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► **To cite this version:**

Mélina Ramos. Characterization of biocontrol products' effect on treated plants: residues fate and plant response. Analytical chemistry. Université de Perpignan; Universitat de Girona, 2022. English. NNT : 2022PERP0005 . tel-03736276

**HAL Id: tel-03736276**

**<https://theses.hal.science/tel-03736276>**

Submitted on 22 Jul 2022

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# THÈSE

Pour obtenir le grade de  
Docteur

Délivrée par  
**UNIVERSITE DE PERPIGNAN VIA DOMITIA**  
et  
**UNIVERSITAT DE GIRONA**

Préparée au sein de l'école doctorale ED 305  
Et des unités de recherche  
CRIOBE, CIDSAV et BABVE

Spécialités : Chimie et Technologies

Présentée par  
Mélina RAMOS

**Characterization of biocontrol products' effect on  
treated plants: residues fate and plant response**

Soutenue le 21 avril 2022 devant le jury composé de :

Mrs. Frédérique COURANT, Associate Pr., <i>Université de Montpellier</i>	reviewer
M. Pierre PETRIACQ, Associate Pr., <i>Université de Bordeaux</i>	reviewer
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*« N'oublie jamais, celui qui croit savoir n'apprend plus. »*

*« Le doute est une force. Une vraie et belle force.  
Veille simplement qu'elle te pousse toujours en avant. »*

**Pierre Bottero, Le Pacte des Marchombres.**





## ACKNOWLEDGEMENTS

I would like to express my gratitude to the members of the Jury, Dr. Frédérique Courant and Dr. Pierre Pétriacq, who have given me the honor of reviewing the present manuscript. I would also like to thank Pr. Anna Bonaterra and Dr. Christophe Calvayrac for their acceptance to examine the present work and to participate in the Jury of my thesis. This is a great honor for me.

I would like to express my gratitude for the five persons who offered me the opportunity to access this thesis, and supervised my work during those four years. They let me take initiatives, helped me focusing on the targets, and were always open to discuss new ideas and propositions. Despite all the difficulties and the critical moments, they always supported my work, they transferred their knowledge and experience to me, which improved my scientific, technical and personal skills. They gave me their trust, and finally guided me towards the accomplishment of this manuscript with a lot of satisfaction from my part. They also helped me at a personal level towards the administrative adventures that constituted a part of this cotutelle experience between France and Spain. Thus, I express my deepest thanks to my supervisors Pr. Cédric Bertrand, Pr. Emilio Montesinos, Pr. Mercè Llugany, Dr. Marie-Virginie Salvia, and Dr. Esther Badosa.

I would like to thank Dr. Anne-Emmanuelle Hay de Bettignies, Pr. Josep Allue Creus, and once again Dr. Christophe Calvayrac for their acceptance to participate to my “Comités de Suivi Individuels” (CSI), and for their appreciated support, guidance, and advices that helped me focusing my targets and orientating my perspectives.

I would like to thank the European Regional Development Fund (ERDF) through the Interreg V-A Spain France Andorra program ([POCTEFA 2014-2020](#)) that co-financed my thesis that is part of the PALVIP project. The assistance and expertise provided by all the partners of the PALVIP project were greatly appreciated. I wish to show my appreciation to Sophie Gabolde who perfectly coordinated the project and to Dr. Vanessa Andreu who offered me her expertise on Akivi product. I am particularly grateful to Dr. Hikmat Ghosson with whom we shared very interesting conversations and solved all the problems we could with our metabolomics research.

I also acknowledge the “Réseau Francophone de Métabolomique et Fluxomique” (RFMF) for allocating me grants during these four years of doctoral work, that allowed me to participate and to communicate in the national congresses that they organized in 2018, 2019, and 2021 and to the international congress organized in 2019 by the Metabolomics Society. These participations helped me improving my knowledge, my skills and my network that were necessary for the accomplishing of the present thesis, and for my aimed future career in scientific research as well.

I wish to extend my special thanks to the three laboratory teams for their warm welcome, their help, their expertise, and their funny company during all these hours spent within the laboratory. I am grateful to Hikmat (again), Mélanie, Christian, Slimane, Christelle, Mathieu, Chandra, Delphine, Jennifer, and all the CRIOBE team for their humor and every good moment we had during formations and congresses. I would like to thank Laura, Nuria, Bea, Mireia, Aina, Hector, Gemma, Josep, Anna, Lluís and all the CIDSAV team as well as Laura, Maria, and all the BABVE team who welcomed me in Spain with good mood and helped me through the difficult times of the pandemic.

Un grand merci à mes proches et à ma famille sans qui je n’aurai pas pu participer à cette aventure qu’est la thèse. En particulier mes parents qui m’ont supportée toutes ces années d’études ainsi que Manou, Manon, Abel, mes amis de longue date Claire, Margaux, Audrey, Aurore, Coralie, Pauline, Cécile, Cannelle, Charlotte, Juliette, Lucie, et Marine qui m’ont soutenue tout au long de ces années, qui ont partagés mes joies et qui ont su me changer les idées dans les moments difficiles.

