁻¹)	$266.7 (\pm 3.0\%)$	$560.6 (\pm 4.0\%)$

¹ M_n : number average molecular weight. ² M_w : weight average molecular weight. ³ \bar{D} : index of Polydispersity. ⁴ AGB: sulfated glycoprotein (crude extract) from *C. decorticutum*. ⁵ AGP: sulfated glycoprotein (purified extract) from *C. decorticutum*.

The dispersion characterization of molar masses in polysaccharide samples exhibited a polydispersity index of 3.1 for AGB and 1.6 for AGP. This indicated that the purified fractions

are monodisperse polymers, meaning that the samples are composed of a single macromolecular species with slight degradation during the extraction and purification processes. However, the crude extracts seemed to be a polydisperse polymer with $\mathcal{D} > 2$, indicating the presence of macromolecular species of different molar masses. The measurement of the intrinsic viscosity revealed that AGB represented a $[\eta]$ value of $266.7 \text{ mL}\cdot\text{g}^{-1}$ and $560.6 \text{ mL}\cdot\text{g}^{-1}$ for AGP (**Table III. 2**).

3.3. Monosaccharide Compositions of AGB and AGP Fractions

The monosaccharide compositions were performed by GC-EI/MS analysis. Based on the chromatograms (**Figure III. 2**), the molar ratio (%) was calculated, and the results were summarized in **Table III. 3**. The sulfated glycoproteins were essentially composed of 46.07% and 51.55% of galactose, 38.78% and 24.56% of glucose, and 12.08% and 17.32% of arabinose, for AGB and AGP, respectively, with minor amounts of xylose and rhamnose.

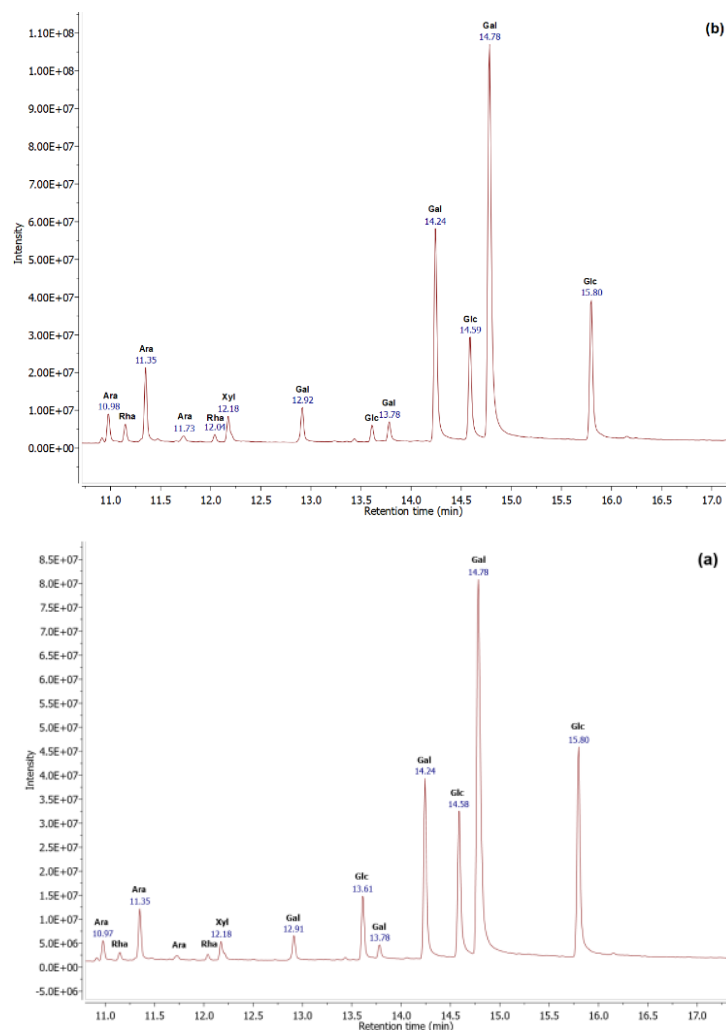


Figure III. 2. GC-EI/MS profiles of the sulfated glycoproteins AGB (**a**) and AGP (**b**), obtained using OPTIMA-1MS (30 m, 0.25 mm, 0.25 μm) column at a flow rate of 1.75 mL/min.

Table III. 3. Monosaccharide compositions determined by GC-EI/MS of sulfated glycoproteins AGB and AGP extracted from the green seaweed *C. decorticum*.

Monosaccharides (% molar ratio)	AGB ¹	AGP ²
Galactose (Gal)	46.07 ± 1.46	51.55 ± 2.67
Glucose (Glc)	38.78 ± 0.24	24.56 ± 1.05
Arabinose (Ara)	12.08 ± 1	17.32 ± 1.33
Xylose (Xyl)	1.70 ± 0.23	3.93 ± 0.61
Rhamnose (Rha)	1.37 ± 0.13	2.63 ± 0.15

¹ AGB: sulfated glycoproteins (crude extract) from *C. decorticum*. ² AGP: sulfated glycoproteins (purified extract) from *C. decorticum*.

The chemical analysis and the monosaccharide compositions of the sulfated glycoproteins extracted from *C. decorticum* indicate that the studied polysaccharides seem to be a sulfated arabinogalactan type II (AGPs).

3.4. Fourier Transform Infrared Spectroscopy

The FTIR spectroscopy was performed to identify the functional groups of polysaccharide samples (**Figure III. 3**) The IR spectrum of isolated crude and purified fractions showed bands at $\approx 3270\text{--}3300\text{ cm}^{-1}$ and 2920 cm^{-1} , indicating the presence of -OH and -CH stretching, respectively [36]. The intense band in the range of $1620\text{--}1631\text{ cm}^{-1}$ might correspond to amide I, which is mostly a carbonyl (C=O) stretching. The peak at 1537 and 1541 cm^{-1} could be attributed to the amide II (N-H) band. The signals extending from 1449 to 1377 cm^{-1} might contribute to the amide III bands [37,38], and absorption in the range of $1141\text{--}1216\text{ cm}^{-1}$ was attributed to the sulfate ester functional groups. The strong signal at $\approx 1019\text{--}1022\text{ cm}^{-1}$ indicated the stretching vibrations of sulphoxides (S=O). The wavenumber at $930\text{--}934\text{ cm}^{-1}$ could correspond to the absorbance of galactopyranose [39]. Around 840 cm^{-1} to 850 cm^{-1} , the signals indicate the sulfate location bonded in the axial or equatorial positions [39]. The intense band in the range of $570\text{--}594\text{ cm}^{-1}$ is characteristic of the bending vibration of C-O-SO₃. Based on the peak intensities, the AGB and AGP fractions contained a significant content of sulfate groups, indicating that the extracts were confirmed as sulfated polysaccharides.

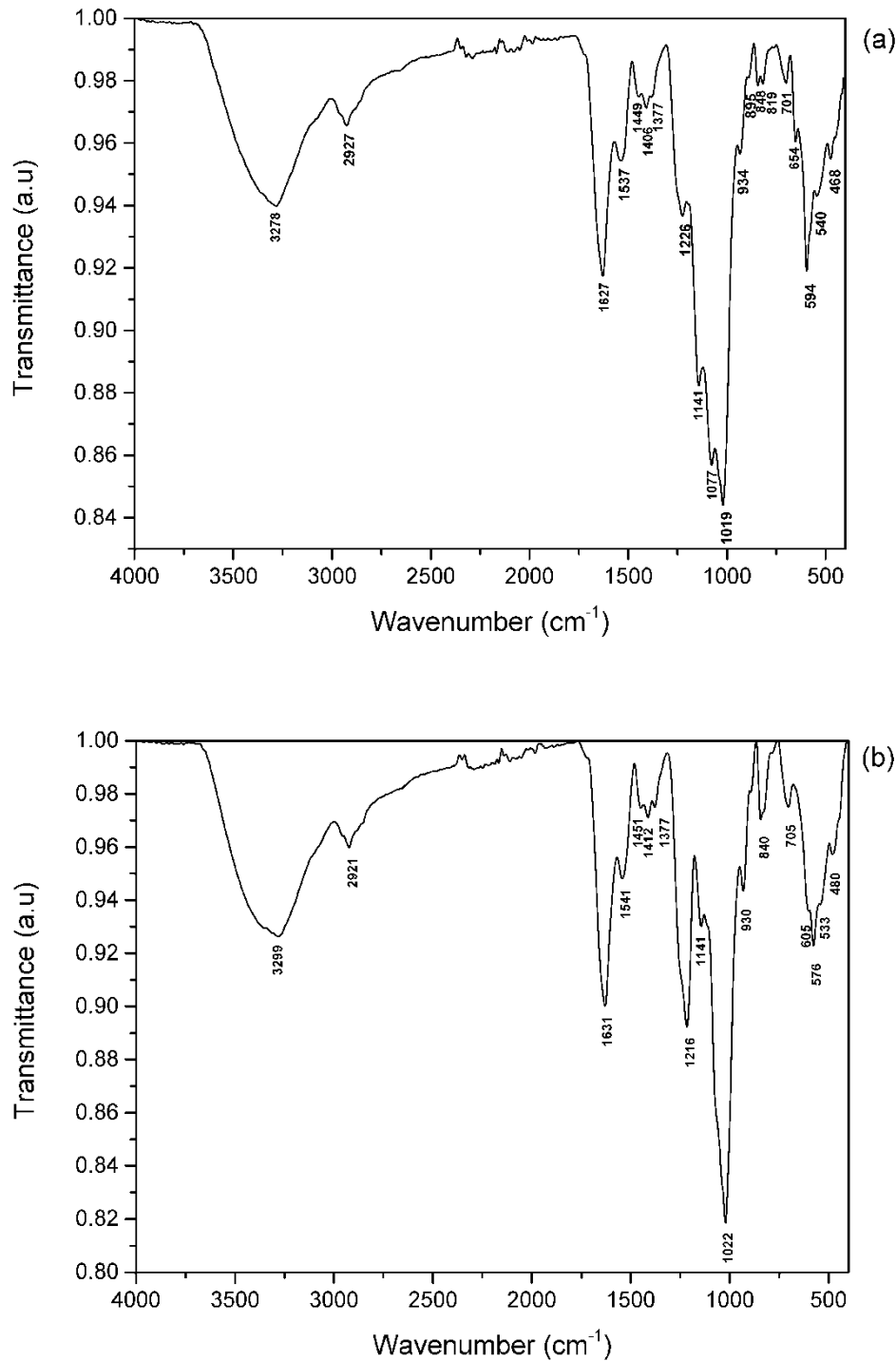


Figure III. 3. ATR-FTIR spectra of sulfated glycoproteins from *C. decorticatorum*, AGB (a), and AGP (b).

3.5. Effect of AGB and AGP Fractions on the Induction of PAL Activity in the Roots and the Leaves of Tomato Seedlings

AGB and AGP extracts were applied using two modes of treatment, foliar spray, and syringe infiltration of 1 g/L of elicitors. Control plants were treated with water. The PAL activity was

measured in the roots and two apical leaves (n and $n - 1$) of 45-day-old tomato seedlings. The sulfated glycoproteins significantly induced the PAL activity in the roots and the leaves of treated plants, depending on the incubation period ($p < 0.05$). After the injection of AGB, the PAL activity showed an upward trend in the leaves from 24 h, achieving a peak value 1.6-fold higher after 72 h. In response to AGP, the activity in the leaf tissue was induced after 24 h and 72 h (**Figure III. 4a**). In the roots, levels rose slowly after 48 h and 72 h after the treatment by both fractions (**Figure III. 4b**). Based on this, both fractions exhibited considerable induction of the PAL activity. The crude fractions (AGB) showed an elicitor capacity more effective in the leaf tissue; however, the purified polysaccharides (AGP) exhibited an efficient activity in the roots of tomato seedlings (**Figure III. 4**).

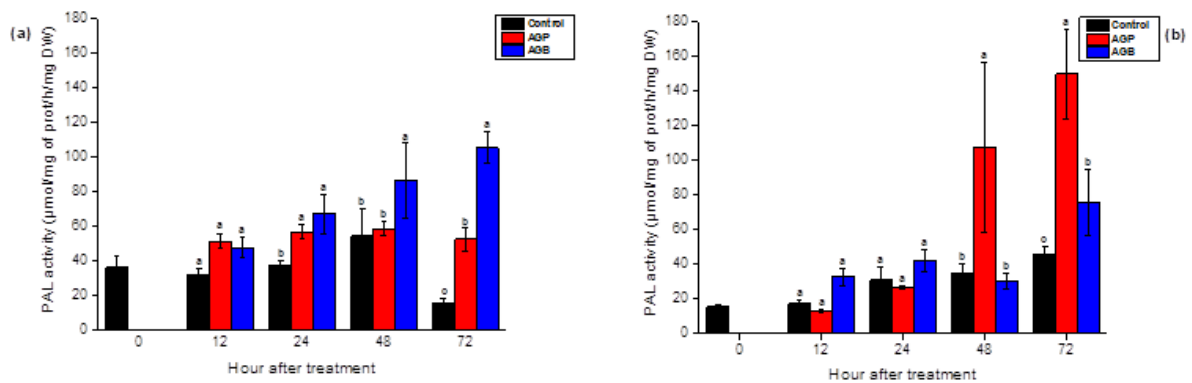


Figure III. 4. Stimulation of PAL activity in the leaves (a) and the roots (b) of tomato seedlings after injection (10 μ L) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b, o) indicate significant differences between treatments (Control, AGB, and AGP) in each point time using Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

The foliar spray of the AGB and AGP extracts revealed globally similar responses to PAL activity. As illustrated in **Figure III. 5**, a significant increase was observed after 72 h of treatment by both sulfated polysaccharides in the leaves and the roots. After 48 h, the elicitor activity was more important in the roots in responses to AGP treatment. In the leaves, the stimulation of the PAL was precocious after the injection of elicitors (24 h) compared to foliar spray. The sulfated glycoproteins showed fluctuations in the elicitor effect depending on the elicitation method (injection and foliar spray) (**Figure III. 4** and **Figure III. 5**).

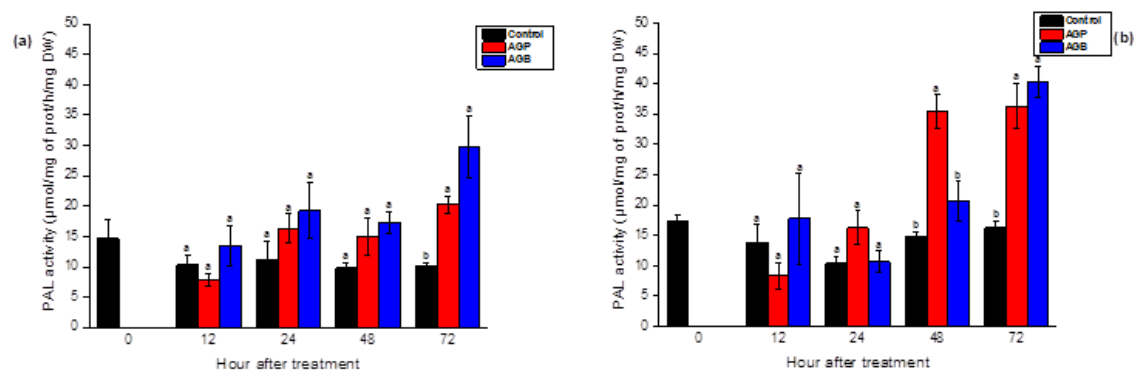


Figure III. 5. Stimulation of PAL activity in the leaves (a) and the roots (b) of tomato seedlings after foliar spray (20 mL) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b) indicate significant differences between treatments (Control, AGB, and AGP) in each point time using a Tukey-HSD test at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

3.6. Effect of AGB and AGP Fractions on the Accumulation of Phenolic Compounds in the Roots and the Leave Tissues of Tomato Seedlings

The accumulation of polyphenols in the leaves and the roots of tomato plants was significantly ($p < 0.05$) triggered after treatment by AGB and AGP compared to the control. These responses depended on the incubation period. Compared to the control, the injection of AGB strongly increased the levels of polyphenols, reaching peak values 5 and 2 times higher in the leaves and the roots, respectively, after 48 h (Figure III. 6). In response to AGP infiltration, the total phenolic compounds rose sharply after 12 h and 48 h in the leaves, and after 12 and 24 h of treatment in the roots (Figure III. 6).

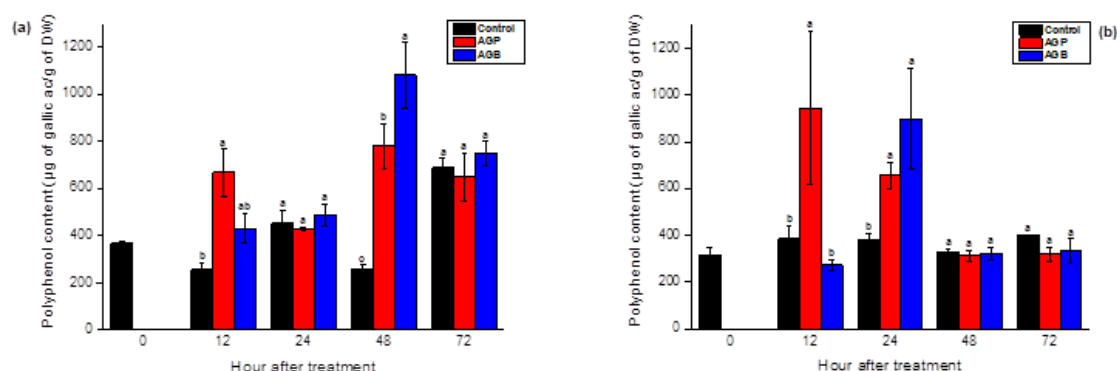


Figure III. 6. Accumulation of phenolic compounds in the leaves (a) and the roots (b) of tomato seedlings after injection (10 μ L) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b, o) indicate significant differences between treatments (Control, AGB, and AGP) in each point time using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

After foliar treatments, both sulfated polysaccharides showed a substantial accumulation of total polyphenols in the leaves after 12 h of incubation, to achieve a significant drop in the following days. For plants treated by AGB fractions, levels hit the lowest point after 24 h and 48 h of treatment compared to the control (**Figure III. 7a**). Nevertheless, the synthesis of these metabolites was mobilized to the root tissue, reaching higher values after 24 h and 48 h in response to AGP application and after 48 h in the plants treated by AGB extracts (**Figure III. 7b**). Compared to the AGB, the elicitor capacity of AGP was more efficient in the roots (**Figure III. 7b**). Globally, the AGB fractions showed a considerable increase of polyphenols in the leaves, whereas the AGP showed an important accumulation of these metabolites in the roots (**Figure III. 6** and **Figure III. 7**).

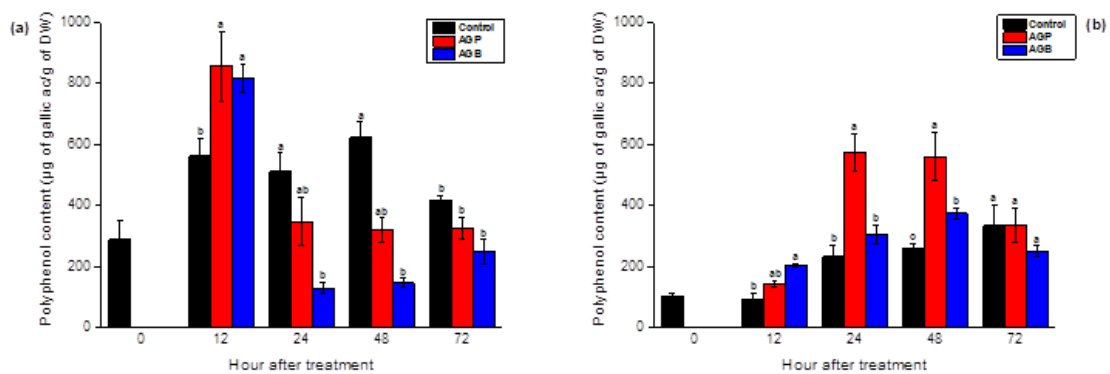


Figure III. 7. Accumulation of phenolic compounds in the leaves (**a**) and the roots (**b**) of tomato seedlings after foliar spray (20 mL) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b, o) indicate significant differences between treatments (Control, AGB, and AGP) in each point time using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

3.7. Effect of AGB and AGP Fractions on the Lignin Content in the Root and the Leaf Tissues of Tomato Seedlings

The AGB and AGP fractions showed significant elicitor activity by stimulating the lignin synthesis in the leaves and the roots of the 45-day-old tomato seedlings. The responses were related to the incubation period ($p < 0.05$). The lignin contents were initially increased in the leaves at 24 h and 72 h after the injection of purified polysaccharides (AGP) (**Figure III. 8a**). These metabolites were promoted in the roots after 24 h and 72 h in response to AGP and AGB, respectively (**Figure III. 8b**). The AGB did not show a significant response in the leaves. The treatment by AGP fractions exhibited an elicitor activity more effective in the leaves and the roots compared to crude extracts (AGB).

The foliar spray of purified extracts (AGP) exhibited an accumulation of lignin polymers after 48 h and 72 h of incubation. Levels were enhanced slowly (72 h) after the elicitation by AGB, which were slightly lower compared to AGP (**Figure III. 9a**). In the roots, the responses were more promoted for both sulfated glycoprotein fractions. The values initially rose from 12 h and remained higher throughout the following days, to reach the control levels after 72 h of treatment (**Figure III. 9b**). The lignin contents were more induced in the roots compared to the leaves (**Figure III. 8** and **Figure III. 9**).

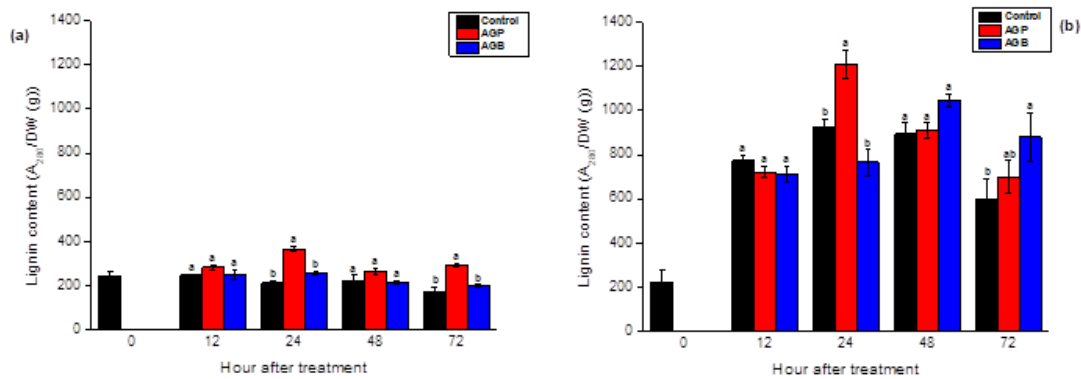


Figure III. 8. Lignin content in the leaves (a) and the roots (b) of tomato seedlings after injection (10 μ L) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b) indicate significant differences between treatments (Control, AGB, and AGP) in each point time using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

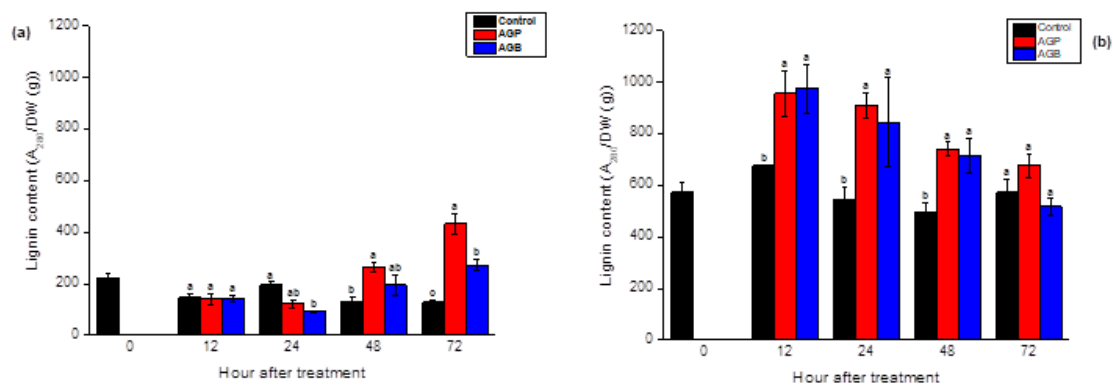


Figure III. 9. Lignin content in the leaves (a) and the roots (b) of tomato seedlings after foliar spray (20 mL) of AGB and AGP fractions (1 g/L). Each value is the mean of four repetitions \pm SE. The letters (a, b, o) indicate significant differences between treatments (Control, AGB, and AGP) in each point time, using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

4. Discussion

The induction of the natural defenses of plants in response to polysaccharides from marine seaweed is one of the new promising alternative strategies in crop protection. Fucans, fucoidans [7], alginates [40], and laminarins [41] from the Phaeophyceae, Ulvans, and glucuronan [5] from the Chlorophyceae, and carrageenans [42] from the Rhodophyceae are the principal carbohydrates studied for their elicitor activities to control plant disease. A recent review has discussed the application of polysaccharides derived from macroalgae in crop protection [43]. The present work shows for the first time the ability of sulfated glycoproteins extracted from the Moroccan green seaweed *C. decorticum* to enhance the natural defense mechanisms of tomato seedlings.