Enfin je souhaite remercier ces personnes, hormis mes parents, qui ont été particulièrement présents durant ces années de thèse.

Marie, milles mercis pour toutes ses années de belle amitié, pour ta présence en toutes circonstances, pour ces escapades le temps de quelques jours pour décompresser, pour ces mini-aventures qui peuplent notre quotidien en particulier quand on est ensemble, pour ton soutien, pour ton aide à organiser mes pensées qui se mélangent, pour tous ces appels, ces fous rires et ces larmes. Merci de faire partie de ma vie.

Pierre, un grand merci pour ton aide et ton expertise sur la réglementation qui m’a bien donnée du fil à retordre. Merci pour ta porte toujours ouverte, tes blagues pas drôles qui me font rire quand même, et ces randonnées en montagne pour prendre l’air et me changer les idées.

Merci à la fine équipe de joueurs invétérés de Perpignan avec qui j'ai partagé de nombreux moments épiques de JDR, des fous rires, des sorties plages, des balades, des jeux de sociétés, des soirées films nuls hilarants, des voyages (Team Ecosse représente !), et des apéros ! Eléonore, ma MJ préférée qui supporte mon comportement chaotique peuplé de bonnes intentions, merci de ton soutien à travers cette thèse qui n'a pas été un long fleuve tranquille. Tu es ma référence BD de qualités, merci pour ces supers découvertes posée sur ton canapé toujours prêt à accueillir ! J'apprécie tout particulièrement nos conversations nocturnes inopinées mais toujours extrêmement intéressantes autour d'un verre de bon rhum ! Mélody, ma référence astronomie ! J'ai hâte de tester ton télescope ! Merci pour ces soirées films/ séries (Kaaaarl, et Tomeeer) en parallèle timées à l'horloge mondiale qui ont toujours été de supers moments d'un grand réconfort surtout pendant les confinements. Stephen, merci pour l'épisode de Lukas et sa boussole qui restera un de mes meilleurs fous rires, j'en ri encore. Merci pour toutes ses discussions et ces jeux en ligne d'un grand réconfort durant cette période compliquée de pandémie. Sylvain et Nélia, merci pour ces super moments de convivialité chez vous et au resto toujours autour d'un bon repas, merci de continuer à nous inviter malgré la destruction de la planète plate. Meryl, ma deuxième MJ préférée, merci pour ta cuisine exquise, ton accueil, ton humour, ton côté mamie Nova. Merci pour ta présence et ton dynamisme. Alicia, j'aime notre passion pour les différents types de fenêtres, il faut être précises ! Merci pour tous ces films « cultes » que je n'aurais jamais vu sans ton intervention, il y en a tant à découvrir après tout ; « Tout a commencé bien avant ma naissance ! ». Chrystelle, l'afficionados des soirées films « Il va t'arriver des bricoles ! » et surtout le cri de guerre « Ninja ! ... ». Mégane, la professionnelle de la vidéo, j'ai hâte de revenir voir passer un train avec toi !

Un très grand merci à toutes les personnes qui m'ont soutenue et je prie celles que j'ai oublié de citer ici de bien vouloir m'excuser.



## ABSTRACT

A more sustainable use of plant protection products (PPPs) is promoted by European Union governments. Indeed, each country is encouraging the development of complements and alternatives to chemical PPPs in order to reduce their use. From this initiative, PPPs from natural sources are promoted, namely biocontrol products (BPs). These new BPs are complex mixtures or microbial strains that are difficult to monitor in complex environmental matrices. Besides, they present modes of action that are not fully understood. For instance, they can enhance plant defense mechanisms against the infection that can trigger plant resistance to the pathogen. More knowledge is therefore needed to better use and regulate these BPs. Thus, this thesis focuses on (1): the investigation of BPs fate on treated plants through a kinetics study monitoring their residues and (2): the plant response to the treatment; in order to better understand the mechanisms involved in BPs efficacy and how they interact with the plant and the environment. Along the investigations, two BPs candidates currently in development were studied, a formulated prototype botanical extract (Akivi, AkiNaO) and a bacterial strain (*Bacillus* UdG, UdG) that gave promising field efficacy results. Firstly, a new tool was developed in order to study residues fate. It is based on an innovative approach (Environmental Metabolic Footprinting, EMF) developed in the CRIOBE laboratory on soil and sediments to study the impact of BPs on the environment. This EMF approach was optimized during the thesis in order (1): to be adapted to fruit matrix (peach peels) and (2): to target only the xenometabolome (residues fate) and then compare treated and untreated samples. Optimized EMF was proven reliable for BPs residues dissipation monitoring on peach peels, and it is currently tested on other plant matrices in order to extend its use, even if the approach still needs to be improved to overcome field experiments variabilities. Secondly, plant response to two treatments (Akivi or *Bacillus* UdG) at transcripts and metabolites levels was evaluated. RNA sequencing gave data about the different genes expression following treatments with BPs compared to the untreated controls. Differentially expressed genes (DEGs) were investigated for both treatments and highly influenced DEGs were selected and were tested in the three cultivars as treatment gene markers using RT-qPCR. These data were completed with metabolic analysis (phytohormones, phenols, and organic acids). Strong hints were found on grapevine defense induction by the treatments, but further investigations are necessary in order to confirm these first results. For each BP treatment, genes markers were identified; these markers could be used to monitor the activity of the products in field treatments for further investigations. In conclusion, this thesis used transcriptomics together with metabolomics to better understand the interaction between the BPs and the treated plant; it also allowed the development of new promising tools to monitor BPs residues on treated plants that could be used for regulation processes.