The crude and purified polysaccharides AGB and AGP were extracted from *C. decorticum* with deionized water. The purification was carried out by ultrafiltration using 100 kDa molecular weight cut-off membranes. The yield of AGB and AGP fractions was about 11% and 5% (w/w). The chemical and structural analysis revealed the presence of a significant rate of proteins and sulfates (**Table III. 1**) and the presence of galactose, arabinose, and glucose as the principal constitutive monosaccharides with a minor amount of xylose and rhamnose (**Table III. 3**). The sulfated glycoproteins extracts were characterized by a high molecular weight (M_w) (>of 2000 kDa) (**Table III. 2**). Additionally, the FTIR spectroscopy has confirmed the presence of sulfates and proteins in both extracts by a strong signal extending from 1000 to 1600 cm^{-1} . Basically, the proteins are known by the presence of bands between 1720 and 1600 cm^{-1} (intense peaks at 1627 cm^{-1} for AGB and 1631 cm^{-1} for AGP) corresponding to the amide I, amide II band, ranging from 1600 and 1500 cm^{-1} (peaks at 1537 and 1541 cm^{-1} for AGB and AGP, respectively), and the amide III band between 1450 and 1200 cm^{-1} (peaks extending from 1449 cm^{-1} to 1377 cm^{-1} for both AGB and AGP) [37,38]. This result ties approximately with previous work reported by Zhou et al. wherein the quality of AGPs extracted from green tea were studied [38]. Based on these, the AGB and AGP fractions seem to be a sulfated arabinogalactan-rich protein in the cell wall of the green seaweed *C. decorticum*.

One of the features that distinguishes seaweed from other resources is its large content of sulfated polysaccharides (SPs). In contrast to brown and red seaweed, green algae are still insufficiently explored as a source of SPs. The Ulvans derived from *Ulva* sp. are the most studied SPs because of their widespread application in agriculture, human health, and biomaterials [10]. Recently, attention has been devoted to the investigation of the SPs derived

from the *Codium* genus of the order Bryopsidales to hunt for different SPs present in green seaweed. The chemical and structural analysis of the cell wall polysaccharides from *Codium* sp. has shown the presence of three different types of SPs, sulfated galactans, sulfated arabinans, and sulfated mannans, as well as hydroxyproline-rich glycoproteins (HRGPs), also known as AGPs [11]. Love and Percival (1964) have explored for the first time the cell wall polysaccharides of *C. fragile*, and they have found that the cold and hot water extracts contained galactose and arabinose as major sugars with minor amounts of glucose, mannose, xylose, and rhamnose [44]. The yield of different fractions was in the range of 0.6–5%. In addition, the extract, which was considered sulfated arabinogalactans, contained 22% of sulfates and 25% of proteins [44]. These chemical and structural features are approximately similar to those provided in the present study. It was reported in another work that the polysaccharides extracted with water at room temperature from *C. fragile* (Argentina) were composed mostly of galactose and arabinose with minor quantities of xylose, mannose, and glucose, and with a high content of sulfates (20.6%) and proteins (11.3%). This indicates the occurrence of low amounts HRGP epitopes as well as pyruvate arabinogalactan sulfates (pAGS) [16]. Similar cell wall structures have been found in the green seaweed *C. vermilara*, which was composed of small amounts of hydroxyproline-rich glycoprotein-like (HRGP-L) [21]. The yield, structure, chemical composition, and content of polysaccharides in seaweed varied among each species, depending on the age, season, geographic location, and climatic conditions, as well as the extraction processes [45]. Conventional extraction of the cell wall SPs from *Codium* sp. is typically performed using water as a solvent under specific temperature conditions for several hours. As discussed earlier, when the aqueous extraction was performed at room temperature, sulfate AGPs were the main obtained polysaccharides from *C. fragile*; however, when hot water was used (i.e., 90 °C for 3 h), the major components were sulfated mannans [16]. The extraction of sulfated mannans with hot water was also confirmed in previous works using *C. vermilara* [14] and *C. fragile* [46]. The difference in the monosaccharide composition of *Codium* sp. extracts seems to be related to the extraction procedure; thus, the sulfated arabinogalactans and arabinans seem to have a high solubility in water at room temperature and the sulfated mannans in hot aqueous temperature [11,46]. It was reported that the M_w of water-soluble polysaccharides isolated from *C. fragile* was in the range of 148–4879 kDa, including a subfraction with an M_w of 2017 kDa [46]. These are in accordance with the M_w of the AGB and AGP fractions from *C. decortatum* obtained in this work. However, several studies revealed that the SPs extracted from *C. vermilara*, *C. fragile*, and *C. isthmocladum* were characterized

by a low M_w , ranging from 10 to 140 kDa [6,11,14,47,48]. The green seaweed *Ulva* sp. is commonly known as a source of ulvans; nevertheless, Přerovská et al. reported for the first time the presence of AGP-like glycoproteins in *Ulva lactuca* with an M_w ranging from 100 to 250 kDa [49]. The structural features of cell wall polysaccharides from *C. decorticum* collected along the Patagonic coast (Argentina) revealed the presence of 6.9% of soluble and 32.9% of fibrillar carbohydrates [50]. Four water soluble polysaccharides were detected, as well as pyruvylated and sulfated galactans, sulfated arabinans, and mannans with a low degree of sulfation. Besides this, HRGPs were co-localized with the fibrillar components [50]. In line with the present work, a glycoprotein (GLP) has been isolated from *C. decorticum* (India) containing 36.24% of carbohydrates [51]. It was characterized by a molecular mass of 48 kDa and the presence of rhamnose, galactose, glucose, and mannose as the main monosaccharides [51]. Compared to the terrestrial plants, the M_w of the type II arabinogalactans varied from 10.4 to 3780 kDa [19]. The glycoproteins purified from *C. decorticum* showed some similarity in terms of chemical and structural compositions with the AG-type II found in land plants [19]. Therefore, the green alga *C. decorticum* could be exploited as a source of sulfated arabinogalactans-rich protein and might be an alternative to terrestrial plants, due to its abundance, the important yield of cell wall AGPs, and the facility of the extraction procedure, which uses only water as an extractant.

In order to investigate the elicitor capacities of the sulfated glycoproteins isolated from *C. decorticum*, the crude and purified extracts (AGB and AGP) were foliar sprayed and injected in 45-day-old tomato seedlings. The results showed that both extracts induced the natural defense mechanisms by enhancing the PAL activity, the phenolic compounds, and the lignin contents in the upper leaves (n and n-1) and the roots depending on the incubation period. PAL is the key enzyme associated with the natural defenses of plants; it is one of the most studied enzymes in plant secondary metabolisms, and it catalyzes the first reaction of the phenylpropanoid pathway through the deamination of phenylalanine to trans-cinnamic acid, which is an essential precursor to the biosynthesis of the majority of polyphenols and lignin [52]. Lignin is a complex polymer derived from the polymerization of monolignols in the presence of peroxidases and laccases. Lignin is involved in plant resistance to pathogens by providing rigidity to the cell walls to avoid permeability to phytopathogens [53]. To the best of our knowledge, this is the first report indicating the ability of sulfated arabinogalactans-rich protein isolated from the green seaweed *C. decorticum* to act as a potent elicitor of the natural defenses of plants. It has been previously reported that aqueous (hot/cold) extracts from *C.*

decorticatum, harvested from the Karachi coast, were found to be active elicitors capable of inducing browning and secondary metabolites in chickpea tissues [54,55]. The extracted polysaccharides were characterized by the presence of glucose, rhamnose, arabinose, xylose, fucose, and glucuronic and galacturonic acids as the main monosaccharides, with a low degree of sulfates (<4 to 8%) and a negligible amount of proteins (0.5%) [55]. These features are completely different from those found in this work using the same species, which illustrates the influence of the geographic location and the extraction processes, as discussed above.

At present, alginates, fucoidans, laminarins, carrageenans, glucuronans, and ulvans are the main polysaccharides, isolated from marine macroalgae, that have been reported as bio-elicitors of defense mechanisms in diverse plants [3,5,7,8,40,41,56]. Currently, the only polysaccharide from the macroalgae released in the market as an elicitor is laminarin from the brown algae *Laminaria digitata*, known as Iodus 40 [57]. This product is solely used to control the powdery mildew on wheat in the field [58]. As has been previously reported in the literature, glycoproteins (GP) isolated from several microorganisms are known as elicitors and are capable of stimulating plant defense responses. A peptide-galactoglucomannan isolated from the cell wall of the fungal plant pathogen *Cladosporium fulvum* has enhanced the accumulation of phytoalexins in treated tomato leaves and fruits. The monosaccharide composition was mannose, galactose, and traces of glucose, and the protein moiety was rich in alanine, asparagine/aspartic acid, glutamine/glutamic acid, proline, serine, and threonine [59]. GPs extracted from other fungi, namely *Verticillium dahliae*, *Phytophthora palmivora*, *Phytophthora megasperma*, *Botrytis cinerea*, and *Alternaria tenuissima*, have been found to induce phytoalexin formation in cotton cell suspension cultures [60], expression of defense genes, and accumulations of H₂O₂, SA, scopoletin (Scp), and abscisic acid (ABA) in rubber, tobacco, and tomato leaves [61-64]. On the other hand, GPs derived from bacteria have also exhibited plant immunity-inducing effects [65].

In this study, the tomato seedlings were treated by two different modes of application: the injection in the midstem, in which the molecules are penetrated directly into the plant system, and the foliar spray, in which elicitors are uptaken by both the stomata and cuticle of the leaves. Whatever the elicitor application, the responses followed globally similar patterns and were mobilized throughout the plant. The PAL activity, the phenolic compounds, and the lignin contents were promoted in roots after foliar application and in both roots and leaves after infiltration of elicitors in tomato plants, which were not directly treated. In this case, the

responses were systemic and seemed to be related to the salicylic acid (SA). This form of defense reaction, known also as systemic acquired resistance (SAR), is a type of induced resistance wherein the SA signaling pathway is involved [66]. A previous study has shown systemic effects after syringe infiltration of polysaccharides and oligosaccharides from the green seaweed *Ulva lactuca* in tomato plants. These responses were accompanied by the accumulation of salicylic acid in the leaves located above and below the elicitation site [5]. The application of these elicitors to plant foliage is a practical method in the field and could be used to protect against foliar, stem, and vascular diseases, since the responses are expressed in the shoot and root systems of the plant. Furthermore, the foliar application is more prompt and target-oriented than other modes of application such as soil application because the molecules are directly in contact with the plant without dilution or degradation that can happen in soil.

Comparing the elicitor activities of the crude (AGB) and purified (AGP) extracts from *C. decorticum*, the results have not shown a considerable difference in responses. Both fractions presented the same monosaccharide composition (Gal, Glc, Ara, Xyl, and Rha) and the same M_w (>to 2000 kDa), as well as an important amount of proteins and sulfates in both fractions AGB (>to 20% (w/w)) and AGP (< to 14% (w/w)). The carbohydrates, the molecular weight, and the presence of proteins and sulfates groups in this polymer seemed to be the main factors influencing the eliciting responses. To further understand this, additional studies are required. Several studies have demonstrated that the sulfate groups of some polysaccharides and oligosaccharides have the main role in various biochemical and biological activities. In tobacco and *Arabidopsis thaliana*, the treatment with sulfated laminarin induced an SA signaling pathway, leading to resistance against Tobacco mosaic virus infection compared to the native unsulfated laminarin [67]. In tomato seedlings, the desulfation of ulvans extracted from *Ulva lactuca* reduced the PAL activity in the leaves of treated plants. Compared to glucuronan, an unsulfated polysaccharide, the sulfated ulvans exhibited a more efficient elicitor effect, this difference in responses was related to the sequences of aldobionic acids and particularly the sulfate substituent [5]. In contrast, Caillot et al. have investigated the effects of sulfation degree variation of oligoglucuronans on H_2O_2 production and defense-related genes induction in grapevine cells; they found that the sulfated oligoglucuronans did not possess any significant induction of the oxidative burst whatever their degrees of polymerization [68]. On the other side, Schaffrath et al. have demonstrated the importance of carbohydrate moiety in the elicitor capacity. After the digestion of the glycoprotein from *Pyricularia oryzae* with pronase, the activity was not affected; however, when the molecule was altered with sodium periodate, the

elicitor capacity was totally suppressed, indicating that the responses were associated with the saccharide part of the polymer [69]. As well as this, the elicitor responses of a high molecular weight glycoprotein isolated from *Colletotrichum falcatum*, were found to reside in the carbohydrate moiety [70]. Otherwise, others have shown that in some glycoprotein elicitors, only the protein moiety is responsible for the induction of the natural defenses of plants [60,71]. This difference could be attributed to many factors, such as plant species, monosaccharides, amino acid compositions, or the molecular weight of the molecules.

As discussed above, elicitation by the crude and the purified extract of *C. decorticutum* manifested globally similar responses in tomato seedlings. These results showed the importance of crude extracts in the stimulation of plant immunity. In this case, the purification steps could be skipped to avoid the yield and the algal raw material losses, as well as to minimize the process costing. The high purity of the molecule is essential for structural determination in order to investigate its biological properties and bring to light new elicitors with particular features. Using the aqueous extraction of polysaccharides is a simple and green optimization method. Additionally, it can decrease the yield loss of polysaccharides and ensure that there are no changes to its physical and chemical characteristics. For industry, this extraction process seemed to be adequate, since it fulfills all the criteria needed, such as being time-saving and cost-effective. Several studies have demonstrated the elicitor potential of aqueous extract from marine seaweed. A crude extract prepared from *Ulva armoricana*, essentially enriched in ulvans, has enhanced resistance against three powdery mildew pathogens on the common bean, grapevine, and cucumber [72]. Extracts of the brown alga *Ascophyllum nodosum* are the most commonly explored in agricultural commercial production, and are used to improve plant tolerances to abiotic and biotic stresses, to promote plant growth, and to ameliorate the root and microorganism interactions [73].

5. Conclusions

In this work, sulfated glycoproteins, identified as sulfated arabinogalactan-rich protein, was extracted for the first time from the Moroccan green seaweed *C. decorticutum*. This molecule was characterized by high molecular weight and a significant degree of sulfates and proteins. The application of the crude (AGB) and the purified extracts (AGP) revealed their capacity to trigger the natural defense mechanisms by inducing PAL activity, the accumulation of polyphenols, and the lignin content in the roots and the upper leaves of tomato seedlings. These responses were systemic and were mobilized beyond the plant tissues after foliar spray and

infiltration of elicitors. These results highlighted the novel elicitor potential of the sulfated glycoproteins isolated from seaweed, as well as their new application in agriculture. The hunt for new bioactive compounds from marine algae becomes very noticeable and challenging, which can lead to the discovery of new drugs with various applications.

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Chapitre III

6. Résultats complémentaires

Dans l'article précédent les fractions brutes (AGB) et purifiées (AGP) des arabinogalactanes sulfatés ont montré leur effet éliciteur en induisant l'activité PAL, l'accumulation de composés phénoliques et la teneur en lignine dans les racines et les feuilles des plants de tomate. Ces résultats ont ouvert la voie à une nouvelle application de ces molécules en agriculture comme stimulateur des défenses naturelles des plantes. Dans le **chapitre II**, comme nous avons également mis en évidence la capacité des alginates (polysaccharides non sulfatés) à induire le métabolisme phénolique des plants de tomate, il nous a paru intéressant d'étudier l'effet éliciteur d'un mélange des deux polysaccharides afin de chercher une synergie d'action.

Pour cela, un protocole similaire a été appliqué sur les plants de tomate. Le mélange de polysaccharides (Mix) a été préparé en utilisant les fractions AGP (extrait purifié des arabinogalactanes sulfatés de *C. decorticum*), ALSM (alginate extrait de *S. muticum*) et ALCM (alginate extrait de *C. myriophylloides*). L'ensemble des molécules a été solubilisé dans l'eau à une concentration finale de 1 g/L. Après traitement par pulvérisation, les plantes ont été échantillonnées et analysées dans les mêmes conditions expérimentales décrites auparavant. L'activité PAL, l'accumulation des polyphénols et le contenu en lignine ont été mesurés au niveau des racines et des feuilles des plants de tomate.

Les résultats obtenus sont présentés dans les **Figure III. 10-12**.

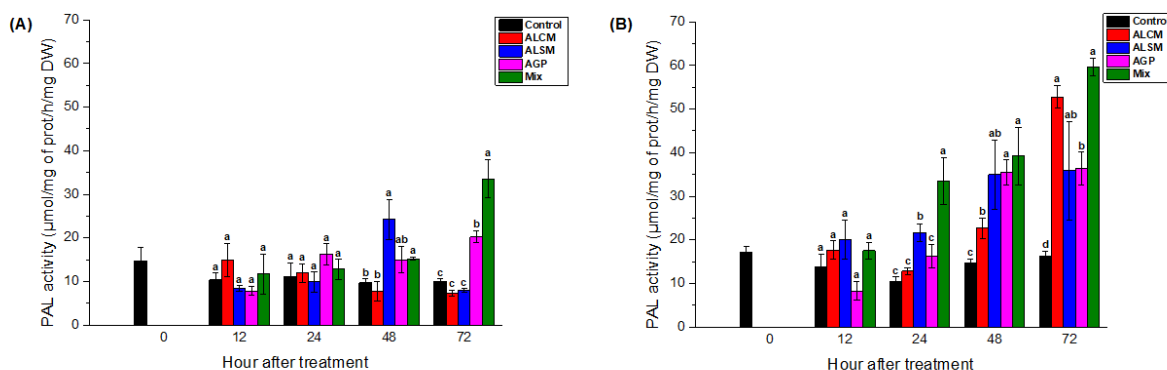


Figure III. 10. Induction de l'activité PAL dans les feuilles supérieures (A) et les racines (B) des plants de tomate après pulvérisation foliaire du mélange de polysaccharides (Mix). Chaque valeur est la moyenne de quatre répétitions \pm SE. Les lettres (a, b, c) indiquent des différences significatives entre les différents traitements.

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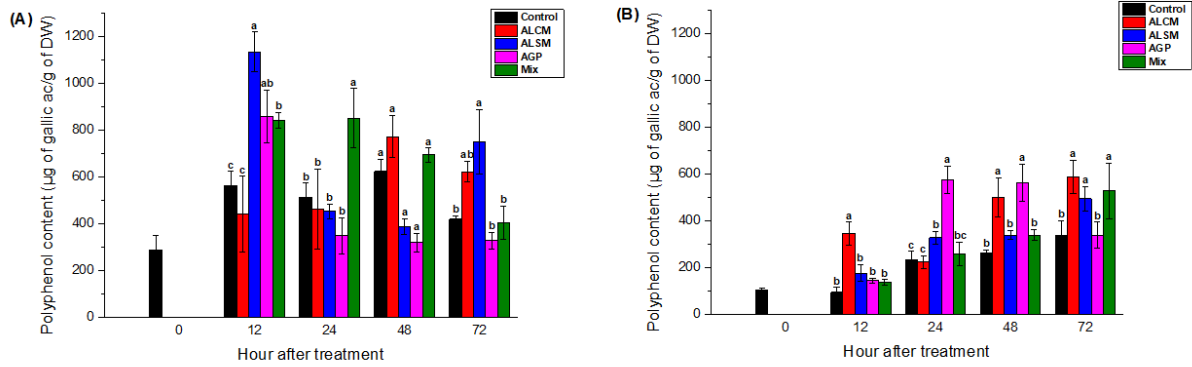


Figure III. 11. Accumulation de composés phénoliques dans les feuilles supérieures (A) et les racines (B) des plants de tomate après pulvérisation foliaire du mélange de polysaccharides (Mix). Chaque valeur est la moyenne de quatre répétitions \pm SE. Les lettres (a, b, c) indiquent des différences significatives entre les différents traitements.

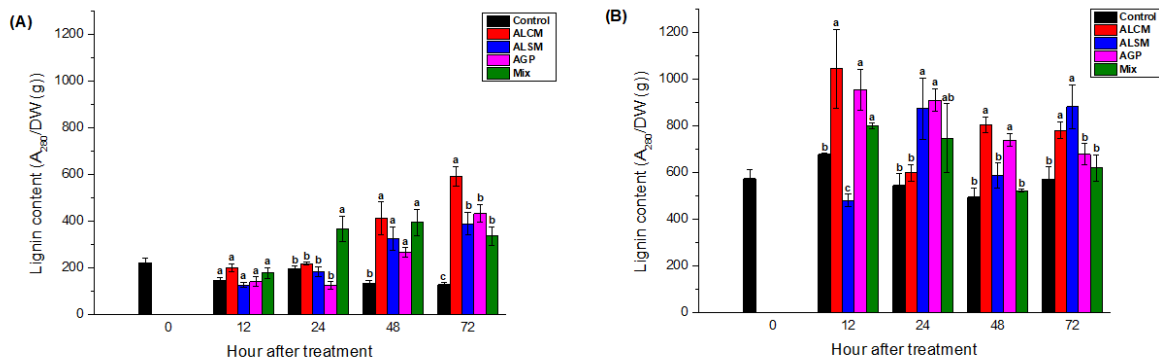


Figure III. 12. Teneur en lignine dans les feuilles supérieures (A) et les racines (B) des plants de tomate après pulvérisation foliaire du mélange de polysaccharides (Mix). Chaque valeur est la moyenne de quatre répétitions \pm SE. Les lettres (a, b, c) indiquent des différences significatives entre les différents traitements.

Les différentes fractions saccharidiques ont stimulé la voie du métabolisme phénolique en fonction de la durée d'incubation ($p < 0,01$). Globalement, aucune différence significative n'a été observée au niveau de l'efficacité électrique en comparant les polysaccharides sulfatés et non sulfatés. Après application du mélange (Mix), les résultats ont montré une induction plus importante de l'activité PAL au niveau racinaire après 24 h et au niveau foliaire après 72 h, par rapport aux traitements individuels (**Figure III. 10**). Les polyphénols et la lignine ont révélé également une forte accumulation au niveau des feuilles supérieures des plants de tomate après 24 h de traitement par le mélange polysaccharidique (**Figure III. 11A** et **Figure III. 12A**). Cependant, au niveau des racines, les traitements individuels ont montré une stimulation plus précoce et plus importante de ces métabolites par rapport au mélange (**Figure III. 11B** et **Figure**

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III. 12B). En se basant sur ces résultats, on peut conclure que le mélange des polysaccharides sulfatés et non sulfatés n'a pas apporté une amélioration significative des réactions de défense chez les plants de tomate.



CHAPITRE IV

EVALUATION DE L'EFFET ÉLICTEUR DES POLYSACCHARIDES SULFATÉS ET NON SULFATÉS EXTRAITS D'ALGUES VERTE ET BRUNE SUR LES FEUILLES DE L'OLIVIER



Article 4 : Accepté en 2022

Meriem Aitouguinane, Zainab El Alaoui–Talibi, Halima Rchid, Imen Fendri, Slim Abdelkafi, Mohamed Didi Ould El–Hadj , Zakaria Boual , Pascal Dubessay, Philippe Michaud, Mounir Traïkia, Guillaume Pierre, Cherkaoui El Modafar, Cédric Delattre. Polysaccharides from Moroccan green and brown seaweed and 2 their derivatives stimulate natural defenses in olive tree leaves. **Appl. Sci.** **2022**, 12(17), 8842, doi: 10.3390/app12178842. **IF (2.838)**.

Contexte scientifique

Face à la complexité du mode d'action des éliciteurs, et avant de pouvoir exploiter de façon fiable et efficace les molécules saccharidiques que nous avons extraits des algues d'intérêt, pour la protection des cultures, il a été nécessaire d'étudier certains paramètres à savoir :

- Evaluer l'efficacité de ces biomolécules en conditions contrôlées pour prouver est ce qu'ils présentent réellement une action de type SDP. Ceci a été démontré et présenté dans les **chapitres II et III**. Toutes les molécules saccharidiques testées ont montré une capacité d'induire le métabolisme phénolique dans les plants de tomate par rapport au témoin.
- Déterminer les facteurs susceptibles d'influencer l'efficacité des éliciteurs notamment leur mode d'application. Ceci a été également évalué et les résultats ont été présentés dans les **chapitres I et II**. Trois modes d'application ont été étudiés : traitement foliaire par pulvérisation, traitement racinaire par trempage des racines dans les solutions électriques (les résultats ne sont pas présentés dans ce mémoire) et l'injection. Les éliciteurs ont montré des profils différents selon le mode de traitement. L'acquisition de ces connaissances pourrait permettre de choisir par la suite les modes adéquats dans le cas d'expérimentations dans les champs.
- Evaluer l'étendue du potentiel d'action des molécules élicitrices en testant la réponse de différentes espèces végétales aux traitements par ces fractions saccharidiques extraites et purifiées. Nous avons ainsi choisi l'olivier (Plante ligneuse pérenne) comme second modèle pour cette étude. Il a été choisi en fonction de son intérêt socio-économique et écologique dans les pays méditerranéens. En outre, la stimulation des défenses naturelles de l'olivier par des éliciteurs saccharidiques est encore insuffisamment explorée. Par conséquent, cette étude pourrait être considérée comme un premier pas vers l'utilisation de cette approche pour prévenir le développement des maladies chez l'olivier.

Dans ce **chapitre IV**, les disques foliaires de l'olivier ont été traités par les alginates purifiés de *C. myriophylloides* et *S. muticum* et leurs dérivés, les arabinogalactanes sulfatés issus de *C. decorticans* et les fucoïdanes extraits de *C. myriophylloides*. Trois concentrations ont été préparées, 0,5g/l, 1g/l et 2 g/l. Les effets éliciteurs ont été évalués par le suivi des activités PAL et tyrosine ammonia-lyase (TAL), le contenu en composés phénoliques et la lignine après 24 h

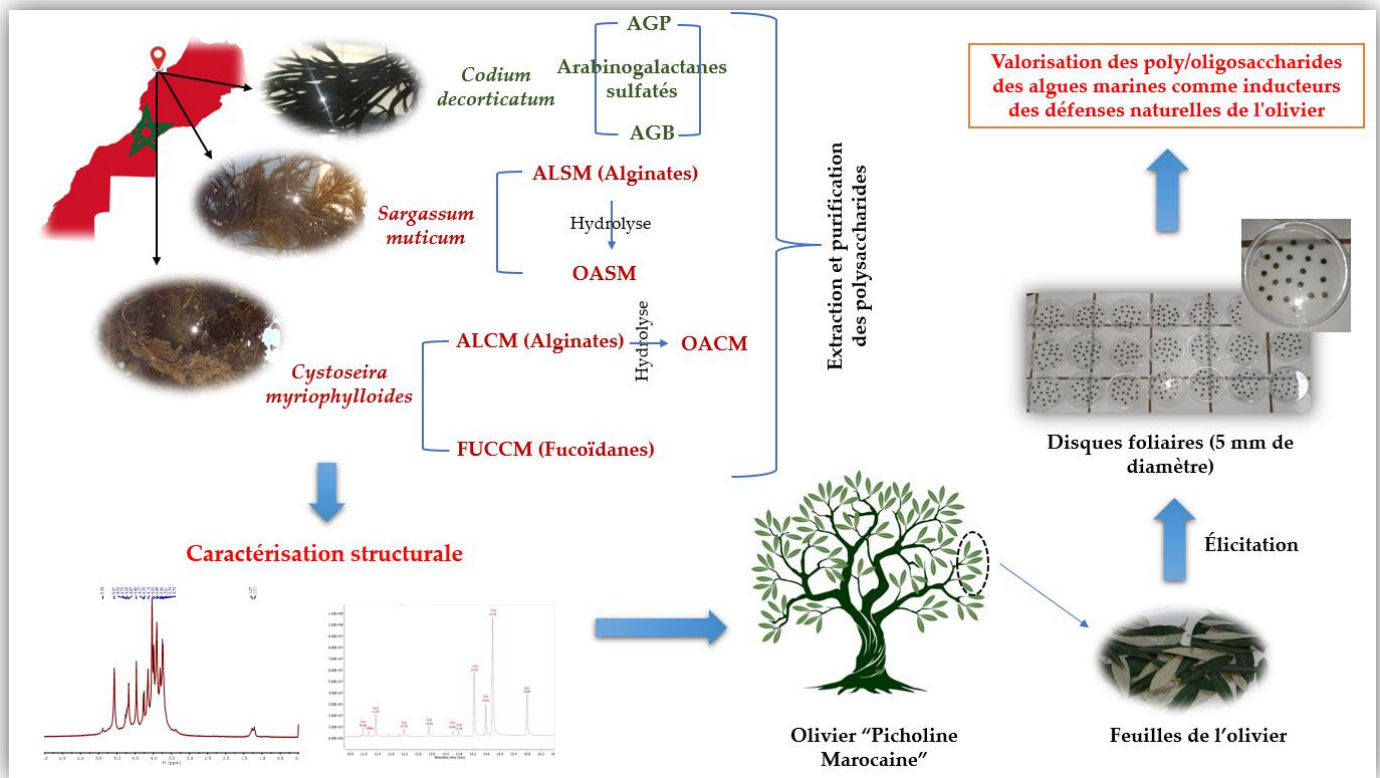
Chapitre IV

de traitement. L'ensemble des résultats obtenus ont fait l'objet d'une publication parue dans le journal « **Applied Sciences** » (**Impact factor : 2.838**). Elle s'intitule « **Polysaccharides from Moroccan green and brown seaweed and their derivatives stimulate natural defenses in olive tree leaves** ».

Polysaccharides from Moroccan Green and Brown Seaweed and Their Derivatives Stimulate Natural Defenses in Olive Tree Leaves

Meriem Aitouguinane, Zainab El Alaoui-Talibi, Halima Rchid, Imen Fendri, Slim Abdelkafi, Mohamed Didi Ould El-Hadj, Zakaria Boual, Pascal Dubessay, Philippe Michaud, Mounir Traïkia, Guillaume Pierre, Cherkaoui El Modafar and Cédric Delattre

Résumé Graphique :



Polysaccharides from Moroccan Green and Brown Seaweed and Their Derivatives Stimulate Natural Defenses in Olive Tree Leaves

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Abstract

This study aims to assess for the first time the ability of marine polysaccharides and their derivatives to stimulate natural defenses in olive tree leaves. Alginates, ALSM, and ALCM were isolated from the brown algae *Sargassum muticum* and *Cystoseira myriophylloides*, respectively. The OASM and OACM fractions were obtained after radical depolymerization of ALSM and ALCM, respectively. Three sulfated polysaccharides, arabinogalactans (AGB and AGP) and fucoidans (FUCCM) were extracted from the green alga *Codium decorticans* and the brown seaweed *C. myriophylloides*, respectively. The extraction yield of different extracts was in the range of 0.82–32% and the average molecular weight (M_w) varied from 3000 Da to 2173 kDa. The M/G ratios of ALSM and ALCM were 0.87 and 1.12, respectively. FUCCM contained 53% of fucose and 12.83% of sulfates. The AGB and AGP fractions were characterized by the presence of a high degree of sulfation and protein (12–23% (w/w)) and were composed mainly of galactose, glucose, and arabinose. The aqueous saccharide solutions

were applied to the leaf discs of the olive tree at 0.5 g/L, 1 g/L, and 2 g/L, for 24 h. These molecules triggered defense responses, by showing a differential capacity to induce the activity of the phenylalanine and tyrosine ammonia-lyase (TAL and PAL), polyphenols, and lignin contents in the leaves of the olive tree. Alginates and their derivatives as well as arabinogalactans exhibited an important induction of TAL activity compared to the PAL. The sulfated polysaccharides were more effective compared to the unsulfated polysaccharides (alginates) which were active at a low concentration. The optimum concentration for most of the studied elicitors was 2 g/L. These results suggest the valorization of these molecules derived from marine biomass as inducers of natural defenses of the olive tree to protect against phytopathogens in the context of sustainable development.

Keywords: olive tree; seaweed; polysaccharides; oligosaccharides; elicitors; PAL; TAL; polyphenols; lignin

1. Introduction

The olive tree (*Olea europaea* L.) plays a key socio-economic and ecological role in Mediterranean countries. It is one of the most cultivated crop species and the only one of the *Oleaceae* family that produces comestible fruits processed for oil and table olive production [1]. In the European Union (EU), the olive-growing area was about 4.59 million hectares (ha) in 2017. Three-quarters of the utilized area were concentrated in Spain, with 2.5 million ha (55%), followed by Italy with 1.07 million ha (23%), and Greece with 15% of the total EU area under olive trees [2]. Spain is the biggest producer of olive oil, providing 59% of total EU production, followed by Italy, Portugal, and Greece with 24.8%, 8.4%, and 7.3%, respectively [3]. Morocco, after Spain, Italy, and Greece, has the fourth-largest olive tree cultivation, covering 78,400 ha and producing 1.48 million tons of olives per year [4]. It is the 2nd largest producer of table olives and the 6th of olive oil [5]. Unfortunately, the olive tree is subjected to several biotic stresses, influencing the health, yield, and quality of the oil and fruits [6-8]. *Verticillium dahlia* (Verticillium wilt) and *Spilocaea oleagina* (olive leaf spot) are the major pathogenic fungi of the olive tree in the world [7,9]. Anthracnose, caused by *Colletotrichum* spp., is also one of the most severe diseases of this woody crop [10]. Furthermore, the olive fruit fly poses a serious economic problem. It reduces about 15% of production, equivalent to an average of 800 million dollars per year [11]. Today, pesticides are an important input in the protection of the olive tree [7]. Despite their effectiveness, the residues of these agrochemicals in fruits and olive oil present a major risk to public health [7,12]. Recently, researchers have

focused on using biological control agents (BCAs) such as arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), plant growth-promoting fungi (PGPF), and endophytes as an eco-friendly alternative to control olive diseases [12]. However, the use of BCAs is qualified by some restrictions, namely negative impact on non-target microorganisms, issues of adaptation to the target ecological niche, and especially direct effects leading to resistance increases in pathogen communities [9]. Therefore, sustainable approaches such as the stimulation of natural defenses of plants using natural elicitors could be a promising alternative to preventing disease development in the olive tree and reducing dependence on pesticides. This strategy aims to induce plant resistance using molecules, such as lipids, glycoproteins, peptides, proteins, oligosaccharides, or polysaccharides [13]. Systemic acquired resistance (SAR) and induced systemic resistance (ISR), are two types of plant resistance involved depending on the nature of the elicitors and the regulatory pathway, leading to protection against a wide range of phytopathogens [14]. Seaweed or marine macroalgae constitute an important source of bioactive products [15]. Due to their abundance, research has been focused on exploiting seaweed extracts and seaweed-derived polymers in agriculture as inducers of plant defense against a wide range of phytopathogens [16,17]. Currently, a great deal of attention has been directed at using algal polysaccharides [16]. Their applications have shown to activate natural defenses in a variety of plants, including tomato, palm date, apple fruits, and tobacco [18-24]. However, only a few studies have been conducted on the olive tree. This study aims to characterize the cell wall polysaccharides of the Moroccan brown seaweed *S. muticum* and *C. myriophylloides*, and to evaluate for the first time, the ability of alginates, and their derivatives, fucoidans, and glycoproteins from the green seaweed *C. decortdatum* to induce natural defenses in the leaves of the olive tree (Picholine Marocaine). To our knowledge, this is the first report investigating the elicitor capacity of fucoidan, low M_w alginates, and glycoprotein from algae in the olive tree.

2. Materials and Methods

2.1. Algal Samples

In July 2019, the green seaweed *C. decortdatum* (CD) and the brown macroalgae *S. muticum* (SM) and *C. myriophylloides* (CM) were harvested from the Atlantic coast of El Jadida City, Morocco. The raw material was thoroughly rinsed with distilled water, then oven-dried at 50 °C and finely ground into a fine powder.

2.2. Extraction and Purification of Polysaccharides and Preparation of Oligomers Extracts

The alginates and fucoidans were extracted from (SM) and (CM) according to the Mazumber et al. method with slight modifications [25]. Briefly, 200 g of algal powder were soaked in 2% of formaldehyde for 24 h to 48 h at room temperature under stirring to discard all phenolic compounds and pigment. The dried residues were treated with HCl (pH: 2–3.5) for 3 h at 60 °C to convert alginate salts into alginic acid. The precipitates were suspended in 2–3% of sodium carbonate solution for 3 h at 80 °C to transform the alginic acid into sodium alginates, and the filtrates were used to extract fucoidans (FUCCM) from (CM). Alginates (ALCM and ALSM) and FUCCM fractions were purified by rinsing with ethanol several times at 96° followed by ultrafiltration using the Vivaflow 50R-200 system (Sartorius Lab Instruments GmbH & Co., KG, Goettingen, Germany). The final samples were then lyophilized for 72 h (**Figure IV. 1**).

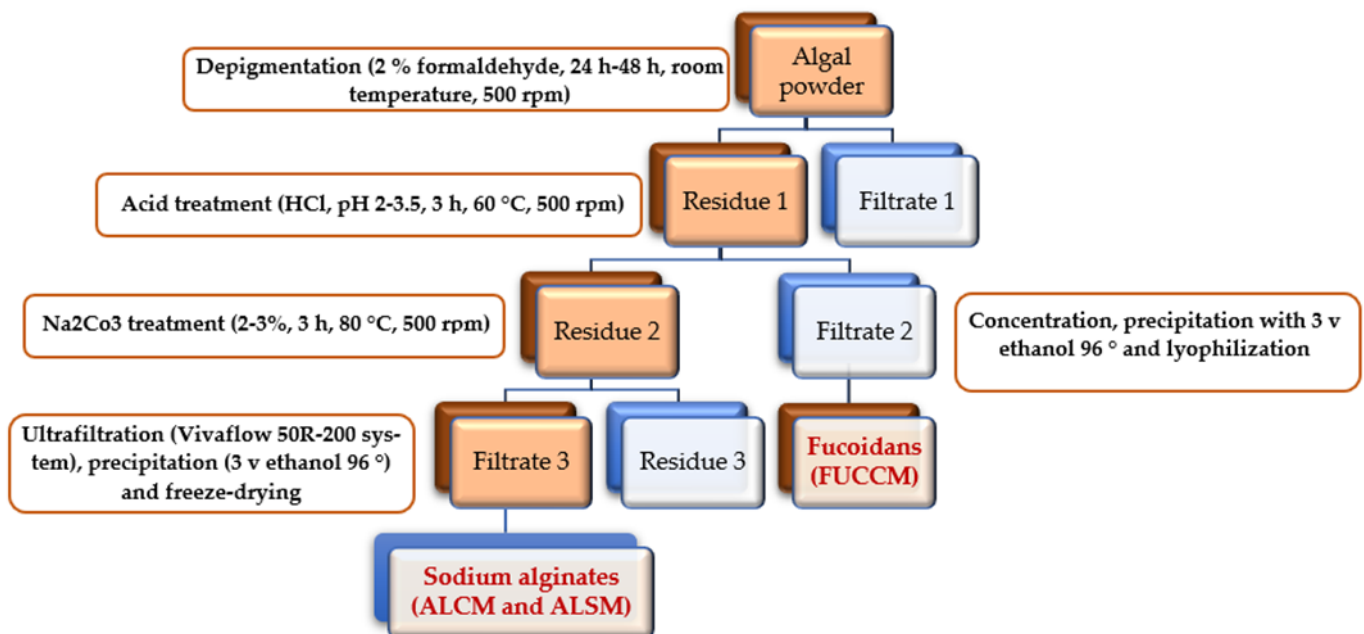


Figure IV. 1. Extraction and purification of sodium alginates (ALCM and ALSM) and fucoidans (FUCCM) from the brown algae *S. muticum* (SM) and *C. myriophylloides* (CM).

The LMW alginates OACM and OASM were obtained from ALCM and ALSM, respectively. The radical hydrolysis was performed using hydrogen peroxide (H₂O₂) [26]. Briefly, alginates were dissolved in distilled water at a concentration of 10 g/L and shaken for 1–2 h at 70 °C. H₂O₂ was added to the solution in a 1:1 (w/w) ratio, and the mixture was incubated for 6 h (70 °C, 300 rpm). The reaction was stopped by adding 96° (1/10, v/v) ethanol and stirring for 1 h at room temperature to remove the residual H₂O₂. The samples were lyophilized, dissolved in

distilled water, and washed several times with ethanol (96°). At the end of the experiment, the H₂O₂ was quantified to ensure its elimination. The oxidative fractions were finally re-dissolved in distilled water and freeze-dried for 72 h. The sulfated arabinogalactan-rich-protein, crude, and purified fractions (AGB and AGP) were extracted from the green seaweed (CD). As described in the previous study [27], the milled seaweed was pretreated with ethanol and the crude fraction (AGB) was extracted with 20 volumes of distilled water and precipitated with 96° ethanol. The AGP was obtained under the same conditions described above, followed by a step of purification by ultrafiltration using a membrane of 100 kDa MWCO. The resulting AGB and AGP fractions were dissolved in distilled water and lyophilized.

2.3. Biochemical Characterization of Extracted Polysaccharides

2.3.1. Total Sugar Content

The total sugar content of the polysaccharide fractions was measured using the phenol-sulfuric acid method using D-glucose as a standard [28]. Briefly, 200 µL of saccharide solutions (10 g/L) were added to 200 µL of phenol (5%) and 1 mL of sulfuric acid (80%) and then incubated for 30 min at 90 °C. The absorbance was read at 485 nm.

2.3.2. Neutral Sugar and Uronic Acid Content

The sulfuric resorcinol and the m-hydroxydiphenyl (mHDP) methods were used to estimate the neutral and acid carbohydrates, respectively [29,30]. The calibration curve was prepared using D-galactose, D-glucose, L-fucose, and D-glucuronic acid. For the neutral sugar, 200 µL of samples were added to 200 µL of resorcinol (6 g/L) and 1 mL of H₂SO₄ (80%). The solutions were incubated for 30 min at 90 °C and measured at 450 nm. The quantification of uronic acid was performed by adding 200 µL of polysaccharide solutions to 1 mL of sodium tetraborate (0.12 M) and kept at 90 °C for 1 h, then 200 µL of mHDP were added. After incubation, the absorbance was read at 520 nm. The corrective equations described by Montreuil and Spick were used to calculate sugar contents [31] (Equations (1) and (2)).

$$[\text{ON}] = \frac{\text{Abs}_{\text{mHDP}} - \beta'[\text{AU}]}{\alpha'} \quad (1)$$

$$[\text{AU}] = \frac{\text{Abs}_{\text{resorcinol}} - \left(\frac{\alpha}{\alpha'}\right) \text{Abs}_{\text{mHDP}}}{\frac{\alpha'\beta - \alpha\beta'}{\alpha'}} \quad (2)$$

[ON]: neutral sugar concentration; [AU]: uronic acid concentration; α : slope coefficient of D-galactose, D-glucose, or L-fucose obtained after resorcinol assay; β : slope coefficient of D-glucuronic acid obtained after resorcinol assay; α' : slope coefficient of D-galactose, D-glucose, or L-fucose obtained after mHDP assay, β' : slope coefficient of D-glucuronic acid obtained after mHDP assay, Ab_{mHDP} : absorbance of the sample after mHDP assay, $Ab_{Resorcinol}$: absorbance of the sample after resorcinol assay.

2.3.3. Sulfate Contents

Barium chloride-gelatin assay was performed to determine the degree of sulfation [32]. Briefly, 120 mg of polysaccharides were treated with HCl (3 mL, 2 M) and heated for 2 h at 100 °C, then the hydrolysate was centrifuged at 13000 g for 30 min. One milliliter of the recovered supernatant was added to 1 mL of HCl (0.5 M) and 500 μ L of the BaCl₂/gelatin reagent already prepared. The mixture was incubated for 30 min at room temperature, and the absorbance was measured at 550 nm. The calibration curve was prepared using K₂SO₄.

2.3.4. Protein and Phenolic Contents

The proteins were quantified according to the Lowry assay using bovine serum albumin (BSA) as a standard [33]. In this case, 500 microliters of samples were added to 700 μ L of the Lowry-forming solution (sodium hydroxide + sodium carbonate + copper sulfate + sodium tartrate), then the mixture was incubated in darkness for 20 min at room temperature. Afterward, 100 μ L of Folin-Ciocalteu reagent were added, and the samples were kept again for 30 min at room temperature in the dark before measuring absorbance at 750 nm.

The phenolic content was determined based on the Blainski et al. method with slight modifications [34]. In this case, 100 microliters of polysaccharide solutions were added to 250 μ L of Folin-Ciocalteu reagent and 500 μ L of sodium carbonate (20%). After incubation at 40 °C for 30 min, the absorbance was read at 760 nm. The calibration curve was made using gallic acid.

2.4. Structural Analysis of Saccharide Fractions

The average molecular weight was determined by High-Performance Size-Exclusion Chromatography coupled with on-line Multi-angle Laser Light Scattering (HPSEC- MALLS, Paris, France), using He-Ne laser (HELEOS II, Wyatt Technology Corp., Santa Barbara, CA, USA), differential refractive index (RID 10A, Shimadzu, Japan), viscosimeter (Viscostar II,

Wyatt Technology Corp., USA), and OHPAK SB 804-806 HQ columns (Shodex). The average degrees of polymerization (DPs) of alginate and their derivatives were estimated considering the uronic acid (Guluronic acid or mannuronic acid) repeat units of sodium alginates and calculated using the Equation (3):

$$DP = \frac{M_w}{216} \quad (3)$$

As described previously, the monosaccharide composition was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) with cold electron ionization (EI) (GC/MS-EI) (Shimadzu, GCMS-QP2020 NX) [27]. Briefly, polysaccharide extracts were hydrolyzed with Trifluoroacetic acid (2M), then treated with pyridine and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (v/v). The final products were dissolved in dichloromethane to be injected into the GCMS-GP2020 NX instrument using the OPTIMA- 1MS (30 m, 0.25 mm, 0.25 μ m) column (Macherey-Nagel). The standard monosaccharides were determined under the same conditions and the results were expressed in a molar ratio (%). The ^1H NMR analysis was carried out using a Bruker AVANCE III HD 500 MHz spectrometer equipped with a Bruker 5 mm inverse probe TXI ($^1\text{H}/^{13}\text{C}/^{15}\text{N}$) with z-gradient coil probe. Samples were dissolved in deuterium oxide (D_2O) (99.99 % D) at 100 g/L and then lyophilized. This step was repeated 3 times to exchange the hydrogen atom for deuterium. ^1H NMR spectra were recorded with a number of 128 scans, an impulsion relaxation and acquisition time of 9.7 μ s, 4 s, and 3.3 s, respectively, a power of 14 W and a spectral window of 20 ppm, and TD = 64 K data points zero-filled to SI = 131 K before Fourier transformation with 0.3 Hz line broadening. For alginates, the ratios (M/G) and the frequencies of structural blocks (F_G , F_M , F_{GG} , F_{MM} , F_{GM} , and F_{MG}) of mannuronic (M) and guluronic acid (G) were estimated based on the signals I, II, and III of the relative area of anomeric regions (G-1, GG-5G, GM-5, and M-1). The following equations were used according to Grasdalen [35]:

$$F_G = \frac{A_I}{A_{II} + A_{III}} \quad (4)$$

$$F_{GG} = \frac{A_{III}}{A_{II} + A_{III}} \quad (5)$$

$$F_M = 1 - F_G \quad (6)$$

$$F_{GM} = F_{MG} = F_G - F_{GG} \quad (7)$$

$$F_{MM} = F_M - F_{MG} \quad (8)$$

$$\frac{M}{G} = \frac{1 - F_G}{F_G} = \frac{F_M}{F_G} \quad (9)$$

$$\eta = \frac{F_{GM}}{F_M \times F_G} \quad (10)$$

2.5. Treatment of Leaf Discs of the Olive Tree

In vitro treatments of the olive tree were conducted on the leaf tissue. Healthy leaves from the same olive tree (Picholine Marocaine) were collected, washed with distilled water, dried, and then cut with a sterile cork borer to small discs of 5 mm in diameter. In this case, 20 discs were placed on their abaxial leaf surfaces in Petri dishes containing Whatman filter paper soaked in 3 mL of saccharide solutions at various concentrations (0.5 g/L, 1 g/L, and 2 g/L) and 0.01% of Tween 20 (**Figure IV. 2**). Afterward, the samples were incubated for 24 h at 30 °C under light. The control discs were immersed in distilled water and Tween 20 (0.01%). Three repetitions were performed for each treatment and concentration.

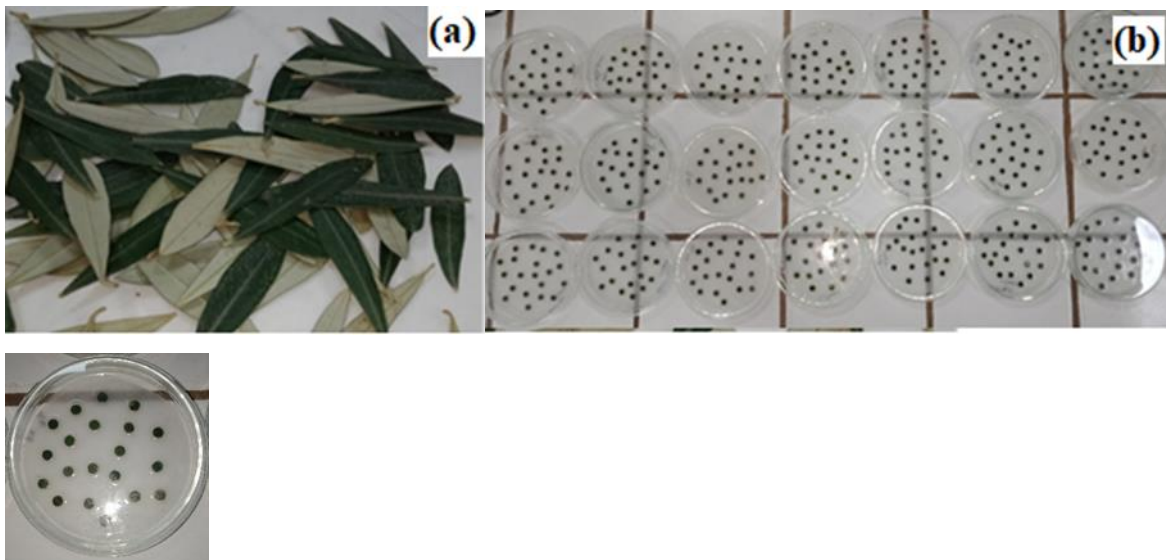


Figure IV. 2. Detached leaves of the olive tree “Picholine Marocaine” **(a)**; Leaf discs (5 mm in diameter) placed in the Petri dishes containing saccharide solutions **(b)**.