## RÉSUMÉ

La France et l'Espagne sont les plus grands consommateurs de produits phytosanitaires (PPPs) de l'Union Européenne (UE) (European Commission, 2020). Les PPPs sont des produits utilisés en agriculture pour éradiquer, ou pour contrôler tous types de maladies ou ravageurs qui peuvent avoir un impact sur la production agricole (FAO, 2006). Cependant les PPPs de synthèse peuvent avoir un fort impact négatif sur l'environnement et sur la santé, c'est pourquoi, une utilisation plus durable des PPPs est plébiscitée par les gouvernements de l'UE (European Parliament and Council Of The European Union, 2009). Chaque pays de l'UE encourage le développement d'alternatives et de solutions complémentaires pour réduire l'utilisation des PPPs de synthèse. Parmi ces solutions les PPPs d'origine naturelle sont en plein essor, il s'agit de produits composés de molécules ou d'organismes déjà présent en tant que tels dans la nature, *i.e.* les produits de biocontrôle (BPs).

Ces BPs sont des mélanges complexes ou des souches microbiennes difficiles à détecter et à suivre dans les matrices environnementales. En effet, les méthodes de suivis actuelles utilisées et validées pour les PPPs de synthèse, comme l'utilisation du temps de demi-vie (DT50) ne sont pas applicables au BPs. De plus, les BPs présentent des modes d'action différents de ceux des PPPs de synthèses ce qui offre de belles perspectives pour lutter contre les souches de pathogènes résistantes aux traitements conventionnels (Villaverde *et al.*, 2016), cependant ces nouveaux modes d'actions ne sont pas entièrement décrits. Par exemple, les BPs comme les substances naturelles ou les microorganismes peuvent avoir une activité de stimulation des défenses de la plante. Cette stimulation des défenses de la plante par application de BPs peut alors directement protéger la plante contre l'infection par un pathogène, ou indirectement préparer la plante à donner une réponse plus forte et plus rapide lors d'une future infection par un pathogène. Dans les deux cas, la stimulation des différentes voies de défenses de la plante peut la protéger contre la maladie. Les voies de défenses de la plante au stress biotique et/ou abiotique reposent sur différents niveaux de reconnaissance de motifs moléculaires ou d'effecteurs spécifiques menant à la synthèse de molécules de défenses qui peuvent avoir une action directe contre le pathogène ou agir directement sur la plante en stimulant ses voies de défense. Ces voies de défenses impliquent différentes phytohormones comme l'acide jasmonique, l'acide salicylique, ou l'éthylène. Les BPs peuvent intervenir à différents niveaux de ces voies de défenses, ainsi, de plus amples connaissances sont nécessaires pour mieux utiliser et réguler ces BPs.



Afin de répondre à ces problématiques, mon travail de doctorat s'est intéressé (1) : à l'étude du devenir des résidus de BPs sur les plantes traitées et (2) à l'étude de la réponse des plantes suite au traitement. L'expression génétique et le contenu métabolique ont été analysés afin de mieux comprendre les mécanismes impliqués dans l'efficacité des BPs et comment ils interagissent avec la plante et l'environnement. Au cours de ces investigations, deux BPs en cours de développement ont été étudiés, un prototype d'extrait botanique formulé (Akivi, *Dittrichia viscosa*, AkiNaO) et une souche bactérienne (*Bacillus* UdG, *Bacillus velezensis*, UdG) qui ont montré des résultats d'efficacité prometteurs au champ.