2.6. Measurement of Enzyme Activities and Protein Assays

After 24 h of incubation, the leaf discs were ground in liquid nitrogen and then weighted. The extraction of phenylalanine and tyrosine ammonia-lyase enzymes (TAL and PAL) was carried

out in a 100 mM boric acid buffer at pH 6, containing 1 mM of ethylene diamine tetraacetic acid (EDTA), and 1% of insoluble polyvinylpolypyrrolidone (PVPP) [36]. The enzymatic reactions were performed using 20 mM of L-phenylalanine and L-Tyrosine as substrates. The PAL and TAL activities were estimated by measuring the formation of *trans*-cinnamic and *p*-coumaric acid at 290 nm and 310 nm, respectively (Jenway 6305 Spectrophotometer, Jenway, Dunmow, Essex, UK). Specific activities were determined using extinction coefficients of 9116 L·mol⁻¹·cm⁻¹ (*trans*-cinnamic acid) and 11558 L·mol⁻¹·cm⁻¹ (*p*-coumaric acid) [37]. Proteins were quantified according to the Bradford assay, using bovine serum albumin (BSA) as a standard [38].

2.7. Quantification of Secondary Metabolites: Phenolic Compounds and Lignin

The leaf disc samples were macerated in acetone (80°) with liquid nitrogen. After centrifugation (10,000× g, 15 min, 4 °C) (UNIVERSAL 320 R, Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany), the recovered supernatants were used to purify the polyphenols, and the pellets were served to extract lignin. The purification and quantification of total phenolic compounds and lignin content were performed based on the methods described previously [27]. The results were expressed as µg of gallic acid/mg of dry weight (DW) and A₂₈₀/g of DW for polyphenols and lignin, respectively.

2.8. Statistical Analysis

The comparison of PAL, TAL, polyphenols, and lignin parameters between each treatment at different concentrations was performed using the ANOVA test in SPSS version 25.0 software (IBM SPSS Statistics for Windows, Armonk, NY, USA) at a significance level of 5%. The findings are presented as a mean value ± Standard error (SE) of three replicates.

3. Results and Discussion

3.1. Chemical Composition of Polysaccharide Fractions

As illustrated in **Table IV. 1**, the extraction yield of the five extracted polysaccharides ranged from 0.87% to 31%. The highest percentage was recorded in alginophytes *C. myriophylloides* and *S. muticum*, representing values of 31% and 23%, respectively. The sulfated arabinogalactans AGP and AGB represented 5% and 11% of the dry weight of the green alga *C. decorticutum*, while the fucoidans yield showed the lowest averages (0.87%). As far as we know, no previous research has investigated the structural features of *C. myriophylloides*. The

ALCM extraction yield was higher than that reported in some of the *Cystoseira* genus [39-44]. By comparing the alginate content of *S. muticum* from previous works [25,45-47], the ALSM yield was in accordance with that reported by El Atouani et al. [45]. The AGB fraction exhibited an important sulfation degree of 22.68%, while lower averages of 13.21% and 12.83% were observed for AGP and FUCCM, respectively. The presence of 7.29% of sulfates in ALCM could be explained by eventual contamination by fucoidans during the co-extraction step. The FUCCM yield was similar to that reported by Benslima et al. [42]. However, it was lower than that obtained from other *Cystoseira* species. Higher averages were recorded in *C. trinodis* (6.34%), *C. barbata* (5.45%) and *C. compressa* (5.2%) [43,44,48]. The content of sulfates of FUCCM was within the range of that reported for other *Cystoseira* species [43,44,49,50]. High averages were found in *Fucus spiralis* (49.53%) and *Bifurcaria bifurcata* (45.49%) [19] and lower amounts were obtained from *C. schiffneri* (4.5–10.7%) [49]. These variations in yields and chemical compositions, even within the same species, could be attributed to a variety of factors, including the temporal and spatial conditions of harvesting and extraction procedures [25,47,49,51].

Table IV. 1. Biochemical characterization and extraction yield of polysaccharides extracted from CE, SM, and CM.

Fractions	Source	Yield (% w/w)	Total Sugar (% w/w)	Neutral Sugar (% w/w)	Uronic Acid (% w/w)	Sulfate (% w/w)	Protein (% w/w)	Polyphenol (% w/w)
ALSM ¹	<i>S. muticum</i>	23	44.46 ± 1.67	2.42 ± 1.96	63.27 ± 0.17	Nd ⁶	Trace	Nd
ALCM ²	<i>C. myriophylloides</i>	31	57.48 ± 0.35	30.27 ± 0.84	44.99 ± 3.70	7.29 ± 0.4	Nd	Nd
FUCCM ³	<i>C. myriophylloides</i>	0.87	52.11 ± 3.07	29.06 ± 0.52	23.19 ± 1.56	12.83 ± 0.06	2.93 ± 0.17	Nd
AGB ⁴	<i>C. decortiatum</i>	11	37.67 ± 1.10	37.56 ± 1.73	3.05 ± 0.29	22.68 ± 2.08	20.03 ± 0.29	Trace ⁷
AGP ⁵	<i>C. decortiatum</i>	5	48.38 ± 1.44	45.68 ± 0.58	4.04 ± 0.35	13.21 ± 0.23	11.89 ± 2.48	Trace

¹ ALSM: alginate extracted from SM; ² ALCM: alginate extracted from CM; ³ FUCCM: fucoidan extracted from CM; ⁴ AGB: sulfated glycoprotein (crude extract) extracted from CD; ⁵ AGP: sulfated glycoprotein (purified extract) extracted from CD; ⁶ Nd: Not determined; ⁷ Trace: % w/w < 1.

3.2. SEC-MALLS Analysis of Saccharide Fractions

The five fractions were analyzed using SEC-MALLS and the results are summarized in **Table IV. 1**. The average molecular weight (M_w) ranged from 3 kDa for oligoalginates OASM obtained from ALSM to 2173 kDa for sulfated arabinogalactans extracted from the green seaweed *C. decorticans* (**Table IV. 2**). The polydispersity index (\mathcal{D}) was less than 2 for the ALCM (1.3), ALSM (1.2), OACM (1.7), OASM (1.04), and AGP (1.6) fractions, and greater than 2 for the AGB (3.1) and FUCCM (4) fractions. These indicate that the purified arabinogalactans (AGP) and alginates polymers and oligomers are monodisperse with minor degradation or contamination with fucoidans in the case of ALSM and ALCM extracts. For the crude extract (AGB) and FUCCM, the \mathcal{D} values were higher, meaning that both are polydisperse polymers presenting impurities of other poly/oligosaccharides such as laminarin and especially alginate for FUCCM. The intrinsic viscosity of ALSM and AGP presented the highest averages, whereas the oligoalginates (OASM) exhibited the lowest values compared to other fractions. Usually, the intrinsic viscosity of polysaccharides is dependent on their molecular weight (M_w) and chain dimension, which explains the fluctuation of values of alginates and their derivatives [52]. It was found that the presence of salts in the solution could also decrease the $[\eta]$, which exhibited a typical polyelectrolyte behavior [53]. This might explain the low intrinsic viscosity of AGB and AGP despite their important M_w . In addition, FUCCM presented an important M_w of 148 kDa, compared to ALCM (76 kDa). However, the $[\eta]$ was lower (84.4 mL/g) than that found in ALCM (224.2 mL/g). These indicated that the presence of sulfates in polysaccharides could decrease the $[\eta]$ values due to the charge screening effects, leading to a more compact structure, hence, reducing the hydrodynamic volume of the molecule [54]. The fractions of OACM and OASM were obtained after 6 h of radical depolymerization of ALCM and ALSM, respectively. The M_w exhibited an important fluctuation in which OASM is characterized by a low M_w (3 kDa) and DP of 14 compared to the OACM fraction ($M_w = 19$ kDa and DP = 88). These indicate that the OASM was hydrolyzed into oligomers in which the DP of ALSM was sharply decreased from 935 to 14, contrary to OACM. As a result, increasing the hydrolysis reaction time is required to generate oligomers from *C. myriophylloides* alginate. The presence of sulfates contamination in ALCM could be the factor influencing the degradation process. Moseley et al. have suggested that the presence of sulfate could protect molecules against ROS hydrolysis [55]. Under the same conditions, oligomers produced by the degradation of alginate extracted from *B. bifurcata*, exhibited an M_w of 5.5 kDa and $2 \leq DP \leq 24$ after 6 h of treatment and 3.5 kDa after 10 h [26], which are different from values found in this study.

Table IV. 2. Average molecular weight determination and DPs distribution of extracted saccharides.

Fractions	Source	M_n^8 (g/mol)	M_w^9 (g/mol)	\bar{D}^{10}	Intrinsic Viscosity (mL/g)	DP ¹¹
ALCM ¹	<i>C. myriophylloides</i>	59 000 (±1.3%)	76 000 (±0.9%)	1.3 (±1.6%)	224.2 (±1.5%)	352
ALSM ²	<i>S. muticum</i>	173 000 (±1.7%)	202 000 (±1.7%)	1.2 (±2.3%)	738.6 (±1.0%)	935
OACM ³	<i>C. myriophylloides</i>	11 000 (±4.9%)	19 000 (±5.6%)	1.7 (±7.4%)	35.8 (±6.0%)	88
OASM ⁴	<i>S. muticum</i>	2 000 (±9.8%)	3 000 (±10.8%)	1.04 (±14.5%)	8.4 (±3.1%)	14
FUCCM ⁵	<i>C. myriophylloides</i>	37 000 (±3.0%)	148 000 (±1.0%)	4.0 (±3.1%)	84.4 (±3.7%)	Nd ¹²
AGP ⁶	<i>C. decortiatum</i>	1 295 000 (±0.9%)	2 042 000 (±0.7%)	1.6 (±1.2%)	560.6 (±4.%)	Nd
AGB ⁷	<i>C. decortiatum</i>	711 000 (±1.4%)	2 173 000 (±0.4%)	3.1 (±1.4%)	266.7 (±3.0%)	Nd

¹ ALCM: alginate extracted from CM; ² ALSM: alginate extracted from SM; ³ OACM: LMW alginates obtained from ALCM; ⁴ OASM: LMW alginates obtained from ALSM; ⁵ FUCCM: fucoidan extracted from CM; ⁶ AGB: sulfated glycoprotein (crude extract) extracted from CD; ⁷ AGP: sulfated glycoprotein (purified extract) extracted from CD; ⁸ M_n : number average molecular weight; ⁹ M_w : weight average molecular weight; ¹⁰ \bar{D} : index of Polydispersity; ¹¹ DP: degree of polymerization; ¹² Nd: Not determined.

3.3. Monosaccharide Composition of Saccharide Fractions

The monosaccharide composition was analyzed by GC-MS-EI and the results are summarized in **Table IV. 3**. For uronic acids, only the molar ratio of mannuronic acid was defined for alginates and fucoidan. ALCM and ALSM were mainly composed of 74.61% and 87.59% of ManA, respectively, with minor impurities of fucose and glucose. FUCCM presented 53.39% of fucose and 45.70% of ManA, which indicates the contamination by alginate, confirming the colorimetric analysis presented in Section 3.1. In contrast to prior research, fucoidans isolated from *Cystoseira* spp. and other species contained monosaccharides other than fucose, including galactose, glucose, mannose, xylose, rhamnose, and uronic acids [42,49,56]. On the other hand, sulfated homofucans were isolated from *C. crinita*, *C. compressa*, and *C. sedoides*, of which fucose constituted 43.4%, 61.5%, and 54.5%, respectively [50]. The monosaccharide composition of fucoidans may differ depending on the source of brown macroalgae. The FUCCM isolated from CM is a sulfated homogenous fucoidan made up mainly of fucose.

Table IV. 3. GC/MS-EI analysis of different saccharide extracts.

Monosaccharides (%Molar Ratio)	AGB ¹	AGP ²	FUCCM ³	ALCM ⁴	ALSM ⁵
Galactose (Gal)	46.07 ± 1.46	51.55 ± 2.67	Nd	Nd	Nd
Glucose (Glc)	38.78 ± 0.24	24.56 ± 1.05	0.91 ± 0.10	0.55 ± 0.09	1.13 ± 0.57
Arabinose (Ara)	12.08 ± 1	17.32 ± 1.33	Nd	Nd	Nd
Xylose (Xyl)	1.70 ± 0.23	3.93 ± 0.61	Nd	Nd	Nd
Rhamnose (Rha)	1.37 ± 0.13	2.63 ± 0.15	Nd	Nd	Nd
Fucose (Fuc)	Nd ⁶	Nd	53.39 ± 4.57	24.84 ± 4.74	11.28 ± 1.23
Mannuronic acid (ManA)	Nd	Nd	45.70 ± 4.52	74.61 ± 4.81	87.59 ± 0.85

¹ AGB: sulfated glycoprotein (crude extract) extracted from CD; ² AGP: sulfated glycoprotein (purified extract) extracted from CD; ³ FUCCM: fucoidan extracted from CM; ⁴ ALCM: alginate extracted from CM; ⁵ ALSM: alginate extracted from SM; ⁶ Nd: Not determined.

3.4. ¹H NMR Analysis of ALCM, ALSM, and FUCCM

Fractions The alginates and fucoidan were analyzed using ¹H NMR, and the findings are presented in **Figure IV. 3**, **Table IV. 4** and **Table IV. 5**. FUCCM is mainly composed of α -L-fucose presenting intense signals at 1.26–1.47 ppm, which are the characteristic regions of fucopyranose assigned to the CH₃ methyl group of H-6 [56] and at 5.13 ppm confirming the α -linked fucose monomers [57]. Peaks at 1.94–2.15 ppm correspond to the CH₃ proton of *O*-acetyl groups [58]. H-1 and C-H protons of *O*-substituted carbons of α -L-isomers extend from 5.09 to 5.45 ppm, whereas ring proton H-2-H-5 spread out from 3.56 to 4.86 ppm [49]. By comparing with proton chemical shifts of each *O*-sulfated L-fucose isomer [59], the resonance of H-6 in the region of 1.38 ppm indicates that sulfates could be bonded to C-4 of FUCCM [58] (**Table IV. 5**). In order to determine more precisely the sulfate group positions, it is required to investigate as well the desulfated fucoidan residues. Other peaks appearing slightly at 4.73 and 4.77 ppm could be attributed to the mannuronic acid anomeric protons due to the presence of alginate traces in the FUCCM extracts. The ¹H NMR spectra of alginates ALCM and ALSM (**Figure IV. 3**) were used to determine the structural block distribution and the rate of mannuronic and guluronic acid of each fraction through the M/G ratio (**Table IV. 4**). According to Grasdalen et al. [35], signals at 5.07 and 4.46 ppm (signal I and signal III) were attributed to H-1 and H-5 of guluronic acid (G) residues, respectively. The anomeric proton of mannuronic acid (H-1) and the C5 of alternating blocks (GM-5) (signal II) imbricated at 4.76–4.68 ppm. Minor signals at 1.21–1.27 ppm for ALSM and 1.20–1.42 ppm for ALCM correspond to fucopyranose residues, which confirm the results obtained by GC-MS-EI analysis, indicating the presence of slight impurities of fucoidan. Each alginate fraction showed a different M/G

ratio, which was 0.87 and 1.12 for ALSM and ALCM, respectively. This indicates that alginate extracted from Moroccan *S. muticum* is rich in guluronic acid (54%), while ALCM extracted from *C. myriophylloides* exhibits higher levels of mannuronic acid (53%) compared to guluronic acid (47%). The M/G ratio of alginates extracted from *S. muticum* ranged from 0.31 to 1.08 [25,45,47], these variations within the species are related to harvesting conditions (place and period). In contrast to the current finding, the M/G ratios were < to 1 for *C. barbata*, *C. schiffneri*, *C. compressa*, and *C. trinodis* [40–44]. The estimated η parameter was < to 1 for both alginate samples, indicating the abundance of homopolymeric sequences (MM and GG) as shown in **Table IV. 4**.

Table IV. 4. Structural characterization of ALCM and ALSM extracted from *C. myriophylloides* and *S. muticum*.

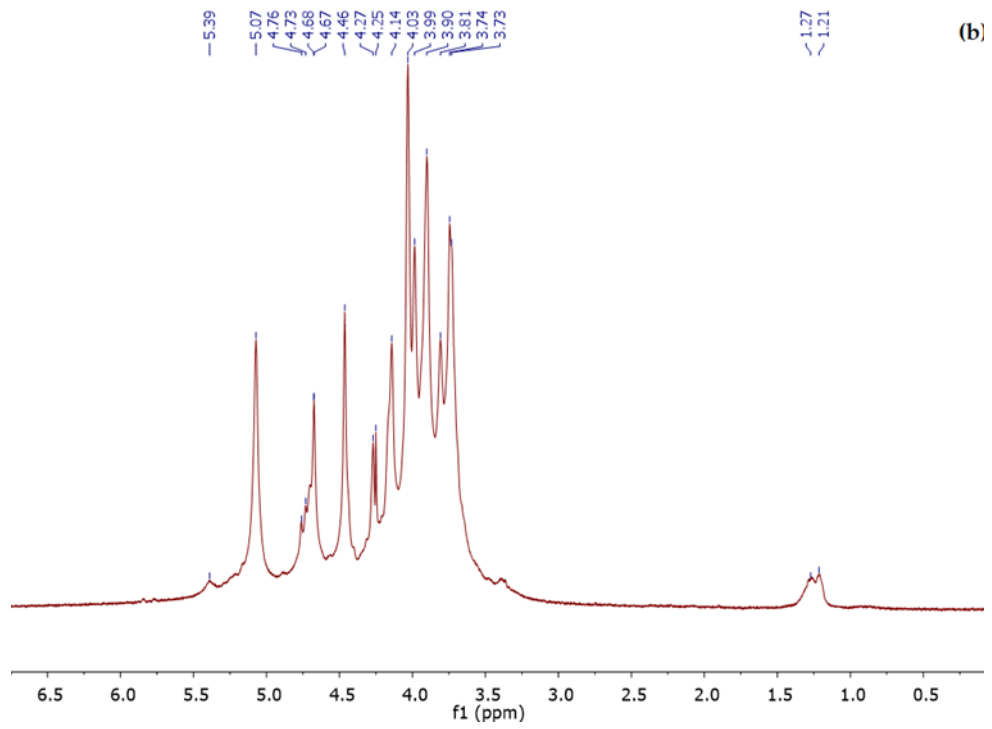
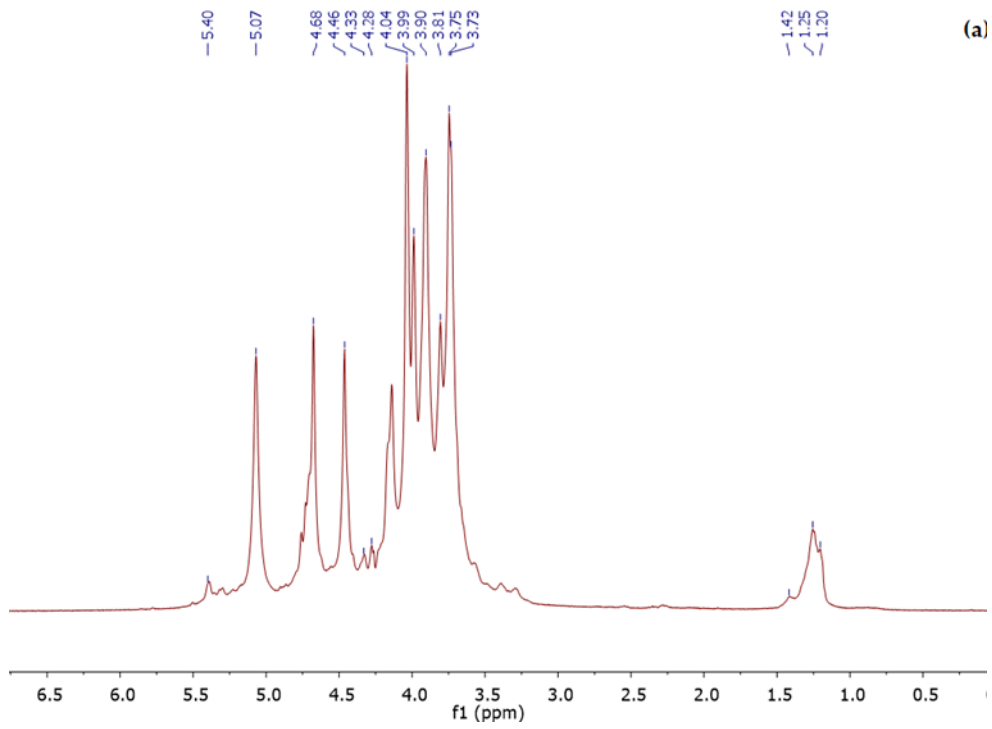
Alginates	Source	Frequencies of Structural Blocks							% M	% G	η
		F _G	F _M	F _{GG}	F _{MM}	F _{MG}	F _{GM}	M/G			
ALSM ¹	<i>S. muticum</i>	0.54	0.46	0.47	0.40	0.063	0.063	0.87	46	54	0.25
ALCM ²	<i>C. myriophylloides</i>	0.47	0.53	0.43	0.49	0.038	0.038	1.12	53	47	0.15

¹ ALSM: alginate extracted from SM; ² ALCM: alginate extracted from CM.

Table IV. 5. ¹H NMR chemical shifts of fucoidan extracted from *C. myriophylloides* (FUCCM) and sulfated fucopyranoses standard.

Fucose	H-1	H-2	H-3	H-4	H-5	H-6
α -L-Fuc _p [59]	5.20	3.77	3.86	3.81	4.20	1.21
α -L-Fuc _p (FUCCM ¹)	5.13 (-0.07)	3.76 (-0.01)	3.89 (0.03)	3.83 (0.02)	4.17 (-0.03)	1.26 (0.05)

¹ FUCCM: fucoidan extracted from CM.



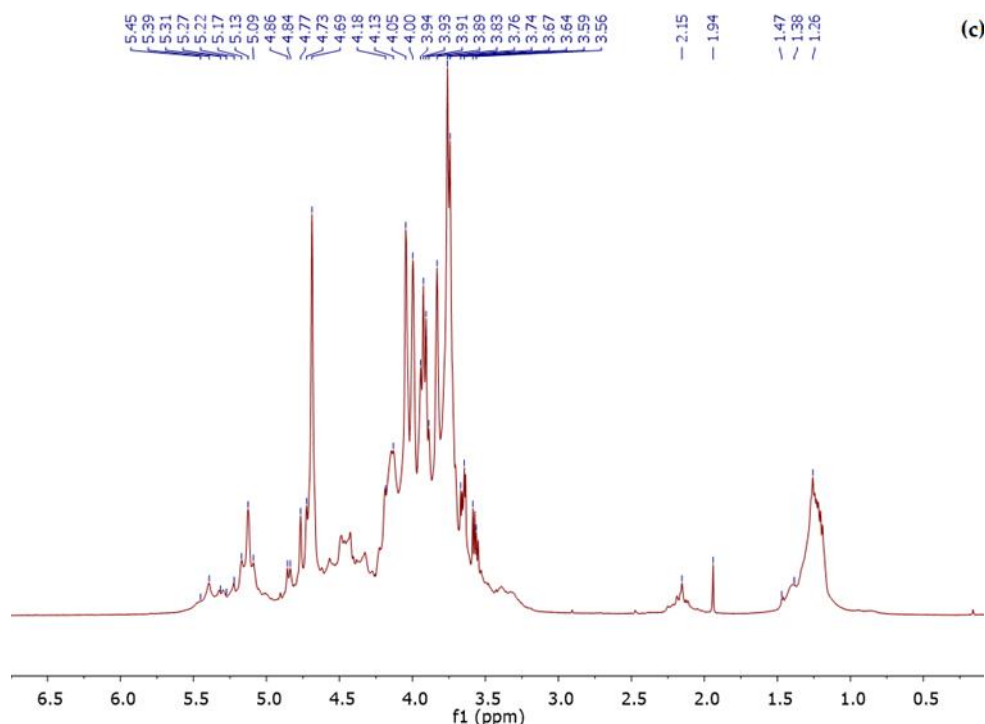


Figure IV. 3. ^1H NMR analysis of alginate (ALCM) (a) and fucoidan (FUCCM) (c) co-extracted from brown seaweed CM, and ALSM isolated from SM (b).

3.5. Sulfated and Unsulfated Polysaccharides and Oligosaccharides induce Natural Defenses in Olive Tree Leaves

To evaluate the potential effect of the seven saccharide fractions on the natural defenses of the olive tree, leaf discs of 5 mm were used as a model system and were treated for 24 h with different concentrations. The PAL and TAL activities, the phenolic compounds, and the lignin content were then measured. To our knowledge, no study has reported the capacity of alginate (unsulfated polysaccharides), LMW alginates, fucoidan, and sulfated arabinogalactan-rich protein to induce the natural defense of olive trees.

PAL and TAL are monofunctional enzymes belonging to the plant aromatic aminoacid-lyase family [60]. The PAL catalyzes the deamination of L-phenylalanine into trans-cinnamic acid, while the TAL converts the L-tyrosine yielding *trans-p*-coumarate. PAL is the most widely studied enzyme in plant phenolic synthesis. However, TAL is another enzyme involved in this pathway, that has rarely been investigated. This is the first report exploring the activity of TAL in the leaves of the olive tree.

3.5.1. Evaluation of Enzyme Activities and Secondary Metabolism Synthesis in the Leaf Discs of the Olive Tree in Response to Alginates and Their Oligomers

The application of alginates with different molecular weights, ALCM (202 kDa), ALSM (76 kDa), OACM (19 kDa), and the oligomers OASM (3 kDa), significantly induced the PAL and TAL activities, the phenolic compounds, and the lignin content in the leaves of the olive tree depending on the concentration of saccharide solutions ($p < 0.05$). After treatment with OASM, the PAL and TAL activities revealed a considerable increase at different concentrations, reaching an upward trend at 2 g/L. The polyphenols followed a similar pattern and rose at 0.5 g/L and 1 g/L, while no significant increment was observed in lignin content. Compared to their native polymer (ALSM) at 2 g/L, the oligoalginates (OASM) showed the induction of PAL and TAL activities, 1.4–1.6 times higher (**Figure IV. 4**), as well as efficiency at a low concentration (0.5 g/L). On the other hand, the accumulation of polyphenols and lignin in the leaves was significantly increased after the treatment with ALSM polymers. The elicitor activity of the LMW alginates (OACM) was less important compared to the other fractions. In response to the native alginate polymers, ALCM showed significant stimulation of PAL and TAL activities at a low concentration (0.5 g/L) compared to ALSM, while the secondary metabolites (lignin and polyphenols) were strongly promoted after the application of ALSM (**Figure IV. 5**). Lignin was only promoted in response to high molecular weight alginates polymers (ALCM and ALSM). In this study, the PAL and TAL activities showed a similar trend. In contrast, a considerable fluctuation was observed. All fractions reported a PAL/TAL ratio $<$ to 1, ranging from 0.34 to 0.77, indicating the high expression of TAL in the leaves of the olive tree compared to PAL. As mentioned before, PAL and TAL are the key regulatory enzymes associated with the biosynthesis of various secondary metabolites via the shikimic pathway [61]. Nevertheless, PAL is the most occurs in plants [60], while TAL enzyme is commonly found in Gramineae such as wheat and maize [62-65]. Recently, TAL has been detected in other plants such as orchids, strawberry fruits, and leaves, ginger and soybean leave in response to biotic and abiotic stresses [61,66-68]. As reported by Khan et al., the treatment of soybean by chitin and chitosan oligomers significantly induced the PAL, TAL, and phenolic compounds in leaf tissues, which the specific activity of TAL was lower compared to PAL [67].

In this study, the elicitor capacities of alginate polymers and their derivatives depended on many factors such as concentration, nature of polymers, and chain length. The OASM (3 kDa), ALCM (76 kDa), and OACM (19 kDa) were more effective due to their abilities to activate the PAL

and TAL at a low concentration (0.5 g/L) compared to ALSM (202 kDa). However, the OASM (3 kDa) exhibited the highest levels of enzyme activities at 2 g/L. Alginate is an anionic polysaccharide isolated from brown algae. Recently, it has been widely utilized in agriculture as an inducer of the natural defenses of plants. The application of alginates extracted from *B. bifurcata* and *F. spiralis* triggered PAL activity and polyphenol synthesis in leaves of tomato seedlings and roots of date palm [18-20]. Few research has focused on the use of LMW alginates as inducers of natural defenses. Partial hydrolysis with HCl of alginates extracted from *Lessonia vadosa* enhanced the PAL and peroxidase (POD) activities in wheat plants, higher levels were recorded after the treatment with polymannuronic enriched fraction [69]. A recent study has reported the effectiveness of oligoalginates (5.5 kDa), obtained after oxidative degradation, compared to their native polymer, showing their capacity to stimulate the PAL activity as well as the phenolic compounds in the leaves of tomato seedlings at a concentration of 1 g/L [18]. It has been conclusively shown that oligoglucuronans and oligoulvans with low DP (2–3) enhanced the activation of PAL, antioxidant-related enzymes as well as the accumulation of lignin and phenolic compounds, protecting apple fruit against *Penicillium expansum* and *Botrytis cinerea*. These oligomers were more effective than their native polymers [22,23]. No previous study to date has examined the elicitor effects of alginates and their derivatives in leaf tissues of the olive tree.

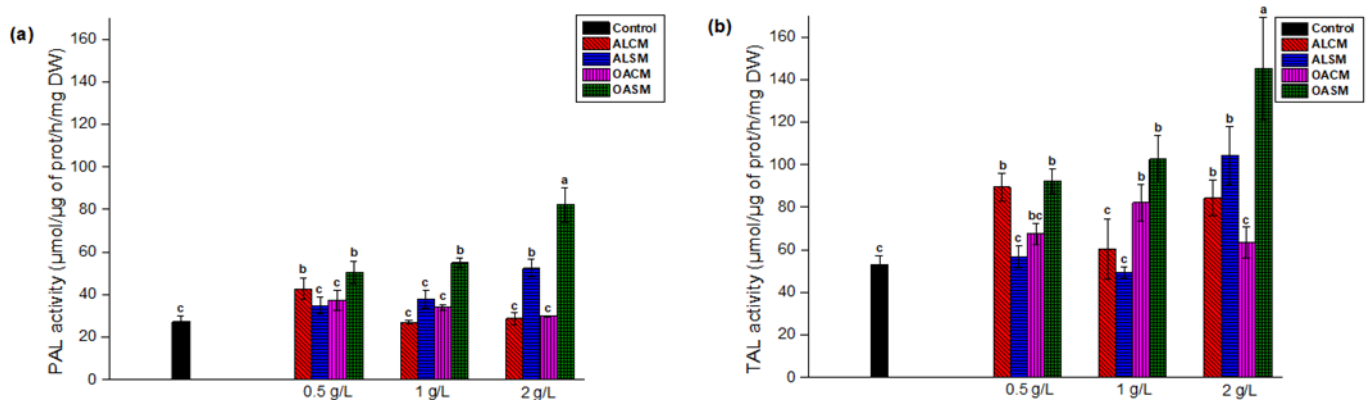


Figure IV. 4. Effect of alginates extracted from CM (ALCM) and SM (ALSM) and their derivatives (OACM and OASM) on the induction of PAL (a) and TAL (b) activities in the leaves of olive tree after 24 h of treatment. The letters (a, b, c) indicate significant differences between treatments at each concentration using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

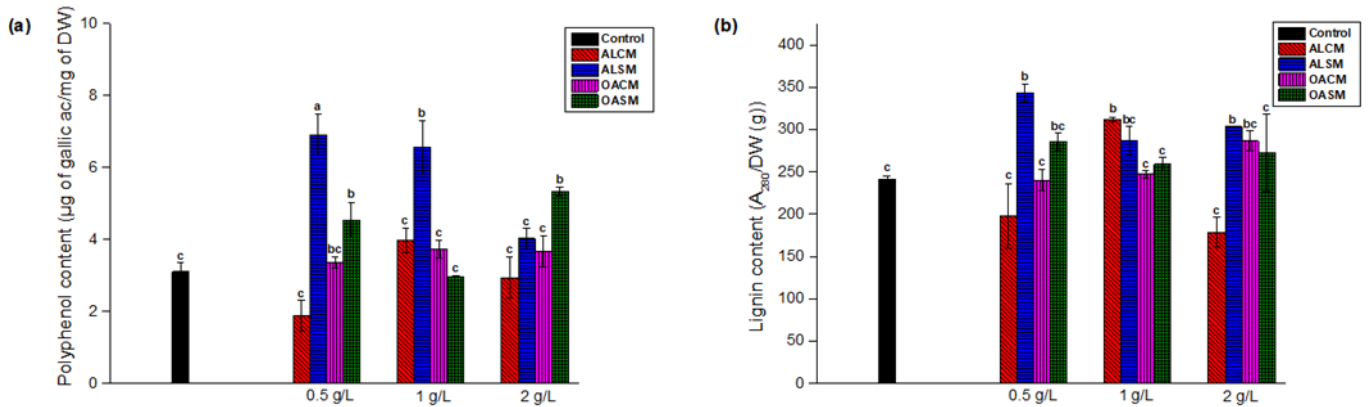


Figure IV. 5. Effect of alginates extracted from CM (ALCM) and SM (ALSM) and their derivatives (OACM and OASM) on the induction of polyphenol (a) and lignin (b) contents in the leaves of olive tree after 24 h of treatment. The letters (a, b, c) indicate significant differences between treatments at each concentration using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

3.5.2. Evaluation of Enzyme Activities and Secondary Metabolism Synthesis in the Leaf Discs of the Olive Tree in Response to Sulfated Polysaccharides AGB, AGP and FUCCM

The sulfated polysaccharides, arabinogalactan, and fucoidan extracted from the brown and green seaweed were applied to the olive leaves at different concentrations. The results showed significant induction of the PAL and TAL activities accompanied by the synthesis of lignin and polyphenols ($p < 0.05$). These responses depend on the nature of the polymers and their concentrations. In response to the tree fractions, the phenolic metabolism was significantly stimulated, at the low concentration (0.5 g/L). FUCCM revealed an important elicitor capacity at 0.5 g/L, showing high levels of PAL, polyphenols, and lignin compared to arabinogalactan (crude and purified extracts). The TAL was less expressed than PAL after the treatment by the fucoidan, indicating a PAL/TAL ratio higher than 1. On the other hand, for the crude (AGB) and the purified (AGP) extracts, the PAL/TAL ratio was in the range of 0.45–0.75, showing high activity of the TAL enzyme. No considerable difference was observed in the elicitor effects between AGB and AGP (Figure IV. 6 and Figure IV. 7). A similar conclusion was reached in the previous work, after the application of sulfated arabinogalactan AGB and AGP for the first time as inducers of PAL activity, phenolic compounds, and lignin content in leaf and root tissues of tomato seedlings [27]. The application of sulfated polysaccharides from marine algae as stimulators of natural defenses of plants has been largely reported. Carrageenan, a sulfated

galactan presented in the red algae, was found to be capable of activating the defense mechanisms against a large wide of pathogens in *Arabidopsis thaliana*, tobacco, and tomato plants [70-75]. The sulfated heteropolysaccharides, ulvan, from the green seaweed have been shown to trigger the pathogenesis-related proteins gene expression, reactive oxygen species metabolism, and the octadecanoid pathway, leading to protection against *Zymoseptoria tritici* in wheat [76]. The treatment of *A. thaliana* plants by ulvans with sulfate content of 18.9 to 36.6% reduced the severity of *Alternaria brassicicola* and *Colletotrichum higginsianum* independently of sulfation degree [77]. These findings are in accordance with those reported in this work and the previous work [27], in which the AGB and AGP showed similarities in responses despite their sulfation variation. Furthermore, several studies have investigated the elicitor effects of ulvans in several plants [21,23,78-80]. The ability of fucoidan to provide protection and enhance natural defenses in plants has been previously assessed only to a very limited extent. Fucoidans from the brown macroalgae *B. bifurcata* and *F. spiralis* stimulated PAL activity, polyphenols, and lignin content in the roots of the date palm [19]. Detached leaves of *Datura stramonium* L. and *Nicotiana tabacum* L. infected by potato virus X (PVX) and tobacco mosaic virus (TMV), respectively, exhibited an inhibition of the development of the virus during the first stage of infection after treatment with fucoidan extracted from the brown alga *F. evanescens* [81,82]. In addition, fucoidan from *Lessonia vadosa* activated the PAL, lipooxygenase and glutathioneS-transferase activities in tobacco plants [24].

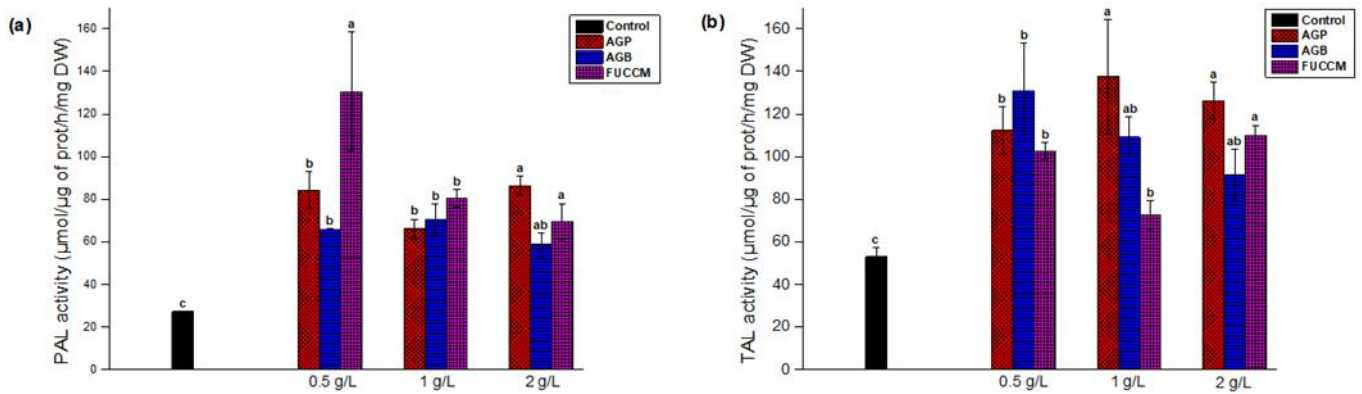


Figure IV. 6. Effect of sulfated polysaccharides AGB and AGP extracted from CE and FUCCM isolated from CM on the induction of PAL (a) and TAL (b) activities in the leaves of olive tree after 24 h of treatment. The letters (a, b, c) indicate significant differences between treatments at each concentration using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

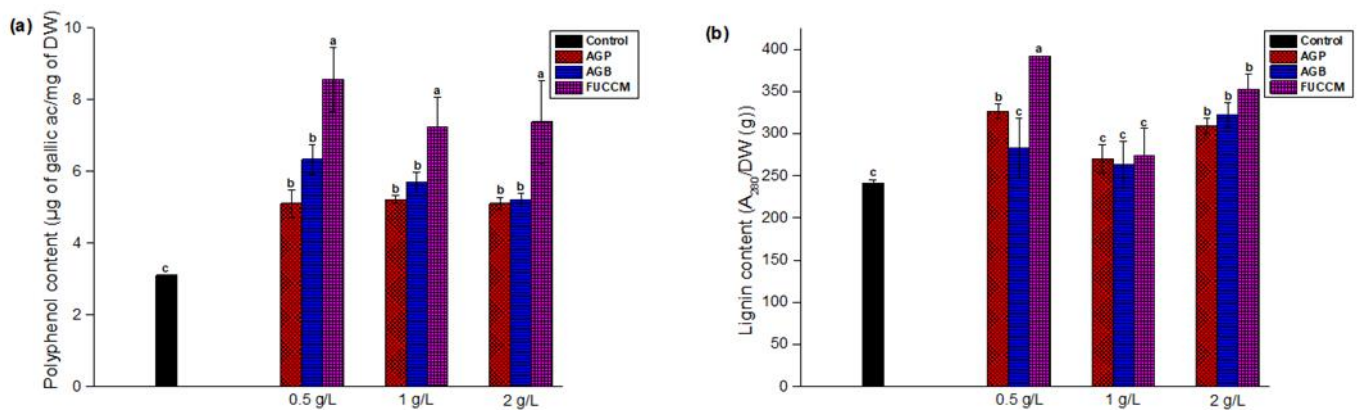


Figure IV. 7. Effect of sulfated polysaccharides AGB and AGP extracted from CE and FUCCM isolated from CM on the induction of polyphenol (a) and lignin (b) contents in the leaves of olive tree after 24 h of treatment. The letters (a, b, c) indicate significant differences between treatments at each concentration using the Tukey-HSD test, at ($p < 0.05$). Treatments presented with the same letter are not significantly different.

Compared to alginates (ALCM and ALSM), the sulfated polysaccharides (AGP, AGB, and FUCCM) exhibited an elicitor capacity more effective at low concentrations (0.5 g/L and 1 g/L). In response to fucoïdan at 0.5 g/L, the levels of the PAL activity, phenolic compounds, and lignin content were significantly higher compared to AGB, AGP, ALCM, and ALSM. The sulfate group and the low M_w seem to be the principal factors in this fluctuation of plant defense responses. It has been shown before that the elicitation by glucuronan (unsulfated polysaccharide) had no considerable effects on PAL activity of tomato seedlings, while ulvan

(sulfated polysaccharides) exhibited significant levels [21]. In addition, the desulfation of ulvan has shown suppression of the elicitor effect in the leaves of tomato plants, indicating the main role of sulfate substituent in the elicitation efficiency [21]. Several studies have found that desulfation reduces biological activities [83-86]. The use of polysaccharides extracted from seaweeds as olive resistance-inducers has been previously assessed only to a very limited extent. Tziros et al. have reported the evaluation of laminarin (Vacciplant 4.5SL) efficiency, against olive leaf spot (OLS) disease, caused by *Fusicladium oleagineum* on six-month-old olive plants [87]. The application of laminarin suppressed the disease severity and no symptoms were detected, compared to copper pesticides used as reference treatments. Moreover, the expression of PAL, copper amine oxidase (Cuao), and major pollen allergen (Mpol) genes was induced in laminarin-treated plants [87]. Another study has involved the ability of ulvan to control verticillium wilt of olive (VWO) caused by *V. dahlia* [88]. In response to this elicitor, the phenolic metabolism (PAL, polyphenols, and lignin) was significantly enhanced, reducing the verticillium wilt in the twigs (10 cm) of the olive tree (Picholine Marocaine) [88]. To the best of our knowledge, no study to date has examined the fucoidan, alginates from the brown macroalgae *S. muticum* and *C. myriophylloides* and their derivatives, as well as the sulfated glycoprotein from the green seaweed *C. decorticum*, as inducers of natural defenses in the leaves of the olive tree. Nevertheless, further studies are required to evaluate the efficiency of these polysaccharides in stimulating and protecting against vascular and foliar pathogens of the olive tree under field conditions.

4. Conclusions

In the current work, alginates, LMW alginate derivatives, fucoidans from the brown seaweed *S. muticum* and *C. myriophylloides*, and the glycoproteins isolated from *C. decorticum* were characterized chemically and structurally. These saccharide extracts were investigated for their ability to induce the PAL and TAL activities, polyphenol, and lignin contents in the leaves of the olive tree (Picholine Marocaine). Sulfated polysaccharides (FUCCM, AGB, and AGP) were found to be the most active at a low concentration (0.5 g/L) compared to alginates (unsulfated polysaccharides), indicating the main role of the sulfate group in the efficiency of the elicitor activity. Fucoidan, with an M_w of 148 kDa and 12.83% of sulfates was more effective than the other polymer fractions. The activity of alginate polymers and their derivatives was related to their concentrations and chain length. When compared to ALSM (202 kDa), the low molecular weight alginates OASM (3 kDa), ALCM (76 kDa), and OACM (19 kDa) were more efficient

in inducing PAL and TAL activities at low concentrations. In addition, oligoalginates (OASM) manifested the highest levels at 2 g/L. PAL/TAL ratios were < to 1, indicating that the TAL enzyme was highly expressed in the leaves of the olive tree in response to all saccharide fractions except for fucoidan. The expression of the TAL enzyme in olive tree leaf tissue was examined for the first time. These results highlight the use of alginates, LMW alginates, fucoidan, and glycoprotein from marine macroalgae, as inducers of natural defenses of the olive tree, to protect against phytopathogens in the context of sustainable development. However, future research should consider the protective effects of these molecules against biotic and abiotic stresses under field conditions.

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CHAPITRE V

EVALUATION DE L'EFFICACITÉ DES MÉCANISMES DE DÉFENSE INDUITS PAR LES DIFFÉRENTS ÉLICITEURS SUR LE PATHOSYSTÈME TOMATE/*VERTICILIUM DALLIAE*



Article 5: Prochainement soumis

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Contexte scientifique

Les travaux présentés dans les chapitres précédents ont permis de montrer la capacité des différentes fractions saccharidiques à mobiliser les mécanismes des défenses naturelles des plants de tomate et des disques foliaires de l'olivier. Cependant il était nécessaire de vérifier l'impact de l'induction des voies métaboliques impliquées dans la défense de la plante sur ses capacités à se protéger contre le dépérissement généré par l'attaque d'un bioagresseur. Ainsi, l'objectif de ce dernier chapitre est l'étude de la capacité des saccharides extraits des algues brune et verte issues des côtes marocaines à protéger les jeunes plantules de tomate contre le développement du pathogène vasculaire *Verticilium Dahliae*, ainsi que les symptômes destructeurs qui lui sont associés. Cette étude pourrait argumenter une nouvelle orientation pour la gestion de la verticilliose.

La verticilliose causée par *V. dahliae* est une maladie cryptogamique, particulièrement difficile à contrôler. Ceci est dû à la nature du pathogène qui lui confère la capacité de survivre dans le sol pendant de longues périodes et d'attaquer une large gamme d'hôtes [1]. Plus de 200 espèces végétales sont sensibles à *V. dahliae*, notamment le coton, l'olivier, la pomme de terre, l'aubergine, le poivron, le chou et la tomate [2]. Considérée comme l'une des cultures les plus importantes sur le plan économique au monde, la tomate est une riche source de minéraux, de vitamines, d'acides organiques, d'acides aminés essentiels et de fibres. Sa production réussie au champ et en serre dépend principalement du contrôle des agents pathogènes et d'une nutrition minérale adéquate. La verticilliose de la tomate constitue une contrainte majeure dans la production de cette culture pour plusieurs raisons telles que sa distribution mondiale et l'existence de différentes races pathogènes [3]. Actuellement, seules des mesures préventives sont utilisées pour gérer cette maladie, telles que la fumigation chimique à base de dichloropropène, de chloropicrine, de dazomet et de diméthyle disulfure (DMDS), le développement de variétés résistantes de tomates, le greffage et le traitement par les agents de lutte biologique (BCA) [3]. Cependant, leur utilisation présente plusieurs limites, notamment l'augmentation des préoccupations environnementales et sanitaires ainsi que le développement d'une résistance de l'agent pathogène, inconvénient qui rejoint celui des pesticides. Par conséquent, l'accent est de plus en plus mis sur les technologies respectueuses de l'environnement pour contrôler cette maladie. Il s'agit notamment de stimuler les défenses naturelles de la plante par des éliciteurs naturels, telle que les polysaccharides extraits d'algues marines et leurs dérivés.

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Dans le présent chapitre, les travaux ont été menés sur des jeunes plantules de tomate âgées de 3 semaines. Les solutions saccharidiques ont été appliquées par pulvérisation ou par irrigation du sol avec un volume de 10 mL. Le premier traitement a lieu 3 semaines après la transplantation et avant l'inoculation du pathogène. La seconde application a été effectuée 3 semaines plus tard. Deux jours suivant la première élicitation, les plants de tomates ont été inoculés avec 10 mL de la suspension conidienne de *V. dahliae* (souche : OMV₆) après avoir blessé le collet avec une aiguille stérile. L'efficacité des produits testés a été comparée à celle d'un témoin non traité ainsi qu'à un référent SDP qui est la laminarine (substance active de l'Iodus 40®). Le développement des symptômes de la verticilliose a été évalué tous les 7 jours pendant 5 mois. Enfin, les molécules élictrices ont été évaluées pour leur activité sur la croissance de *V. dahliae* (OMV₆) dans le but de vérifier la possibilité de la contribution d'une action directe des fractions saccharidiques sur le pathogène à l'effet protecteur observé sur la plante.

Les résultats de cette étude sont présentés sous forme d'un article, en préparation pour publication, intitulé « **Saccharide fractions extracted from marine seaweed induce immunity against *Verticillium dahliae* in tomato: new orientation for verticillium wilt management** ».