En premier lieu, un nouvel outil a été mis au point pour étudier le devenir des résidus. Il est basé sur une approche innovante (Environmental Metabolic Footprinting, EMF) développée au laboratoire CRIOBE sur sols et sédiments afin d'étudier l'impact de BPs sur l'environnement (Patil *et al.*, 2016; Salvia *et al.*, 2018). Cette approche EMF est basée sur de la métabolomique non ciblée en chromatographie liquide couplée à la spectrométrie de masse. L'EMF permet d'étudier le méta-métabolome de la matrice traitée, c'est-à-dire l'endométabolome regroupant les métabolites provenant de la matrice elle-même mais aussi le xénométabolome regroupant les métabolites provenant du produit appliqué et de leurs produits de dégradation. L'approche repose sur une étude cinétique de l'évolution du méta-métabolome des échantillons en comparant à chaque pas de temps les échantillons traités (endométabolome et xénométabolome) et les échantillons non traités (endométabolome). L'objectif de l'approche EMF est d'obtenir le temps de résilience au bout duquel la matrice traitée n'est plus impactée par le traitement, autrement dit à partir de quel point cinétique il n'y a plus de différences entre le méta-métabolome des échantillons traités et celui des échantillons non traités.

L'EMF a été optimisé au cours de la thèse pour être adapté à la matrice fruits (peaux de pêches) ainsi que pour cibler le xénométabolome, c'est-à-dire les résidus du produit appliqué. Pour cela l'approche EMF « d'origine » a été optimisée pour séparer le xénométabolome provenant du BP et l'endométabolome provenant du fruit. L'EMF optimisé s'est avéré être une approche fiable pour le suivi de la dissipation des résidus de BPs sur les peaux de pêches. Le suivi de la dissipation d'Akivi et de ses produits de dégradation a pu être effectuée et une cinétique a été dégagée montrant une dissipation de la plupart des résidus d'Akivi entre 7 et 14 jours après le dernier traitement. Cependant, l'échantillonnage n'a pas duré assez longtemps pour identifier une dissipation complète des xénométabolites. De plus, l'approche doit encore être améliorée pour mieux

maîtriser la variabilité induite par les expérimentations au champ. Ces résultats indiquent que l'EMF, précédemment développé sur sol et sédiments, est applicable aux matrices végétales, en particulier aux fruits, et qu'elle peut être utilisée pour suivre le devenir de BP complexes. L'EMF optimisé pourrait être utilisé pour suivre l'efficacité de produits au champs, qui découle de la dissipation du produit, et décider du calendrier de traitement le plus adapté. De plus, l'approche pourrait être envisagée pour une utilisation à large échelle dans le cadre des études de résidus nécessaires à l'autorisation de mise sur le marché de BPs par exemple. La prochaine étape serait de cibler l'effet du traitement sur l'endométabolome de la matrice végétale et de combiner les suivis de l'endométabolome et du xénométabolome pour évaluer l'impact du traitement sur la plante traitée et ses microorganismes associés. En effet, comme expliqué précédemment, l'approche EMF globale permettrait d'obtenir le temps de résilience de la plante après le traitement.

En second lieu, l'évaluation de la réponse de la vigne après le traitement avec les BPs, Akivi ou *Bacillus* UdG, a été menée sur trois cépages locaux Méditerranéens : Garnacha Blanca, Garnacha Tinta et Macabeo. Une combinaison d'approches transcriptomiques et métabolomiques a été choisie afin d'étudier l'expression des gènes dans les feuilles de vigne et d'identifier de possibles gènes marqueurs du traitement, ainsi que pour déterminer les métabolites des feuilles de vigne présentant une concentration différente en réponse aux traitements. Les plants de vigne ont été cultivés dans des conditions contrôlées sous serre et soumis aux traitements par pulvérisation, puis les feuilles ont été échantillonnées 24 heures après le traitement afin de réaliser une étude de l'expression des gènes par séquençage massif de l'ARN (ARN-seq) sur les extraits de feuilles du cépage Garnacha Blanca et par RT-qPCR sur une sélection de gènes pour les trois cépages. Les résultats de l'ARN-seq ont été analysés et des gènes exprimés de manière différentielle (DEGs) ont été obtenus pour les deux traitements, une sélection de certains DEGs spécifique de chaque traitement a été menée pour tester leur expression dans les trois cépages comme gènes marqueurs du traitement. En outre, une extraction des composants foliaires a été effectuée pour quantifier la teneur des feuilles en certains métabolites ciblés tels que les phytohormones, les acides organiques et les phénols.

En considérant tous les gènes surexprimés et sous-exprimés ainsi que les concentrations plus importantes de certains métabolites dans les échantillons traités, de solides indices ont été trouvés en faveur de l'hypothèse de la stimulation des défenses naturelles de la vigne par les traitements. Plus précisément, les traitements ont stimulé certaines voies de défense de la vigne telles que celles de l'acide jasmonique, de l'éthylène