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Références

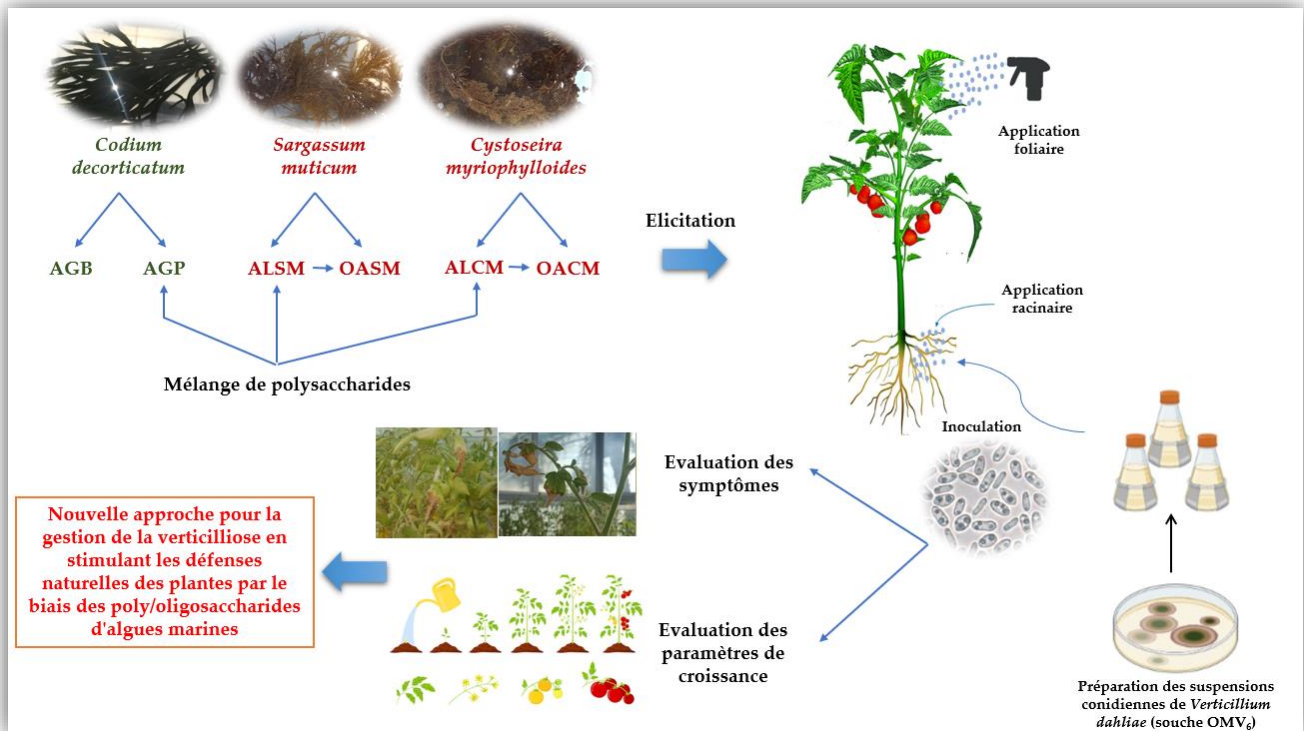
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Article 5 : prochainement soumis

Saccharide fractions extracted from marine seaweed induce immunity against *Verticillium dahliae* in tomato: new orientation for verticillium wilt management

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Résumé graphique :



Saccharide fractions extracted from marine seaweed induce immunity against *Verticillium dahliae* in tomato: new orientation for verticillium wilt management

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Abstract

This study aims to evaluate for the first time the ability of polysaccharides and oligosaccharides from marine seaweed to manage verticillium wilt caused by the soil-borne fungus *Verticillium dahliae* (OMV₆ strain) in tomato plants. Alginates (ALCM and ALSM), low molecular weight alginates derivatives (OACM and OASM), sulfated glycoproteins (AGB and AGP), and their combination were used as elicitors. Young seedlings were treated by foliar spray and root-drenching 48 h before pathogens inoculation. Results showed that saccharide-pretreatment induced OMV₆ resistance and improved growth of tomato plants compared to control and laminarin (commercial polysaccharide from brown seaweed, known as inducers of natural defenses of plants). The different saccharide extracts delayed disease onset. After 13 weeks of inoculation, the disease incidence achieved 100 % in the control group and 75-90 % in laminarin-treated plants. In contrast, in response to other saccharide treatments, the verticillium

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wilt was reduced by about 15-55% after foliar spray and 50-80% after root-drenching. After 5 months, saccharides-treated plants presented a severity of 1.7-2-time and 1.3-1.7-fold lower compared to control and laminarin seedling groups, respectively. In addition, the mortality rate was in the range of 0-25 % in saccharide-pretreated plants and 30 % in laminarin-elicited seedlings. The final mean severity (FMS), area under the disease progress curve (AUDPC), and the dwarfing index (DI) exhibited lower levels in stimulated plants compared to untreated tomato seedlings. The application of different fractions suppresses stunting growth caused by *V. dahliae*, especially, in plants treated by root-drenching. In the presence of the pathogen, saccharide elicitors were found to improve the shoot length and tomato yield with a greater number of fruits depending on the application method. These results highlighted a novel approach to managing verticillium wilt in tomato by inducing innate immunity of plants through polysaccharides and oligosaccharides from marine algae.

Keywords: Verticillium wilt, Tomato seedlings, Elicitors, Polysaccharides, Oligosaccharides, Marine algae.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely consumed and cultivated horticultural crops worldwide. In the world, the production is currently estimated to be around 100 million tons of fresh fruit per 3.7 hectares [1]. Verticillium wilt, caused by the soil-borne fungi *Verticillium dahliae* is one of the fungal diseases affecting the productivity and economic sustainability of tomato plants worldwide [2]. The fungus belongs to the Ascomycota phyla which survive in the soil for years under the difficult condition as resistance structures named microsclerotia [3]. This makes it extremely dangerous, and difficult to manage. Once plants are infected by *V. dahlia*, there are no fungicides available to mitigate the disease [2]. Chemical fumigation of soil using methyl bromide is the main recommended technique for managing verticillium wilt [2]. However, this method presents a negative impact on the biochemical features of soil and microbiome biomass [4-6]. Recently, non-chemical measures have been explored, including biologicals, biocontrol agents [7], anaerobic soil disinfestation [8], and organic Amendments [9]. Despite their environment-friendly manners, their usage is limited because of the variability of effectiveness depending on fields, seasons, and crops. By understanding the mechanisms of plant-pathogen interactions, new alternatives have emerged based on the stimulation of the innate immunity of plants through natural elicitors.

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Polysaccharides and oligosaccharides from marine seaweed have been previously reported as inducers of plant resistance against a wide range of pathogens [10-13]. These biotic elicitors are recognized by plant receptors (pattern recognition receptors (PRRs)) as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) to induce the PAMP-triggered immunity (PTI) [14]. As far as we know, no previous research has investigated the capacity of saccharide elicitors from marine algae to protect against the vascular fungus *V. dahliae* in tomato or other crops.

In the present study, alginates and their derivatives from two brown algae *Sargassum muticum* and *Cystoseira myriophylloides*, sulfated glycoproteins from the green alga *Codium decortdatum*, and their combination were used for the first time to manage verticillium wilt of tomato caused by the soil-borne fungus *V. dahliae*.

2. Materials and Methods

2.1. Preparation of saccharide fractions

Alginates (ALCM and ALSM) and their derivatives (OACM and OASM), sulfated arabinogalactan-rich protein (AGP and AGB), and a mixture of ALCM, ALSM, and AGP were studied. ALCM and ALSM were extracted from the brown algae *S. muticum* and *C. myriophylloides*, respectively. The crude (AGB) and purified (AGP) fractions were isolated from the green seaweed *C. decortdatum* [15]. Low molecular weight alginates (OACM and OASM) were obtained after oxidative degradation of ALCM and ALSM, respectively. The extraction, purification, and characterization of all extracts were previously described in detail [15,16]. The tree species of seaweed were collected from the Atlantic coast of Morocco (El Jadida, Sidi Bouzid) in July 2019.

The saccharide solutions were prepared at a concentration of 1 g/L (pH 6). Commercial laminarin from *Laminaria digitata* (SIGMA) was used as a control. It's a polysaccharide from brown seaweed successfully marketed for crop protection.

2.2. Fungal and plant materials

Verticillium dahliae strain (OMV6) causing Verticillium wilt was provided by the team of Laboratory of Botany, Biotechnology and Plant Protection at the University Ibn Tofail, Faculty of Sciences Kenitra, Morocco. The pathogen was grown on potato dextrose agar medium for 15 days at 25 °C. Fungal colonies were transferred to potato dextrose broth, and incubated for

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2 days at 25 °C, under stirring (100 rpm). The conidial suspension was filtered through two layers of muslin to remove mycelium and adjusted to a final concentration of 1×10^6 conidia. mL^{-1} .

Campbell 33 seeds were sown in peat substrate for 20 days. The young seedlings were then individually transplanted into pots containing soil and peat (v/v) and cultivated under greenhouse conditions.

Saccharide solutions were applied twice to the young seedlings with 10 ml via foliar spray and 10 ml via root-drenching (over roots, around the crown of each plant). The first treatment was performed after 3 weeks of transplantation and the second was carried out 3 weeks later. Two days after the first elicitation, the tomato plants were inoculated with 10 mL of conidial suspension after wounding the upper part of the stem using sterile pins. The positive and negative control were inoculated with 10 ml of fungal suspension and 10 mL of sterilized water, respectively.

2.3. Symptom Assessments

Disease observations were recorded on each plant every 7 days for 5 months from 20 tomato plants/treatment. The seedlings were arranged in completely randomized blocks. According to the percentage of Maximum Intensity Symptoms (MIS), the verticillium wilt was evaluated following a scale ranging from 0 to 4 : 0: 0% (no symptoms); 1: 25% MIS; 2: 50% MIS; 3: 75% MIS; 4: 100% (dead plants) [17]. The mortality rate, disease incidence, disease severity, final mean severity (FMS), area under the disease progress curve (AUDPC), and the dwarfing index (DI) were calculated as follows:

$$\text{Mortality rate} = \frac{\text{Number of dead plants}}{\text{Total number of plants}} \times 100$$

$$\text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

$$\text{Disease severity} = \frac{(n_A \times 0) + (n_B \times 1) + (n_C \times 2) + (n_D \times 3) + (n_E \times 4)}{\text{Total number of plants}}$$

$$\text{AUDPC} = \left[\sum_{i=1}^n \left(\frac{S_i S_{i-1}}{2} \right) \Delta t \right] \left[\frac{100}{S_{\max T}} \right]$$

$$\text{DI} = \frac{M-X}{M} \times 100$$

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Where n_A , n_B , n_C , n_D , and n_E : number of plants belonging to the range 0-4; S_i : mean severity in the observation i ; Δt : number of days between observations; S_{\max} : maximum disease rating (=4); T : experimental duration in days (=147); n : number of observations; M : mean size of negative control plants; X : mean size of infected plants.

FMS and AUDPC were calculated at the end of experiments based on Campbell and Madden [18].

2.4. Antifungal activity of saccharide extracts against *Verticillium dahliae*

OMV₆ strain was cultivated in a PDA medium for 15 days and then the fungal mycelium was cut using a sterile cork borer to disks of 5 mm in diameter. The Disks were placed in the center of Petri dishes (90 mm diameter) containing 20 ml of PDA medium mixed with each saccharide fraction at a final concentration of 1g/L. Samples were incubated at 25 °C and the diameter of the colonies was measured after 5, 10, and 15 days. Five repetitions were performed for each treatment.

2.5. Data analysis

The results were analyzed using SPSS version 25.0 software and presented as mean value \pm standard error (SE). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Efficacy of saccharide elicitors and their combination against verticillium wilt in tomato seedlings

3.1.1. Disease incidence

The *Verticillium* wilt was monitored weekly for 5 months. The first symptoms appeared 6 weeks of inoculation after foliar spray and 11 weeks after root-drenching. As illustrated in **Figure V. 1**, the application of saccharide fractions delayed the disease incidence for both used methods. After foliar spray, the positive control and laminarin treatments achieved a level of 100 and 90 %, respectively, 13 weeks followed inoculation, compared to other treatments. Additionally, the disease incidence was reduced by 55, 40, 25%, and 30 % in response to each of ALSM, ALCM, AGB, and their combination, respectively (**Figure V. 1A, B, and C**). Among treatments, the disease incidence reached maximum value after 5 months in response to all treatments, except for ALCM and the oligosaccharides (OASM) which indicated 5 and 10 % disease reduction, respectively. (**Figure V. 1A**).

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When compared to the foliar spray method, the application of saccharide fractions via root-drenching reduced disease incidence by 80 and 70 % for ALCM and AGB treatments, respectively, 13 weeks after inoculation. The levels decreased by 65 % in response to ALSM, OACM, and AGB, and by 50% after the application of OASM and the polysaccharide mixture. (Figure V. 1D, E, and F). After 5 months, plants treated with laminarin and polysaccharide mixture showed high value as in the control (100%), while the other saccharide treatments reported levels ranging from 85 and 95%.

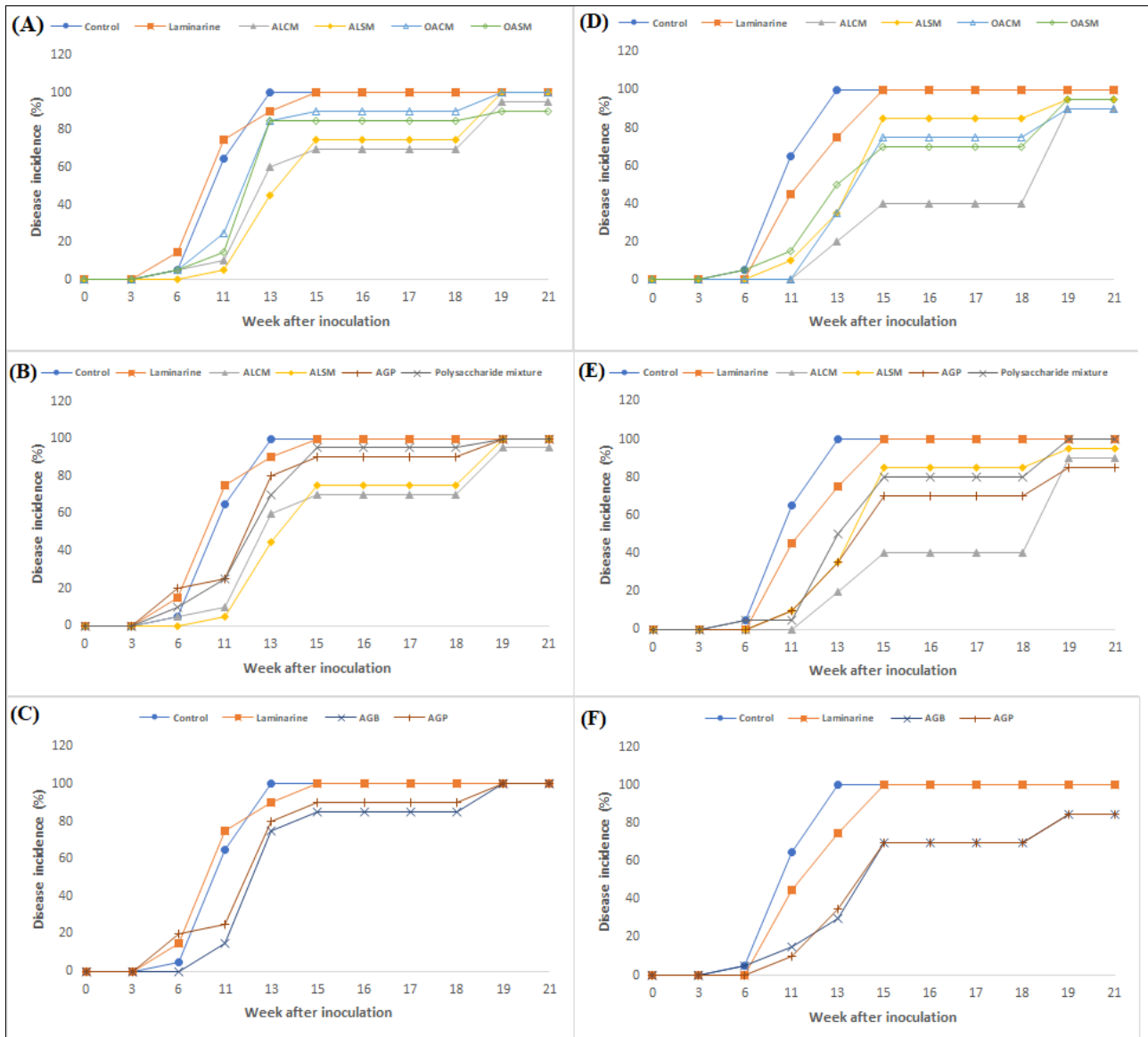


Figure V. 1. Incidence of verticillium wilt in tomato seedlings after foliar spray (A, B, C) and root-drenching (D, E, F) of seaweed saccharide fractions and their combination (1 g/L).

3.1.2. Disease severity

The disease severity was evaluated by monitoring the chlorosis, necrosis, wilting, and dieback of each plant. After 3 months of inoculation, 100% of non-treated plants (positive control)

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showed intense wilting, while for elicited plants the intensity of symptoms was reduced using the two modes of elicitation (Foliar spray and root-drenching) (**Figure V. 2**). In addition, after root-drenching, 5% of plants treated with alginates (ALCM and ALSM) showed moderate wilting, and no severe symptoms were observed after the application of each of OASM and AGB after 3 months (**Figure V. 2D and F**). Over time, the severity progressed sharply to achieve a high value of 4 for untreated plants (positive control) and about 3 after the application of laminarin. In contrast, levels were 2 times lower in response to other saccharide fractions (**Table V. 1**).

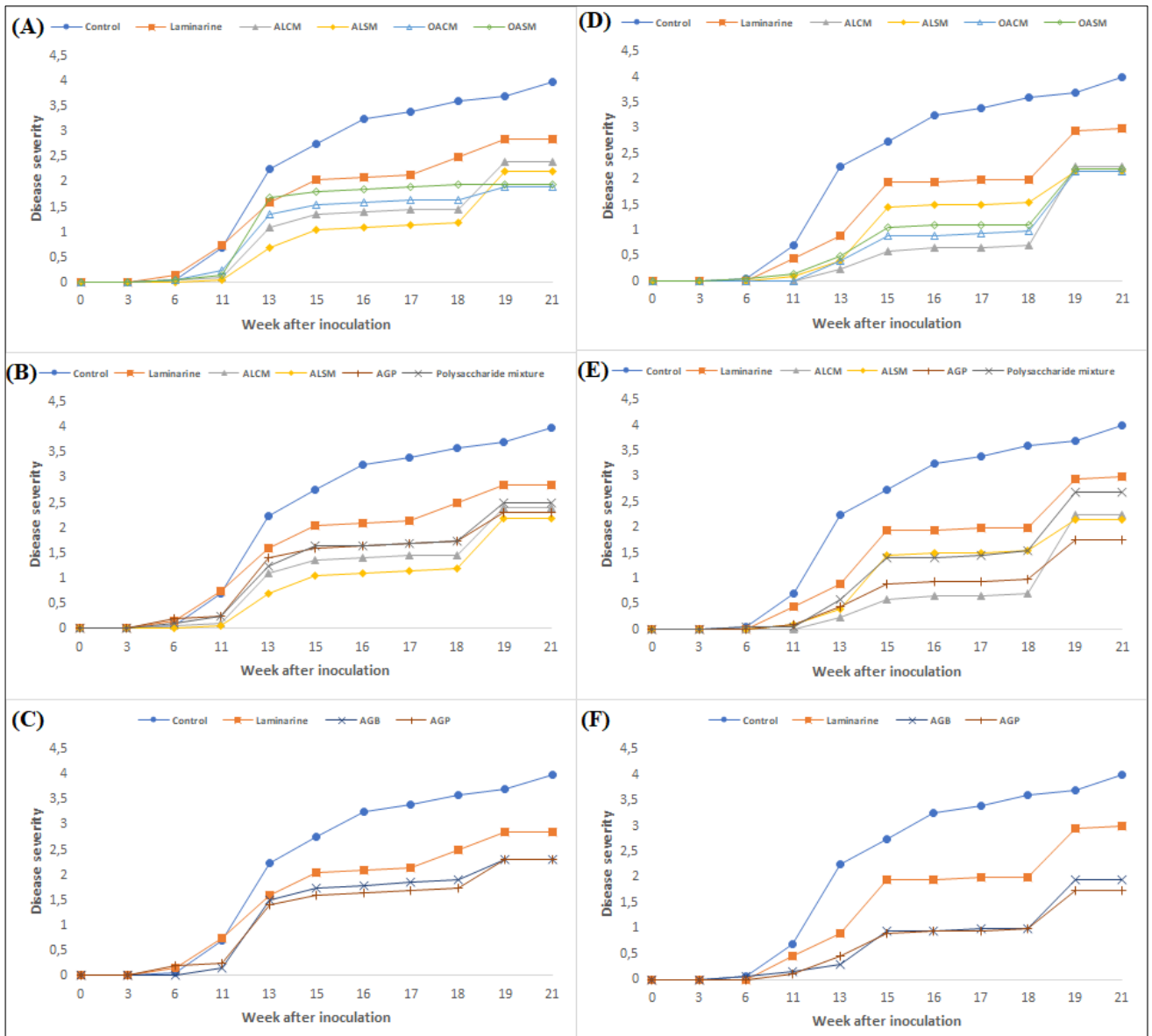


Figure V. 2. Severity of verticillium wilt in tomato seedlings after foliar spray (A, B, C) and root-drenching (D, E, F) of seaweed saccharide fractions and their combination (1 g/L).

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3.1.3. Mortality rate

As shown in (Figure V. 3), the mortality was recorded firstly 3.4 months after inoculation for untreated plants (5%) reaching a percentage of 100% after 5 months. In response to saccharide extract treatments, the rate of dead plants was observed after 4 months following inoculation reaching a maximum value of 30% for laminarin-treated plants using both modes of elicitation. After 5 months, the death rate was in the range of 0-15% after foliar spray application of different extracts. In contrast, the mortality levels were slightly higher using the root-drenching method, which ranged from 5 to 25% (Table V. 1).

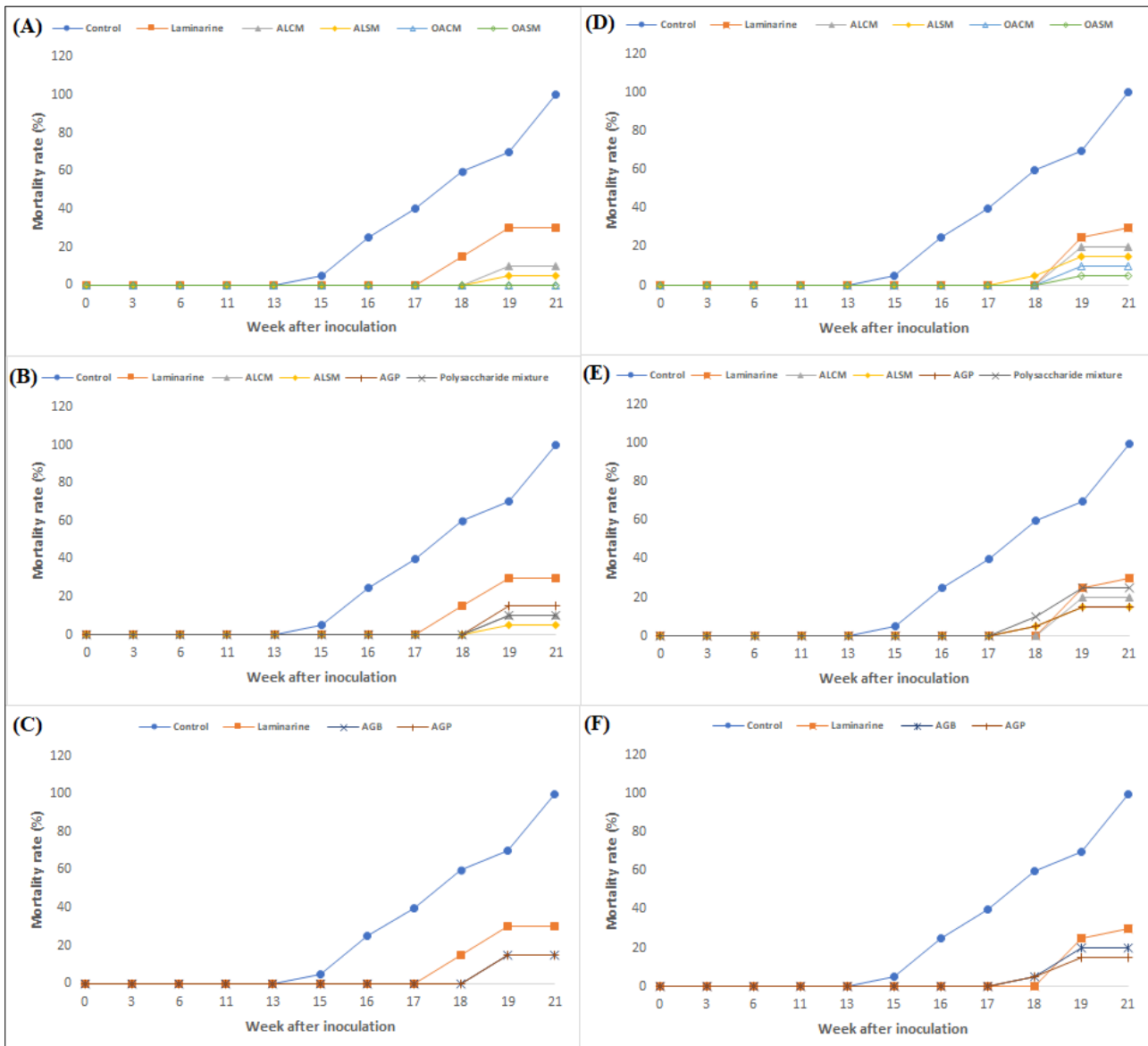


Figure V. 3. Mortality rate of infected tomato seedlings after foliar spray (A, B, C) and root-drenching (D, E, F) of seaweed saccharide fractions and their combination (1 g/L).

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The effects of seaweed saccharides on the reduction of verticillium wilt were estimated by measuring AUDPC, FMS, and DI at the end of the experiment (5 months) (**Table V. 1**). High values were recorded for untreated plants (positive control) followed by laminarin treatment after foliar and root-drenching applications. FMS levels in plants treated with marine saccharides were 1.7-3-time lower than in control plants. ALCM and ALSM-FMS values were reduced by $\approx 50\%$ and 60% after foliar spray and $\approx 70\%$ and 54% after root-drenching methods, respectively. AUDPC was used to evaluate the disease intensity over 5 months. It is a parameter that allows combining repeated observations of disease incidence and severity progress into a single value. In control plants, the AUDPC was important (13.88%), followed by laminarin (9.42-10.62%). Other treatments showed percentages ranging from 4.95 to 8.45%. Lower values were recorded after AGP root-drenching and OACM foliar spray. Dwarfing index (DI) was calculated to assess the effect of saccharide treatments to suppress the stunting growth of tomato seedlings caused by *V. dahliae*. In response to all treatments, the DI was reduced compared to positive control. Lower levels were obtained in response to ALCM foliar spray and OACM root-drenching. AUDPC, FMS, and DI levels were slightly higher after foliar spray mode than after the root-drenching method. However, the mortality rate and disease severity were considerably reduced after foliar spray application. These indicate that both treatments seem to be effective to control the vascular pathogen *V. dahliae*.