et des phénylpropanoïdes. De plus amples recherches seront nécessaires pour détailler l'activité de stimulation de défense de la vigne par les produits Akivi et *Bacillus* UdG. En particulier l'interaction BP / vigne / pathogène devrait être étudiée car certaines voies de défenses peuvent être stimulées jusqu'à un certain point par un traitement, mais le produit peut aussi avoir pour effet de faciliter une réponse plus forte et plus rapide des voies de défense lors de l'infection par un pathogène. Une étude cinétique de la réponse de la vigne aux traitements pourrait aussi être menée pour mieux corrélérer l'expression des gènes avec le contenu métabolique des échantillons. En effet, la réponse à l'échelle transcriptomique ou à l'échelle métabolomique peut avoir lieu à différents moments après application du traitement. En parallèle, plusieurs gènes marqueurs de chacun des traitements ont été identifiés, présentant une surexpression stable après les traitements dans les trois cépages de vigne. Ces marqueurs génétiques pourraient être utilisés lors des expérimentations des produits au champ pour suivre leur activité sur les plantes traitées en conditions non contrôlées.

En conclusion, ce travail de doctorat a utilisé les techniques de transcriptomique ainsi que de métabolomique pour mieux comprendre l'interaction entre les BPs et la plante traitée. Un nouvel outil prometteur pour suivre les résidus de BPs sur les plantes traitées a également été développé et il pourrait, à termes, être utilisé dans les processus réglementaires menant à l'autorisation des BPs.

## RESUMEN

Francia y España son los mayores consumidores de productos fitosanitarios (PPPs) de la Unión Europea (UE) (European Commission, 2020). Los PPPs son los productos que se utilizan en agricultura para erradicar o controlar todo tipo de enfermedades y plagas que pueden afectar a la producción agrícola (FAO, 2006). Sin embargo, los PPPs sintéticos pueden tener un fuerte impacto negativo en el medio ambiente y en la salud humana, por lo que los gobiernos de la UE abogan por un uso más sostenible de estos productos (European Parliament and Council Of The European Union, 2009). Actualmente, todos los países de la UE fomentan el desarrollo y uso de soluciones alternativas y/o complementarias para reducir el uso de los PPPs sintéticos. Entre estas soluciones alternativas destaca el gran desarrollo de los PPPs de origen natural, es decir los productos de biocontrol (BPs), compuestos por moléculas u organismos ya presentes en la naturaleza.

Estos BPs son mezclas complejas o cepas de microorganismos que pueden ser difíciles de detectar y monitorizar en matrices ambientales. De hecho, los actuales métodos utilizados y validados para el seguimiento de los PPPs sintéticos, como la utilización del término de la vida media del producto o (DT<sub>50</sub>) no son aplicables a los BPs. Además, dado que los BPs tienen mecanismos de acción diferentes a los de los PPPs sintéticos, ofrecen buenas perspectivas para el control de cepas patógenas resistentes a los tratamientos convencionales (Villaverde *et al.*, 2016), sin embargo, estos nuevos mecanismos de acción no están completamente descritos ni elucidados. Por ejemplo, los BPs, tanto las sustancias naturales como los microorganismos, pueden provocar una respuesta defensiva de la propia planta. La estimulación de la respuesta defensiva de la planta por la aplicación de estos productos, puede directamente proteger a la planta de un ataque del patógeno o indirectamente, preparar a la planta para dar una respuesta más rápida en un futuro ataque del patógeno. En ambos casos, la estimulación de las distintas vías de defensa de la planta puede proteger a la planta de la enfermedad. Las distintas vías de defensa de la planta que se activan frente al estrés biótico y/o abiótico se basan en diferentes niveles de reconocimiento de patrones moleculares o efectores específicos que conducen a la síntesis de moléculas de defensa que pueden actuar directamente contra el patógeno o que pueden actuar directamente sobre la planta estimulando su respuesta defensiva. Estas vías de defensa implican diferentes fitohormonas como son el ácido jasmónico, el ácido salicílico o el etileno. Los BPs pueden intervenir a distintos niveles de estas vías de defensa, por lo que actualmente es necesario adquirir más conocimiento para una mejor utilización y regulación de estos BPs.

Con el objetivo de dar respuesta a estas problemáticas, esta tesis se centra en: (1) el estudio de la trazabilidad de los residuos de los BPs en las plantas tratadas y, (2) en el estudio de la respuesta de las plantas después del tratamiento con los BPs. Se analizó la expresión génica y el contenido metabólico de las plantas tratadas respecto al control, para comprender mejor la respuesta de la planta al tratamiento con los BPs. En el transcurso de esta investigación, se estudiaron dos BPs en desarrollo, un prototipo de extracto botánico formulado (Akivi, *Dittrichia viscosa*, AkiNaO) y una cepa bacteriana (*Bacillus* UdG, *Bacillus velezensis*, UdG) que mostraron resultados prometedores de eficacia en campo.

En primer lugar, se ha desarrollado y puesto a punto una nueva herramienta para monitorizar los residuos. Está basada en un enfoque innovador (Environmental Metabolic Footprinting, EMF) desarrollado en el laboratorio CRIOBE para suelos y sedimentos con el fin de estudiar el impacto de los BPs en el medio ambiente (Patil et al., 2016; Salvia et al., 2018). Este enfoque EMF se basa en el estudio metabólico no dirigido mediante cromatografía líquida acoplada a espectrometría de masas. Este enfoque EMF permite estudiar el meta-metaboloma de las matrices tratadas, es decir, el endometaboloma que agrupa los metabolitos procedentes de la propia matriz, pero también el xenometaboloma que agrupa los metabolitos procedentes del producto aplicado, así como sus productos de degradación. Con esta aproximación, se lleva a cabo un estudio cinético de la evolución del meta-metaboloma de las muestras, comparando en cada momento las muestras tratadas (endometaboloma y xenometaboloma) respecto a las muestras no tratadas (endometaboloma). El objetivo de la aproximación EMF es determinar el tiempo de resiliencia después del cual la matriz tratada ya no se ve afectada por el tratamiento, es decir, a partir de qué momento no hay diferencias entre el meta-metaboloma de las muestras tratadas y las no tratadas.

El EMF se ha optimizado en el transcurso de la tesis para adaptarla a la matriz de la fruta (pieles de melocotón) así como para el estudio del xenometaboloma, es decir, a los residuos del producto aplicado. Para ello, el enfoque “original” del EMF se ha optimizado para separar el xenometaboloma correspondiente al BP y el endometaboloma del fruto. El EMF optimizado ha mostrado ser un método fiable para monitorizar la disipación de los residuos de los BPs en la piel de melocotón. Se llevó a cabo el seguimiento de la disipación de Akivi y sus productos de degradación en la piel de melocotón y los resultados de la cinética mostraron la disipación de la mayoría de los residuos de Akivi entre 7 y 14 días después del último tratamiento. Sin embargo, el tiempo de muestreo no fue lo

suficientemente largo para identificar la disipación completa de los xenometabolitos. Además, el enfoque aún debe ser mejorado para disminuir la variabilidad observada debido a que los experimentos son experimentos realizados en campo.

Estos resultados indican que la EMF, desarrollada previamente para su uso en suelos y sedimentos, es aplicable también a matrices vegetales, en particular a los frutos, y que puede ser utilizado para monitorizar la disipación de los BPs complejas. El EMF optimizado podría utilizarse para establecer el tiempo en que podría ser eficaz el producto en campo, teniendo en cuenta el tiempo de disipación del producto, y decidir el programa de tratamiento más adecuado. Además, este enfoque podría considerarse para su uso de manera generalizada en la determinación de residuos necesarios para la autorización de la comercialización de los BPs. La siguiente etapa consistiría en determinar el efecto del tratamiento sobre el endometaboloma de la matriz vegetal y combinar el seguimiento del endometaboloma y el xenometaboloma para evaluar el impacto del tratamiento y los microorganismos asociados a la planta tratada. De hecho, como se ha explicado anteriormente, el enfoque global de la EMF permitiría determinar el tiempo de resiliencia de la planta después del tratamiento.

En segundo lugar, la evaluación de la respuesta de la vid tras el tratamiento con BPs, Akivi o *Bacillus* UdG, se llevó a cabo en tres cultivares mediterráneos locales: Garnacha Blanca, Garnacha Tinta y Macabeo. Se ha realizado un estudio combinado de transcriptómica y metabolómica con el fin de estudiar la expresión génica en las hojas de la vid e identificar posibles genes marcadores de tratamiento, así como para determinar si se observan diferencias en la concentración de los metabolitos estudiados en respuesta a los tratamientos. Las plantas de vid se cultivaron en condiciones controladas en invernadero y se sometieron a los tratamientos mediante pulverización. Las hojas se recogieron 24 horas después del tratamiento para realizar un estudio de expresión génica por secuenciación masiva de ARN (ARN-seq) en extractos foliares de la variedad Garnacha Blanca y por RT-qPCR de una selección de genes para las tres variedades. Se analizaron los resultados del ARNseq obteniendo los genes expresados diferencialmente (DEGs) para ambos tratamientos y de esto se seleccionaron algunos DEGs específicos para cada tratamiento para ser estudiados en las tres variedades de vid como genes marcadores de tratamiento. Además, se realizó la extracción de los metabolitos que se querían determinar, fitohormonas, ácidos orgánicos y fenoles, de las hojas para su posterior cuantificación