Table V. 1. Symptomatological parameters after 5 months of inoculation with OMV₆.

	FMS		AUDPC (%)		Mortality rate (%)		Disease severity		DI (%)	
	Foliar spray	Root drench	Foliar spray	Root drench	Foliar spray	Root drench	Foliar spray	Root drench	Foliar spray	Root drench
Control	2.37		13.88		100		4		15.24	
Laminarin	1.7	1.52	10.63	9.42	30	30	2.85	3	0.88	-15.82
ALCM	1.17	0.735	7.50	5.13	10	20	2.4	2.25	-2.76	-17.35
ALSM	0.965	1.08	6.20	6.29	5	15	2.2	2.15	2.00	-10.59
OACM	1.19	0.845	7.32	5.50	0	10	1.9	2.15	3.06	-20.06
OASM	1.33	0.945	8.02	6.11	0	5	1.95	2.2	7.65	-19.47
AGB	1.355	0.83	8.32	5.29	15	20	2.3	1.95	5.53	-18.88
AGP	1.315	0.785	8.26	4.95	15	15	2.3	1.75	2.65	-9.76

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Polysaccharide mixture	1.335	1.19	8.45	7.44	10	25	2.5	2.7	4.65	-9.24
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3.2. Efficacy of saccharide elicitors and their combination on growth parameters of tomato seedlings

In the presence of the pathogen, the effects of seaweed saccharides on the growth parameters of tomato seedlings and fruit yield were evaluated. *Verticillium* infection had a negative effect on the development and growth of tomato plants, reducing shoot length, fruit yield, and size. The application of saccharide elicitors was found to increase the shoot length and tomato yield with a greater number of fruits depending on the application mode (**Table V. 2**). The root-drenching method was found to be more effective to increase shoot length, while foliar spray mode seems to influence fruit number. Despite the presence of the pathogen, the saccharide elicitors showed a considerable increase in growth parameters compared to non-inoculated plants (negative control).

Table V. 2. Effects of seaweed saccharide fractions and their combination on growth parameter of tomato plant after 5 months of inoculation with OMV₆.

	Shoot length (cm)		Number of fruits		Fruit yield (g)		Fruit diameter (mm)	
	Foliar spray	Root drench	Foliar spray	Root drench	Foliar spray	Root drench	Foliar spray	Root drench
Negative control		85		13		83.9		24.38
Positive control		72.05		7		23		15.83
Laminarin	84.25	98.45	25	3	121.4	37.7	20.82	29.87
ALCM	87.35	99.75	20	14	94.6	79.6	23.82	24.89
ALSM	83.3	94	11	6	71.2	45.6	26.75	24.28
OACM	82.4	102.05	16	8	55.4	74.6	19.94	36.73
OASM	78.5	101.55	15	7	34.6	36.2	19.69	26.45
AGB	80.3	101.05	23	13	132.8	132.6	25.73	27.87
AGP	82.75	93.3	27	27	82.3	130.9	22.66	21.70
Polysaccharide mixture	81.05	92.85	36	8	127	75.2	17.55	27.16

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3.3. Effects of saccharide elicitors on the mycelium growth of *Verticillium dahliae* (OMV₆)

In vivo assays were performed to evaluate the direct antifungal activities of saccharide extracts. As shown in **Figure V. 4**, no inhibition effect of the mycelial growth was reported for all fractions, indicating that these molecules had no direct antifungal activities against *V. dahliae* at a concentration of 1g/L. In contrast, a slight increase in OMV₆ growth was observed after 10 days of incubation. The diameter levels recorded for all seaweed extracts, including the control, reached their maximum (90 mm) 15 days later.

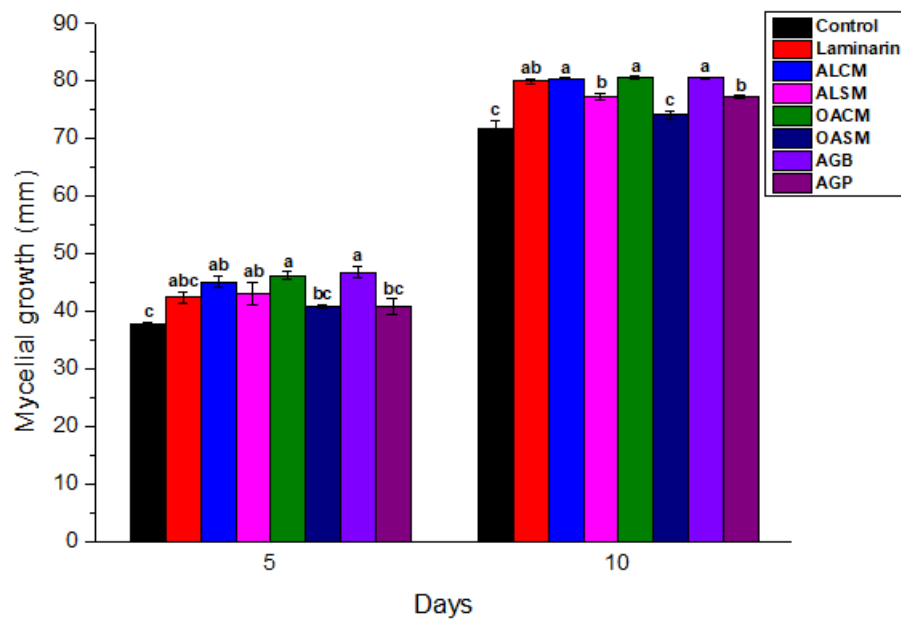


Figure V. 4. Effect of saccharide fractions (1 g/L) on the mycelial growth of *V. dahliae* (OMV₆) for 10 days of incubation at 25 °C.

4. Discussion

Seaweed polysaccharides and oligosaccharides became an alternative to chemical products in agriculture. These molecules can act both as a natural plant growth stimulator and an inducer of natural defenses against biotic and abiotic stresses [19-22]. In this study, the application of polysaccharides and oligosaccharides and their combination as elicitors was effective to control the verticillium wilt of tomato caused by *V. dahliae*. The chemical and structural characterization of these elicitors were previously performed [15,16]. The molecular weights (M_w) of ALCM and ALSM are 76 and 202 kDa, respectively. ALCM is rich in mannuronic acid (53%), whereas ALSM exhibits higher levels guluronic acid (54%). Two LMW alginates, OACM and OASM, were produced as a result of the oxidative degradation of ALCM and ALSM, respectively. The first has a M_w of 19 kDa, while the second has a M_w of 3 kDa. Sulfated

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arabinogalactans (purified and crude extracts) are characterized by an important molecular weight (M_w) > of 2000 kDa and a high degree of sulfation and protein (22.68 and 20.03% for AGB; 13.21% and 11.89% for AGP). They are composed mainly of galactose, glucose, and arabinose [15].

These molecules showed their capacity to delay and reduce disease severity, incidence, and mortality compared to control and laminarin-treated plants. They were also found to promote growth parameters in the presence of pathogens. The elicitation by root-drenching was more efficient rather than foliar spray. Furthermore, no inhibition effect on the growth of *V. dahliae* was observed when applying the seaweed saccharide, which avoids the development of pathogen resistance in soil. The LMW alginates (OASM and OACM) showed their effectiveness to reduce mortality in both applied methods, and to suppress the stunting growth of tomato seedlings, after root drenching, compared to other saccharides. In the previous work, these fractions exhibited a significant induction of the Phenylalanine ammonia-lyase followed by a considerable accumulation of secondary metabolites (polyphenols and lignin) in the roots and the leaves of tomato plants, compared to their native alginates (**Article N°2**). This could explain the present finding. The sulfated arabinogalactan (AGB and AGP) exhibited their efficiency to decrease the severity and AUDPC parameter after root-drenching. In recent years, several carbohydrate structures have been determined but the structure-activity correlations and their effectiveness in crop protection remain inconsistent and poorly understood. However, many factors have been reported to influence the level of poly/oligosaccharides to induce resistance in plants. Among them are the molecule structures (monosaccharide and glycosidic-linkage composition, conformation, M_w , functional groups, and branching characteristics), the application methods, and the recognition of molecules by plants [14,23]. It has been found that only branched β -glucans are recognized by soybean and rice, while tobacco detects β -1,3-glucans in their linear form [24]. Additionally, altering the structure of molecules could change the receptor affinities, which would impact the elicitor activity. The acetylation of chitin is important to be recognized by chitin elicitor receptor kinase 1 (CERK1), However, fully deacetylated chitooligomers have no affinity to CERK1 [25]. The presence of sulfates and the degree of sulfation could also influence the elicitor activities [26-28].

Rather than using a single elicitor, a combination of molecules often appeared to have strong synergistic effects [10,29-31]. Nevertheless, in the present study, sulfated (AGP) and unsulfated polysaccharides (ALCM and ALSM) combination did not improve protective efficacy in either

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applied methods (Foliar spray and root-drenching), compared to individual treatment. Several studies have reported that the application of elicitor mixtures such as methyl jasmonate (MeJA) and salicylic acid (SA) was less effective [32,33].

These findings showed both an increase in tomato growth in the presence of pathogen as well as a reduction of the disease caused by the major vascular fungi *V. dahliae* when foliar and root-drenching application of seaweed saccharide extracts. To our knowledge, this is the first report evaluating the protective effects of seaweed polysaccharides and oligosaccharides on tomato seedlings against *V. dahliae*. Several studies have reported the ability of seaweed polysaccharides to increase plant resistance to phytopathogens and plant growth. Ulvan and oligoulvans from *Ulva lactuca* reduced the development of *Fusarium oxysporum* f. sp. *lycopersici* in tomato seedlings and *Penicillium expansum* and *Botrytis cinerea* in apple fruit [10,11]. Pretreatment of tomato plants with iota (ι), lambda (λ), and kappa (κ)-carrageenan (1 g. L⁻¹) reduced tomato chlorotic dwarf viroid (TCDVd) symptoms by 65 % [34]. Furthermore, the height of λ -carrageenan treated plants was higher compared to the control-infected group [34]. Sodium alginate derived from brown algae has also been found to induce resistance against a wide range of pathogens. In tomato seedlings, the foliar spray of this polysaccharide at 0.6%, significantly reduced the severity of early blight disease caused by the necrotrophic fungal *Alternaria solani* [35]. Alginate from *Bifurcaria bifurcata* (1 g.L⁻¹) has shown the capacity to induce resistance against *Fusarium oxysporum* f. sp. *Albedinis* in date palm, by decreasing the disease incidence and severity as well as the mortality compared to laminarin and control plants [12]. These findings are in accordance with the present work. The use of low molecular weight alginate (LMW) in crop protection has been previously reported only to a very limited extent. For instance, studies were conducted on resistance induction of *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* DC3000 [36], tobacco plants against tobacco mosaic virus (TMV) [37], and rice against *Magnaporthe grisea* [38]. It has been reported that LMW alginate induced tolerance to drought stress, salinity, and heavy metal ions in wheat [39,40], cucumber [41], *Brassica campestris* [42], *Eucomis autumnalis* [43], and *Vicia faba* [44], improving in parallel growth and physiological activity. As far as we know, no prior studies have examined the potent application of LMW against Verticilium wilt. Furthermore, the use of glycoproteins from marine seaweed to enhance resistance against phytopathogens has not yet been reported to date. Previous studies have shown the ability of glycoproteins purified from microorganisms to enhance resistance against TMV, *Botrytis cinerea*, *P. syringae* pv. *tomato* DC3000 in tobacco and tomato leaves [45-47]. A recent study has investigated the

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protective effects of aqueous (ASE) and methanolic seaweed extracts (MSE) (*C. myriophylloides*, *Laminaria digitata*, and *Fucus spiralis*) against *V. dahliae* in tomato seedlings. It has been found that ASE treatment from *C. myriophylloides* and *F. spiralis* was more effective to reduce Verticillium wilt and to improve the number of flowers and fruits [48].

V. dahliae is a soil-borne vascular fungus that causes Verticillium wilt (**Figure V. 6C, D and E**), resulting in serious economic losses worldwide in yield and quality of more than 200 plants including tomato [2,49]. Typically, the infection by verticillium hyphae occurs either at the root tip or the sites of lateral root formation of host plants [3,49]. Once crossing the endodermis, the fungus penetrates the vascular tissues (xylem), in order to sporulate and continue the colonization of stem vessel elements [3]. In tomato typical symptoms starts with yellowing of the lower leaves, wilting on one-half of infected leaves (**Figure V. 6B**), chlorosis, and V-shaped lesions (**Figure V. 5**). As the disease progresses, necrosis starts to develop within lesions leading to severe defoliation and plant death. Once plants are infected with the *V. dahliae*, there is no effective treatment to eradicate the disease. Moreover, no new fungicide is available for use against this pathogen to date. Currently, only preventive measures are used to manage verticillium wilts such as chemical fumigation, development of resistant varieties of tomato, grafting, and biocontrol agents (BCAs) [2]. This is the first report that highlighted the importance of polysaccharides and oligosaccharides from marine seaweed to control verticillium wilt in tomato opening a new orientation for disease management.

Elicitors are mostly applied through seed priming, soil drenching, or foliar spray methods to control diseases. The application of seaweed extracts using these tree modes has been previously reported to enhance growth and disease protection in tomato plants [48,50-52]. The efficiency of protection capacity of these inducers could vary, and the performance is plant species, concentration, and application method dependent. The influence of elicitation mode has been previously demonstrated [48,53] The present study showed that root drenching was slightly effective compared to foliage spray in reduction of verticillium wilt and improvement of tomato plant growth. Ali et al. have shown that the treatment by *Ascophyllum nodosum* (brown seaweed) extract (ANE), showed a reduction of early blight disease caused by the foliar pathogens *Alternaria solani* and *Xanthomonas campestris* pv. *vesicatoria* in tomato seedlings independently of the methods of application (foliar spray or root drench) [51]. It has been demonstrated that Stimplex™ (commercial formulation of seaweed extract from *A. nodosum*) reduced the *Fusarium* root rot incidence in cucumbers. The disease control effect was more

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effective after treatment by root-drenching than foliar spray. Moreover, in *Fusarium*-inoculated plants, the shoot and root biomasses were improved in Stimplex™ (0.5%) drenched plants compared to sprayed seedlings [53]. In contrast, the foliar spray was found to be more effective to control *Botrytis* and *Didymella* blights caused by foliar pathogens *Didymella applanate* and *B. cinerea* [53]. These addresses several further questions about the effectiveness of application methods to manage fungal diseases, in which root-drenching could be more efficient against soil-borne pathogens. Taking into consideration that the applied elicitors have no negative effects on phytopathogens. It has been proven that seaweed extract has increased the total rhizosphere microbial colonies accompanied by increasing their activities as well as soil respiration [51,54,55]. These could contribute to enhancing plant growth and delaying disease development. The foliar application of these molecules has also shown an efficiency to manage the vascular pathogen *V. dahliae*. This could be explained by the systemic effects of the applied elicitors, which have been demonstrated previously [15,16].



Figure V. 5. Leaves of tomato plants infected by *V. dahliae* show typical V-shaped necrosis.

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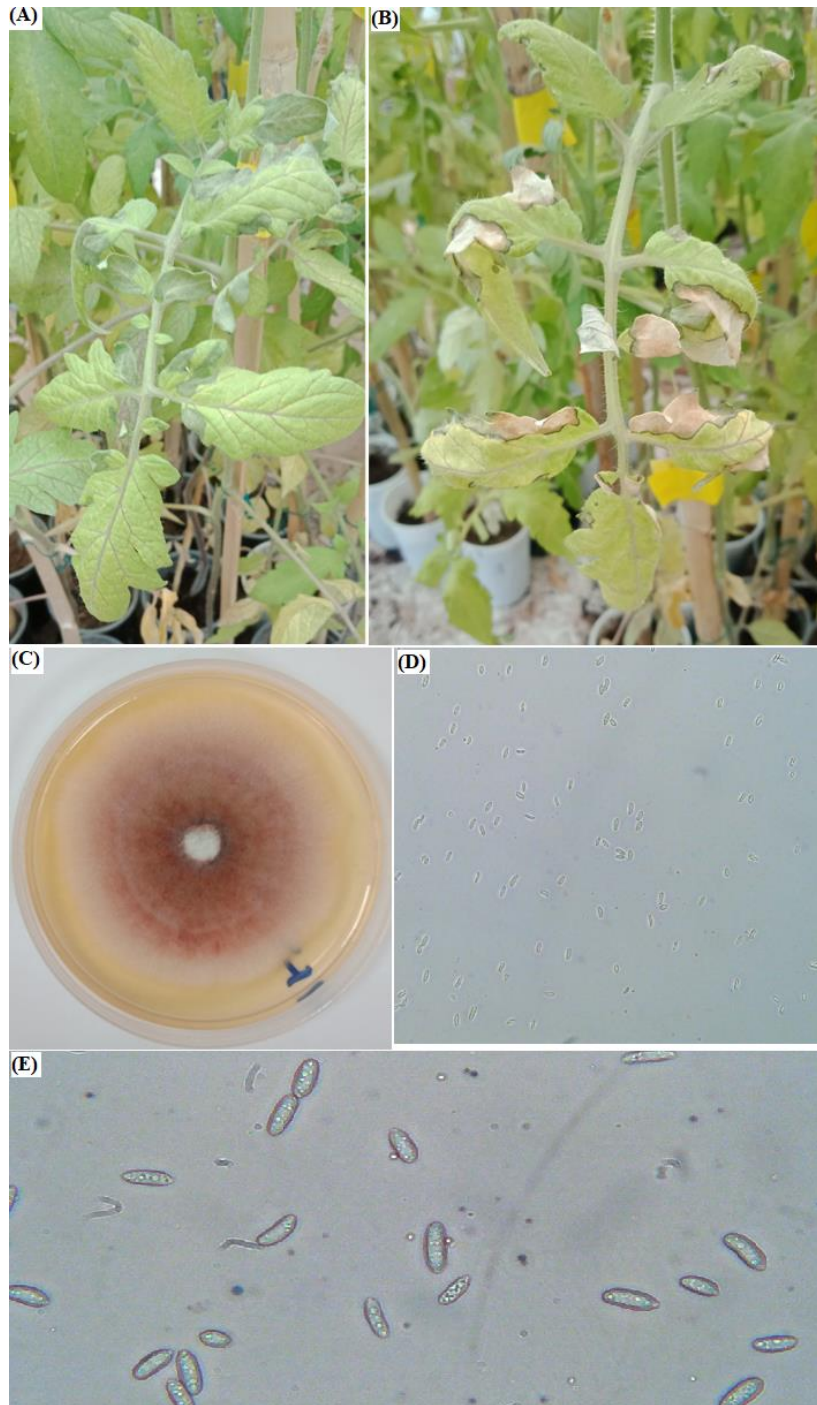


Figure V. 6. Appearance of blotches from the margin of a leaf of infected tomato plant (A); Development of spots to necrotic and brown lesions (B); Cultural feature of *V. dahliae* on PDA after 10 days of incubation at 25 °C (C); Conidia of *V. dahliae* (10 × magnification) (D); Conidia of *V. dahliae* (40 × magnification) (E).

5. Conclusion

Several chemical and non-chemical techniques have been deployed to manage verticillium wilt in tomato and other crops, but their usefulness is still limited. Therefore, this study represents

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an effective and sustainable strategy based on the stimulation of natural defenses using seaweed saccharides to control the soil-borne fungus *V. dahliae* in tomato seedlings. Alginates, LMW alginates derivatives, and sulfated glycoproteins extracted from brown and green seaweed have been shown to induce the disease incidence and severity. In addition, the mortality was decreased to more than 75%. The extracted saccharides were more effective compared to the commercial elicitor (laminarin). The application of these bioelicitors not only induced plant defense mechanisms but also improved tomato growth by increasing shoot length and fruit yield. To the best of our knowledge, this is the first study to involve the pretreatment of tomato plants with marine polysaccharides and oligosaccharides derivatives induced resistance against *V. dahliae*.

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DISCUSSION GÉNÉRALE ET PERSPECTIVES

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L'utilisation des polysaccharides d'origine algale comme stimulateur de défenses naturelles des plantes est l'une des nouvelles approches prometteuses en protection des cultures. Elle permet à la fois de réduire le recours aux intrants chimiques, de valoriser les ressources algales inexploitées et de minimiser les problèmes environnementaux et économiques liés aux efflorescences nuisibles formées par les espèces envahissantes.

Le présent travail s'intéresse particulièrement à :

- ✓ L'extraction et la caractérisation des polysaccharides à partir de deux algues brunes *Sargassum muticum* et *Cystoseira myriophylloides* et de l'algue verte *Codium decorticatum* ainsi que la production de leurs dérivés de faible poids moléculaires. Ces trois espèces, connues pour leur abondance sur les côtes marocaines, sont encore inexploitées à l'échelle nationale.
- ✓ L'évaluation de la capacité de ces molécules à stimuler les défenses naturelles des plants de tomates en cultures sous serre et des disques foliaires de l'olivier. Cette évaluation a ciblé également l'identification des conditions nécessaires pour l'obtention de réponses optimales aux traitements par ces biomolécules, tenant compte d'un certain nombre de paramètres tels que :
 - ❖ La structure des molécules : degré de polymérisation, nature des oses constitutifs, richesse en groupements sulfatés et présence de protéines (**chapitre II et III**).
 - ❖ Synergie d'action par le mélange des fractions saccharidiques de structures différentes (**Chapitre III**).
 - ❖ Le mode d'application : au niveau foliaire (pulvérisation), au niveau racinaire (trempage des racines saillantes dans les solutions saccharidiques ou leur apport dans le sol en contact avec les racines) ou injection des solutions au milieu des tiges des plantules. Ceci est dans l'objectif également d'évaluer l'effet systémique des réponses induites par les éliciteurs (**chapitre II et III**).
 - ❖ Le type de l'espèce végétale : par le choix de deux espèces de plantes de la classe des dicotylédones, importantes économiquement ; la tomate (plante herbacée) de la famille des Solanaceae et l'olivier (plante arborescente pérenne) de la famille des *Oleaceae* (**chapitre IV**).

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- ✓ L'évaluation du potentiel protecteur des différentes fractions saccharidiques extraites sur le pathosystème tomate/*Verticilium dahliae* (**chapitre V**).

Cette étude a révélé que les polysaccharides algaux et leurs dérivés oligosaccharidiques présentent des propriétés stimulatrices des défenses naturelles des plants de tomate et de l'olivier. Les résultats obtenus montrent que le métabolisme de la voie des phénylpropanoïdes était inductible par ces molécules saccharidiques d'une manière variable, dépendamment de la structure moléculaire, du mode d'apport et de la nature de l'espèce végétale traitée.

Les fractions saccharidiques qui ont fait l'objet de cette étude sont, les alginates, les oligoalginates, les arabinogalactanes sulfatés, les fucoïdanes et le mélanges des alginates et des arabinogalactanes sulfatés.

Les alginates ont été extraits à partir de trois algues brunes de la famille des Sargassaceae, *Bifurcaria bifurcata* (BBSA) [1], *Sargassum muticum* (ALSM) et *Cystoseira myriophylloides* (ALCM). Après caractérisation, BBSA, ALSM et ALCM avaient des poids moléculaires de 220, 202 et 76 kDa, respectivement. Le rapport mannuronate/guluronate (M/G) estimé à partir des spectres ¹H-NMR a montré que les fraction BBSA et ALSM sont riches en acide guluronique (BBSA : 68 % ; ALSM : 54 %), tandis que ALCM présente un taux élevé d'acide mannuronique (53 %). ALCM et ALSM sont caractérisés par la richesse en séquences homopolymériques (MM et GG) contrairement à la fraction BBSA.

Le traitement des plants de tomate par ces polysaccharides a révélé leur capacité à induire l'activité PAL, l'accumulation des composés phénoliques et de la lignine au niveau des feuilles et des racines quel que soit leur mode d'application (pulvérisation ou injection). La pulvérisation par la fraction ALSM a montré un effet éliciteur plus efficace comparé aux alginates extrait de *C. myriophylloides* (ALCM). Ceci a également été accompagné par une activité protectrice importante par rapport au ALCM, traduite par une réduction plus significative du taux de mortalité, de la sévérité moyenne finale (FMS) et de l'aire sous la courbe de progression de la maladie (AUDPC), 5 mois après l'infection par *V. dahliae* (OMV₆). Cette différence d'efficacité pourrait être due aux poids moléculaires des polysaccharides ou bien à leurs contenus en acides uroniques (acide guluronique ou mannuronique). Cependant, aucune différence significative au niveau de l'efficacité n'a été observée après application de ALCM et ALSM sur les disques foliaires de l'olivier.

Des études récentes ont montré que la richesse en mannuronate ou guluronate pourrait influencer l'activité élicitrice des alginates. Les plants du blé et de tabac traités par des fractions

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enrichies en acide mannuronique (AM) ont révélé un pouvoir éliciteur plus important comparé aux fractions enrichies en acide guluronique (AG) [2,3]. En revanche, cette étude a montré que les alginates riches en AG étaient les plus actifs sur les plants de tomate. Ceci pourrait être lié à la présence de récepteurs spécifiques selon la structure du polymère et selon l'espèce végétale.