Teniendo en cuenta todos los genes sobre-expresados y reprimidos, así como las mayores concentraciones de algunos de los metabolitos determinados en las muestras tratadas, apuntan a favor de la hipótesis de que los tratamientos estimularan el sistema de defensa de la vid. En concreto, los tratamientos estimularon ciertas vías de defensa de la vid, como la del ácido jasmónico, del etileno y de los fenilpropanoides. Sin embargo, son necesarios más estudios para corroborar esta actividad estimuladora de las defensas de la planta de vid por parte de los productos Akivi y *Bacillus* UdG. En particular, debe estudiarse la interacción BPs/ vid/ patógeno ya que algunas vías de defensa pueden ser estimuladas hasta un cierto grado por el tratamiento con un producto, pero puede haber un efecto de facilitador de una respuesta más rápida y mayor de las vías de defensa después del ataque de un patógeno. Sería también interesante realizar un estudio cinético de la respuesta de la vid a los tratamientos para correlacionar mejor la expresión génica con el contenido metabólico de las muestras, ya que la respuesta transcriptómica y la metabolómica pueden darse en distintos momentos después del tratamiento. Paralelamente, se identificaron varios genes marcadores para cada uno de los tratamientos, escogiendo los genes que habían mostrado una sobreexpresión estable tras los tratamientos en las tres variedades de vid. Estos marcadores genéticos podrían ser utilizados en ensayos de campo para determinar la actividad de los productos en las plantas tratadas en condiciones no controladas.

En resumen, en esta tesis doctoral se han utilizado técnicas de transcriptómica y metabolómica para comprender mejor la interacción entre los BPs y la planta tratada. También se ha desarrollado una nueva y prometedora herramienta para la monitorización de los residuos de los BPs en las plantas tratadas, que podría ser utilizada en los procesos reglamentarios llevando a la autorización de los BPs.

## INTELLECTUAL PROPERTY

The results of this PhD have been obtained under the PALVIP project (Protection Alternative des cultures Végétales Interregionales Pyrénéennes) for **alternative protection of interregional Pyrenean plant culture**, a project under the framework of the Transnational European Cooperation Programme INTERREG POCTEFA (2014-2020). The project brings together six partners: Chambre d'Agriculture des Pyrénées Orientales, Université de Perpignan Via Dolomita (UPVD) from Roussillon and Institut Català de la Vinya i el Vi (INCAVI), Universitat de Girona (UdG), Universitat Autònoma de Barcelona (UAB) and the enterprise Futureco Bioscience, all from Catalonia. The aim of the project is to find new biocontrol solutions to protect the most important crops in the agricultural Mediterranean area.

This PhD has been done thanks to a specific Grant under the PALVIP project, and the research was performed in the laboratory CRIOBE USR3278 at the Université de Perpignan Via Domitia (UPVD), laboratory of Plant Pathology-CIDSAV at the University of Girona (UdG) and laboratory of Plant Physiology-BABVE at the Autonomus University of Barcelona (UAB).

As a consequence, this PhD is submitted to shared intellectual property with researchers that have participate in the PALVIP project in the above-mentioned laboratories (UPVD, UdG, UAB), according to the corresponding regulation in Spain (RD 1326/2003 of 24-10-2003; Intellectual Property Law, RD 1/1996 of 12-04-1996) and France (Code de la propriété Intellectuelle).

In agreement with the above mentioned consideration I undersign the present declaration in Perpignan, February 15th, 2022.