Le système immunitaire des plantes consiste en un réseau complexe de cellules et de molécules qui interagissent les unes avec les autres pour initier les défenses chez l'hôte. Il repose principalement sur la reconnaissance des molécules étrangères en tant que MAMP ou PAMP par des récepteurs de surface de la plante (PRR). De nombreux PRR portent des domaines de lectine capables de reconnaître et d'interagir avec des structures glucidiques spécifiques d'organismes microbiens ou des saccharides dérivés des parois cellulaires végétales [4]. Les lectines, jouent un rôle majeur dans les réponses immunitaires comme des récepteurs de reconnaissance de forme [4]. Il a été établi que les interactions entre les glucides et leurs récepteurs associés peuvent être à l'origine de nombreux événements biologiques importants [5]. Ces récepteurs peuvent se lier également avec les chaînes glucidiques des glycolipides et les protéoglycanes, et interagir avec les polysaccharides et les sucres libres [4]. Les lectines chez les végétaux, peuvent être classées en 12 familles selon la spécificité des sucres et la structure du domaine de reconnaissance conservé des glucides CRD (Carbohydrate-Recognition Domain) [4]. Les PRR pour la chitine et les oligogalacturonides (OGA) ont été identifiés, mais les récepteurs apparentés à d'autres glucides, y compris les β -glucanes, le chitosane, le fucane, l'alginate, etc. sont encore inconnus [6]. Récemment, il a été rapporté que les kinases associées aux parois, ou WAK (Wall-Associated Kinase), spécifiques aux OGAs [7], reconnaissent les alginates en présence de calcium et de conditions ioniques. Ceci permet la formation de ponts Ca^{2+} entre les oligomères et les polymères [8], créant une conformation appelée « boîtes à œufs » qui est nécessaire au système de perception des plantes [9]. Néanmoins, la perception des polysaccharides et des oligosaccharides par les plantes est extrêmement complexe. Des recherches plus approfondies sont nécessaires pour bien élucider les interactions chimiques intimes entre les biomolécules actives et les récepteurs membranaires et qui sont à la base de la transduction des signaux induisant les voies de défense appropriées.

L'activité électrique pourrait aussi être associée à d'autres facteurs, notamment le degré de polymérisation (DP). Dans ce travail, les alginates ont été hydrolysés par l' H_2O_2 pour l'obtention des alginates à faible poids moléculaires ou des oligomères fonctionnelles. Trois fractions oligosaccharidiques ont été produites, OGABs à partir de BBSA, OASM issu de ALSM et OACM obtenu à partir de ALCM. Bien que l'hydrolyse radicalaire soit réalisée dans

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les mêmes conditions, leurs DP étaient variables. OGABs et OASM sont caractérisés par des faibles poids moléculaires de 5,5 kDa ($2 \leq DP \leq 24$) et 3 kDa ($2 \leq DP \leq 14$), respectivement, tandis que OACM avait un M_w plus élevé de 19 kDa ($DP = 88$). L'application de ces fractions sur les plants de tomate et les disques foliaires de l'olivier a révélé des réponses différentes dépendamment de l'espèce végétale, de la durée d'incubation pour les plants de tomate et la concentration pour les feuilles de l'olivier. Les jeunes plantules de tomate subissant une injection par OGABs ont montré une accumulation des composés phénoliques plus importante au niveau des feuilles, comparées à celles traitées par le polymère (BBSA). D'autre part, la pulvérisation des deux fractions OASM et OACM, a stimulé l'activité PAL de manière précoce au niveau des feuilles et des racines. Par contre, l'activité stimulatrice de leurs alginates natifs (ALSM et ALCM) est relativement tardive et plus faible. Cette efficacité élicitrice a été associée à une amélioration de la résistance des plants de tomate vis-à-vis de l'attaque par *V. dahliae*, montrant une réduction de mortalité à 100 %. En outre, le traitement des feuilles de l'olivier avec ces deux dérivés oligosaccharidiques (OACM et OASM) a manifesté un effet éliciteur plus important de OASM (3kDa) même à des faibles concentrations (0,5 g/L). Des séries d'études récentes ont indiqué que les oligosaccharides dérivés d'algues avec un faible DP stimulent fortement les mécanismes de défense des plantes par rapport aux polysaccharides. Comparés à leurs polymères, les oligoulvanes ($DP = 2$) et les oligoglucuronanes ($DP = 3$) préparés par hydrolyse enzymatique du glucuronane et de l'ulvane, ont montré un meilleur effet protecteur des pommes en post-récolte contre *Penicillium expansum* et *Botrytis cinerea*, et des plants de tomate contre *Fusarium oxysporum* f. sp. *lycopersici* [10-12]. Fu et al. (2011) ont reporté que les oligosaccharides de curdlane (CRDO) et de chitosane (COS) avec un faible DP (2-10) induisent des réponses plus rapides que la laminarine avec un DP élevé (25-40) dans les cellules de tabac [13]. Cependant, la laminarine a manifesté des réponses de défense plus persistantes [13], suggérant que les éliciteurs d'origine glucidiques exogènes à faibles DP étaient peut-être facilement reconnus par les cellules de tabac. Bien que les deux oligomères (CRDO) et (COS) avaient des DP similaires, les réponses variaient l'une de l'autre, indiquant que les liaisons glucidiques et les structures des molécules peuvent influencer l'activité stimulatrice d'une manière plus importante que le DP [13].

Parmi les facteurs susceptibles d'impacter le niveau de la résistance induite par les éliciteurs des plantes, nous retrouvons la barrière cuticulaire et stomatique. Une fois pulvérisés, les éliciteurs doivent traverser la barrière cuticulaire pour atteindre la paroi cellulaire et la membrane plasmique et être perçus par la plante. En raison de ses propriétés chimiques, la cuticule est

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perméable aux composés lipophiles mais représente une barrière de diffusion aux composés polaires tels que les glucides [14]. Les molécules polaires peuvent pénétrer la feuille par des pores hydrophiles transcuticulaires ou par les stomates [14]. La voie stomatique permet la diffusion des glucides à hauts poids moléculaires, tandis que la voie cuticulaire présente des limites d'exclusion de taille. Elle est plutôt compatible avec la diffusion de petites molécules glucidiques [15]. Il est possible que le taux de pénétration des oligosaccharides soit plus élevé par rapport à celui des polysaccharides avec poids moléculaires élevés. Ceci pourrait, en outre, expliquer l'efficacité variable des applications des polysaccharides et oligosaccharides par pulvérisation foliaire. Par conséquent, les caractéristiques physico-chimiques des molécules élicitrices en solution aqueuse, telles que leur solubilité, leur pH, et leurs poids moléculaire auront une influence majeure sur le succès de l'absorption par la feuille et de la transduction des signaux vers les organes de la plante. Il a été démontré que certains glucides appliqués par pulvérisation favorisent la fermeture stomatique. Tout comme l'acide abscissique (ABA), la laminarine a induit une réduction significative de l'ouverture stomatique sur les feuilles de la vigne. En revanche, la laminarine sulfatée n'a montré aucun effet inhibiteur quelles que soient les concentrations testées [16]. En outre, Li et al. (2009) ont révélé que l'application des oligochitosanes sur les cellules épidermiques de *Brassica napus* L. induit la fermeture des stomates en fonction de la production de l' H_2O_2 et le monoxyde d'azote (NO) [17]. En se basant sur ce qui précédait, les molécules à faible poids moléculaire bénéficient probablement d'une meilleure perméabilité à la plante, et par conséquent des réactions de défense plus rapides et plus précoces.

Une autre propriété structurale connue pour son impact sur les fonctions biologiques des polymères glucidiques ou glycoprotéiques est la présence des groupements sulfatés. Notre étude porte en outre sur l'évaluation du potentiel des polysaccharides sulfatés (arabinogalactanes et fucoïdanes) à induire les réactions de défense chez les plants de tomate et/ou sur les disques foliaires de l'olivier. L'effet protecteur de ces molécules vis-à-vis de l'infection par *V. dahliae* a été ainsi mis en évidence. Une fraction brute (AGB) a été extraite de l'algue verte *C. decorticans*. Une étape de purification a été ensuite réalisée dans le but d'obtenir un extrait purifié (AGP). L'analyse chimique et structurale de ces deux fractions a montré la présence d'un taux important de protéines (AGB : 20,03 % ; AGP : 11,89 %) et de sulfates (AGB : 22,68 % ; AGP : 13,21 %). Le galactose, l'arabinose et le glucose étaient les principaux monosaccharides constitutifs de l'AGB et l'AGP. Ils sont également caractérisés par un poids moléculaire élevé de plus de 2000 kDa. Sur cette base, et en complémentarité avec les

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autres analyses (GCMS et FTIR), il a été suggéré que ces polysaccharides étaient des glycoprotéines de type arabinogalactanes sulfatés riches en protéines présentes dans la paroi cellulaire de l'algue verte *C. decorticans*.

Afin d'étudier les capacités élicitrices de ces glycoprotéines isolées de *C. decorticans*, les extraits bruts et purifiés (AGB et AGP) ont été appliqués par injection et pulvérisation sur les plants de tomates. Les résultats ont montré que les deux extraits induisaient les mécanismes de défense naturelle en augmentant l'activité PAL, les composés phénoliques et les teneurs en lignine dans les feuilles supérieures (n et n-1) et les racines en fonction du temps après traitement. AGB et AGP ont également montré leur potentiel à protéger les plants de tomate contre la verticilliose. Aucune différence considérable n'a été observée sur les effets éliciteurs et protecteur entre l'AGB et l'AGP. Des réponses similaires ont été observées après leur application sur les disques foliaires de l'olivier. Les deux fractions présentaient la même composition en monosaccharides (Gal, Glc, Ara, Xyl et Rha) et le même M_w (> à 2000 kDa), cependant, la présence de protéines et de sulfates était plus importante dans l'AGB (> à 20 %) que dans l'AGP (< à 14 %). Par conséquent, cette variation au niveau du degré de protéines et de sulfates ne semble pas avoir d'effet notable sur les réactions de défense. La partie glucidique de ce polymère semble être le principal facteur qui pourrait influencer les réponses induites, et probablement, c'est la partie reconnue par les récepteurs de la plante. Pour mieux comprendre cela, des études supplémentaires sont nécessaires, évaluant le potentiel éliciteur des fractions déprotéinées et/ou désulfatées.

Il a été rapporté que les peptidoglycanes (PGN) sont reconnus comme MAMP chez *Arabidopsis thaliana*, le riz et le tabac [18-21]. Les récepteurs membranaires reconnaissent plutôt des longues chaînes du squelette de sucre PGN, bien que la taille exacte des fragments requise pour déclencher l'immunité n'est pas encore établie [20]. Jusqu'à présent, tous les PRR qui appartiennent à la famille des protéines à motif lysine (LysM) sont connus pour interagir avec les polymères saccharidiques contenant des fragments *N*-acétylglucosamine (GlcNAc), tels que la chitine et les peptidoglycanes [22-24]. Cependant, aucune de ces études n'a pu conclure si cette affinité était due au fait que chaque domaine LysM peut se lier à une molécule glucidique spécifique ou au fait que plusieurs domaines LysM peuvent se lier à la même molécule glucidique [23]. Pour déterminer si c'est la partie saccharidique des glycoprotéines extraits de l'algue verte *C. decorticans* qui est perçus par des récepteurs de type LysM de la plante, des études supplémentaires sont nécessaires telles que les marquages à l'échelle moléculaire afin de suivre le devenir de la molécule au niveau des tissus de la plante.

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Ce travail a mis en évidence le potentiel SDP des glycoprotéines sulfatées isolées à partir des algues marines, et protecteur des plants de tomates contre la verticilliose. Il ouvre ainsi d'autres horizons pour la gestion de cette redoutable trachéomyose dans un contexte d'agriculture durable et respectueuse de l'environnement. De surcroît, la mise en évidence de l'efficacité des extraits bruts aqueux dans la stimulation de l'immunité des plantes, permettra de gagner en termes de rendement et de coûts des processus d'extraction, les étapes de purification n'étant pas nécessaires. La purification serait en revanche essentielle pour une meilleure compréhension des mécanismes d'actions de ces molécules aux stades de la reconnaissance et la transduction des signaux. En outre, l'extraction des polysaccharides fonctionnels avec de l'eau est une méthode d'optimisation simple et plus écologique. A l'échelle industrielle, ce processus semble être adéquat, puisqu'il remplit tous les critères requis pour avoir une meilleure rentabilité à moindre coût.

Par rapport aux alginates (ALCM et ALSM), les polysaccharides sulfatés (AGP, AGB) n'ont pas montré une différence significative de l'activité stimulatrice chez les jeunes plantules de tomate. Cependant, sur les disques foliaires de l'olivier, en plus des fucoïdanes extraits de l'algue brune *C. myriophylloides* (FUCCM), les arabinogalactanes sulfatés, ont révélé un pouvoir éliciteur plus efficace même à des faibles concentrations (0,5 g/L et 1 g/L). En réponse aux fucoïdanes à 0,5 g/L, les niveaux d'activité PAL, de composés phénoliques et de teneurs en lignine étaient significativement plus élevés par rapport aux arabinogalactanes (AGB et AGP) et aux alginates (ALCM et ALSM). La fraction des fucoïdanes (FUCCM) est composée principalement de fucose (53,39 %) et elle est caractérisés par un M_w de 148 kDa et un degré de sulfatation de l'ordre de 12,83 % (similaire à celle de l'AGP (13,21 %)). La composition en monosaccharide et/ou le poids moléculaire semble être les facteurs principaux de cette variabilité des réactions de défense sur les disques foliaires de l'olivier. D'autre part, la différence de réponse entre les plants de tomate et de l'olivier pourrait être expliquée par les différentes voies métaboliques de défense prioritairement mobilisées et qui peuvent varier d'une plante à l'autre et selon le modèle expérimental adopté (disques foliaires et plantules). Les mécanismes de défense varient selon les espèces végétales, ils sont souvent le résultat d'une adaptation temporelle à des conditions climatiques ou environnementales spécifiques [25]. Ainsi, les morphologies des feuilles de l'olivier et de la tomate sont très différentes. Les feuilles de l'olivier sont recouvertes d'une cuticule plus épaisse comparée aux feuilles de la tomate. Ceci pourrait impacter la diffusion et la disponibilité des molécules appliquées par voie foliaire et donc leur perception et leur efficacité électrique. L'induction des défenses des plantes étant

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régit par les facteurs génétiques, physiologiques et environnementales, sa modulation demeure très complexe. De nombreuses études ont rapporté l'impact du génotype de la plante dans les réponses aux traitements par les éliciteurs [26-28].

La recherche d'une synergie d'action par la combinaison de différentes molécules stimulatrices des défenses naturelles des plantes a été concluante dans plusieurs travaux [12,29-31]. Néanmoins, dans la présente étude, la combinaison de polysaccharides sulfatés (AGP) et non sulfatés (ALCM et ALSM) n'a pas amélioré l'efficacité stimulatrice et protectrice sur les plants de tomate. Cependant, une synergie d'action a été observée au niveau des paramètres de croissance (fructification). Plusieurs autres études ont montré que l'application d'un mélange d'éliciteurs tels que le jasmonate de méthyle (MeJA) et l'acide salicylique (SA) était moins efficace par rapport au traitement individuel [32,33]. Ceci pourrait s'expliquer au moins en partie par une compétition des différents éliciteurs pour les mêmes récepteurs membranaires qui reconnaissent des groupements fonctionnels communs à ces molécules. Pour vérifier cette hypothèse, il serait toutefois intéressant de réaliser différents marquages à l'échelle moléculaire pour élucider les mécanismes intimes de perception des molécules électrices par les récepteurs membranaire de la plante.

Dans cette étude, les plants de tomates ont été traités par trois modes d'application différents : l'injection au milieu de la tige, au cours de laquelle les molécules pénètrent directement dans le système végétal, le traitement foliaire par pulvérisation qui permet la pénétration des molécules par les stomates et/ou la cuticule, et un traitement racinaire, soit par l'apport des éliciteurs au sol favorisant le contact direct des racines avec les solutions saccharidiques (résultats non présentés). Les résultats obtenus ont montré que la réponse induite varie d'un mode de traitement à l'autre, surtout au niveau de leur précocité, sans affecter l'activité électrice des molécules étudiées. L'induction de l'activité PAL, la première enzyme intervenant dans les défenses naturelles des plantes, était plus précoce après application par voie racinaire (12 h après le traitement). La pulvérisation a stimulé cette enzyme d'une manière relativement tardive (2 jours à trois jours après le traitement). En outre, au niveau de l'activité protectrice, une différence selon les modes de traitement (foliaire et racinaire) a été observée. Il est important de souligner que nos résultats ont montré que quel que soit le site d'application de ces éliciteurs, les réponses induites étaient systémiques. Elles sont exprimées tout au long de la plante, dans les feuilles et les racines. D'autre part, l'application des molécules saccharidiques sur les plants de tomate a montré une efficacité de la pulvérisation dans le contrôle du développement de *V. dahliae*, et qui était comparable à celle du traitement racinaire. Ceci encourage l'utilisation de

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la pulvérisation pour contrôler non seulement les maladies foliaires, mais aussi les maladies vasculaires. De plus, cette méthode est plus simple, rapide et ciblée par rapport à d'autres modes d'application tels que l'application au sol. Les molécules sont directement en contact avec la plante sans dilution ni dégradation qui pourraient être produites par l'activité des micro-organismes du sol.

En plus de leur pouvoir de contrôler le développement de la verticilliose, les fractions polysaccharidiques et oligosaccharidiques ont amélioré en parallèle la croissance des plants de tomate en présence du pathogène. Ceci indique leur propriété polyvalente en tant qu'éliciteur et biostimulant. En outre, cette étude a également mis en évidence l'importance de la stimulation des défenses naturelles des plantes pour la gestion de la verticilliose.

En conclusion, l'ensemble des résultats obtenus dans ce travail a permis d'évaluer les activités électrice et protectrice des polysaccharides et des oligosaccharides d'origine algale en conditions contrôlées, en mettant l'accent sur l'étude de certains critères qui peuvent influencer leur efficacité au champ. Nous avons ainsi identifié des nouveaux polysaccharides algaux dotés d'un pouvoir stimulateur des défenses naturelles des plantes. Les résultats qui émanent de ce travail ouvrent ainsi la voie à l'exploration de cette méthode alternative pour la lutte contre la verticilliose. Ceci dans le but principal de formuler des nouvelles générations des SDPs biosourcés ne présentant pas de toxicité pour l'environnement et n'ayant pas d'effets directs sur les pathogènes ce qui limite les phénomènes de résistance qui affaiblissent l'efficacité des pesticides. Ils peuvent ainsi répondre aux besoins du marché agricole en produits phytosanitaires écologiques et efficaces pour une agriculture durable et innovante.

Bien que ces résultats soient innovants, plusieurs questions et points restent à prendre en considération quant à la continuité des travaux et nous les citerons comme perspectives.

Même si les algues constituent une biomasse hautement renouvelable et abondante, à l'échelle industrielle, il faut prendre en considération les défis qui pourraient être liés à la récolte, la transformation, le stockage et la croissance saisonnière. Pour une récolte à grande échelle, il apparaît important que les industries prennent en compte les quotas établis pour maintenir une utilisation durable de cette ressource. Un système de production pourrait être mis en place pour limiter la surexploitation des algues marines et préserver la biodiversité écologique. Il faut également tenir compte de la contamination de la biomasse algale par des métaux lourds et des polluants organiques qui peuvent menacer leur utilisation dans des applications agricoles. Plusieurs espèces d'algues marines ont tendance à absorber les polluants et les métaux lourds

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présents dans l'eau [34,35]. Une évaluation de la toxicité et de la biodégradabilité est donc nécessaire afin de vérifier l'innocuité des produits dérivés des algues. Ceci pourrait être effectué par marquage de la molécule permettant donc de suivre si des traces de celle-ci persistent dans le produit final destiné à la consommation.

Bien que les molécules dérivées des algues évoquent de multiples réponses chez les plantes contre les phytopathogènes, une grande partie des mécanismes génétiques, physiologiques et biochimiques restent à élucider, ce qui nécessite des recherches plus approfondies et systématiques. Il serait intéressant de compléter ces premières observations par des études transcriptomiques et protéomiques pour mieux clarifier les voies métaboliques prioritairement mobilisées par chaque éliciteur. En outre, un suivi du devenir de la molécule après sa mise en contact avec les tissus de la plante serait également important. Il permettra de connaître le niveau auquel l'éliciteur est perçu et par quel type de récepteur (PRR).

En cas de l'application des solutions saccharidiques SDPs par pulvérisation, il serait nécessaire de prendre en considération certains facteurs environnementaux, physiologiques et biologiques affectant la réponse des plantes à ces molécules. La bonne maîtrise de ces paramètres est essentielle pour optimiser l'efficacité et la reproductibilité des performances des SDPs. La lumière, la température et l'humidité au moment de l'application foliaire peuvent affecter l'état métabolique de la plante et donc influencer directement les processus d'absorption à travers la surface des feuilles. Les pertes dues à l'évaporation des solutions, les ruissellements et les réflexions des gouttelettes en présence des rayonnements solaires sont ainsi des paramètres à tenir en compte. La physiologie des feuilles (âge et surface foliaire) pourrait également impacter l'absorption et l'utilisation des molécules élicitrices par la plante. Pour cela, il faudrait penser à ajouter des adjuvants (surfactants, etc.) afin de faciliter la pénétration. Cependant, il est important de choisir les adjuvants adéquats et d'étudier les interactions éliciteur-adjuvant pour vérifier leur effet sur l'activité stimulatrice.

En cas d'application dans le sol, il est important d'étudier les interactions entre les éliciteurs et le microbiote tellurique pour éviter tout effet indésirable. En outre, une évaluation de la biodégradabilité et du devenir de ces molécules dans le sol serait également très pertinente.

La plupart des recherches ont tendance à se concentrer sur les essais en conditions contrôlées. Cependant, des applications rigoureuses à large échelle en plein champ sont nécessaires avant toute proposition aux producteurs. Des expérimentations sur différents pathosystèmes, saisons

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et localisations géographiques pourraient être également importantes à mener dans les perspectives de notre étude.

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RESUME

Ce travail repose sur les connaissances fondamentales acquises sur les interactions plante-parasite en matière d'induction des mécanismes de défense des plantes en réponse aux agents phytopathogènes afin de développer une nouvelle génération de molécules biosourcées susceptibles de stimuler les défenses naturelles des plantes dans un contexte de développement durable. Il est ainsi admis que le déclenchement du système de défense de la plante résulte d'une reconnaissance par la cellule-hôte d'un éliciteur souvent produit par le pathogène (éliciteur exogène) ou par la plante-hôte (éliciteur endogène). La plupart des éliciteurs sont des polysaccharides et particulièrement des oligosaccharides. Compte tenu de leur richesse en polymères de glucides, les algues constituent une source naturelle pouvant être valorisée en matière de recherche d'éliciteurs des défenses naturelles des plantes. L'objectif principal de ce travail consiste en la recherche et la caractérisation des poly/oligosaccharides d'algues marines, susceptibles de stimuler les défenses naturelles (SDPs) de la tomate et de l'olivier et d'induire la résistance vis-à-vis de la verticilliose.

Le travail a permis tout d'abord l'extraction et les caractérisations chimique et structurale des polysaccharides non sulfatés (alginates) à partir des algues brunes marocaines (*Cystoseira myriophylloides*, *Sargassum muticum* et *Bifurcaria bifurcata*) et la production de leurs dérivés de faibles poids moléculaires, ainsi que les polysaccharides sulfatés (arabinogalactanes et fucoïdanes) à partir des algues verte et brune (*Codium decorticatum* et *C. myriophylloides*). Le pouvoir éliciteur de l'ensemble de ces molécules a été évalué sur les plants de tomate et les disques foliaires de l'olivier à travers le suivi des principaux mécanismes de défense reconnus par leur implication dans la résistance des plantes aux agents phytopathogènes notamment, la phénylalanine ammonia-lyase (PAL) et la tyrosine ammonia-lyase (TAL), enzymes clés du métabolisme phénolique et l'accumulation des polyphénols solubles et de la lignine. Cette étude a permis d'opérer les principaux paramètres conduisant à des réponses optimales (structure des molécules, mode d'application et synergie entre différents motifs éliciteurs). Le traitement des plants de tomate et les disques foliaires de l'olivier par les fractions extraites des algues brunes et verte ainsi que leurs dérivés a révélé un potentiel éliciteur remarquable. Toutefois, les activités électriques ont montré une variabilité en fonction de la durée de l'incubation, la structure des molécules et le mode de traitement. En outre, ces motifs saccharidiques ont montré un potentiel protecteur des plants de tomate vis-à-vis du développement de la verticilliose causée par *Verticillium dahliae* avec une réduction considérable de l'incidence et de la sévérité de la maladie et de la mortalité, comparée à la laminarine qui représente la substance active du SDP commercial Iodus 40[®]. L'ensemble des résultats obtenus ouvre de nouveaux horizons pour la gestion des phytopathogènes vasculaires à travers une stratégie compatible avec les principes d'une agriculture durable respectueuse de l'environnement.

Mots clés : Polysaccharides, Oligosaccharides, Algues marines, SDPs, *Verticillium dahliae*, Agriculture durable.