Signature: Méлина Ramos





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## ABBREVIATIONS

2-OG: Oxoglutaric acid

ABA: Abscisic Acid

Abs: Absorbance

ACC: 1-Aminocyclopropane-1-Carboxylic acid

ACO: 1-Aminocyclopropane-1-Carboxylate Oxidase

Aki: Akivi

ANSES: Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail

Arm: Armicarb®

AS: Active Substance

BF: Fresh culture of *Bacillus* UdG

BHLH TFs: Helix Loop Helix Transcription Factors

BL: *Bacillus* UdG lyophilized

BPs: Biocontrol Products

Bt: *Bacillus thuringiensis*

<sup>14</sup>C: Carbon-14, carbon radioactive isotope

CaM: Calmodulin

CDPKs: Ca<sup>2+</sup> Dependent Kinases

Chi: Chemical reference

CHS: Chalcone Synthase

CFU: Colony-Forming Unit

<sup>35</sup>Cl: Chlorine main stable isotope

<sup>37</sup>Cl: Chlorine stable isotope

Ctr: Control

CO<sub>2</sub>: carbon dioxide

DAMPs: Damage-Associated Molecular Patterns

DEGs: Differentially Expressed Genes

DNA: Deoxyribonucleic Acid

DT<sub>50</sub>: half-life

DT<sub>90</sub>: 90% dissipation time

DW: Dry Weight

EDS1: Enhanced Disease Susceptibility 1

EFSA: European Food Safety Authority  
EMF: Environmental Metabolic Footprinting  
ERDF: European Regional Development Fund  
ESI: Electrospray  
ET: Ethylene  
ETI: Effector-Triggered Immunity  
EU: European Union  
FAO: Food and Agriculture Organization of the United Nations  
FC: Fold Change  
FDR: False Discovery Rate  
FPMK: Fragments Per Kilobase Million  
GA: Gibberillin  
GO: Gene Ontology  
GST: Glutathione-S-Transferase  
H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide  
HAMPS: Herbivore-Associated Molecular Patterns  
HCl: Hydrochloric acid  
HESI: Heated Electrospray  
HILIC: Hydrophilic Interaction Liquid Chromatography  
H<sub>3</sub>PO<sub>4</sub>: Phosphoric acid  
HPLC: High Performance Liquid Chromatography  
HR: Hypersensitive Response  
HS TFs: Heat Shock Transcription Factors  
IBMA: International Biocontrol Manufacturers' Association  
INIA: Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria  
ISR: Induced Systemic Resistance  
JA: Jasmonic Acid  
JAZ: Jasmonate-Zim domain  
KEGG: Kyoto Encyclopedia of Genes and Genomes  
KHCO<sub>3</sub>: Potassium hydrogen carbonate  
KH<sub>2</sub>PO<sub>4</sub>: Potassium dihydrogen phosphate  
LAR: Leucoanthocyanidin dioxygenase  
LC50: median lethal concentration

LC-MS: Liquid Chromatography paired with Mass Spectrometry

LOQ: Limit Of Quantification

MAMPs: Microbial-Associated Molecular Patterns

MAPKs: Mitogen-Activated Protein Kinases

MeJA: Methyl Jasmonate

MRM: Multiple Reaction Monitoring

N<sub>2</sub>: Nitrogen

NBS-LRR: Nucleotide Binding Site and Leucine Rich Repeat

NCBI: National Center for Biotechnology Information

NCED: 9-Cis-Epoxy-carotenoid Dioxygenase

NIM1: Non-Inducible immunity 1

NPR1: Nonexpressor of Pathogenesis-Related genes 1

NTC: Non-Treated Control

OA: Organic Acids

OECD: Organization for Economic Co-operation and Development

OPLS-DA: Orthogonal Projections to Latent Structures-Discriminant Analysis

<sup>32</sup>P: Phosphorus-32, phosphorus radioactive isotope

PAMPs: Pathogen-Associated Molecular Patterns

PAL: Phenylalanine Ammonia Lyase

PC: Principal Component

PCA: Principal Component Analysis

PDS: Plant Defense Stimulation

PEC: Predicted Environmental Concentration

PGPR: Plant Growth Promoting Rhizobacteria

PNEC: Predicted No Effect Concentration

PPPs: Plant Protection Products

PR: Pathogenesis-Related

PRRs: Pattern Recognition Receptors

PTI: PAMP-Triggered Immunity

QC: Quality Control

R: Resistance

RBOHF: Respiratory Burst Oxydase Protein

RCR: Risk Characterization Ratio



R.I.N.: RNA Integrity Number

RNA: Ribonucleic Acid

RNA-seq: RNA sequencing

ROS: Reactive Oxygen Species

RPLC: Reverse-Phase Liquid Chromatography

RPM: Revolution Per Minute

RT: Retention Time

RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction

<sup>35</sup>S: Sulfur-35, sulfur radioactive isotope

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

SD: Standard Deviation

S/N: Signal-to-Noise

STS: Stilbene Synthase

TCA: Tricarboxylic Acid

TRR: Total Radioactive Residues

UdG: Universitat de Girona

UAB: Universidad Autónoma de Barcelona

UHPLC: Ultra High Performance Liquid Chromatography

UHPLC-HRMS: Ultra High Performance Liquid Chromatography paired with High Resolution Mass Spectrometry

UPVD: Université de Perpignan Via Domitia

UV: Ultra Violet

WRKYs TFs: Transcription Factors with domain WRKYs

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**Figure 2.** Heatmap of Akivi xenometabolites abundance (the darker is the red, the higher is the intensity).

*“Aki” treated samples from 1 day (dark blue) to 14 days (light blue) after treatment, vs. “Ctr” untreated control samples from 1 day (dark green) to 14 days (light green) after treatment.*

*(A, B, C, D): Blocks of features' dissipation patterns from the less persistent (A) to the most persistent (D).*

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**Figure 4.** Heatmap of Armicarb® xenometabolites abundance (the darker is the red, the higher is the intensity).

*“Arm” treated samples from 1 day (dark yellow) to 14 days (light yellow) after treatment, vs. “Ctr” untreated control samples from 1 day (dark green) to 14 days (light green) after treatment.*

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### Chapter 3. Characterization of the Plant Response to Treatment with Biocontrol Products

**Figure 1.** Venn diagram showing the relationship between up-regulated (A) and down-regulated (B) differentially expressed genes (DEGs) identified in leaves of cv. Garnacha Blanca grapevine. Data correspond to 24h after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC).

**Figure 2.** Upregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). Bar graphs show the number of upregulated DEGs in each GO term cluster. Clusters that showed less than 10 DEGs were included under the term “other”, indicating in parenthesis the number of clusters that represent. Venn diagrams show the total upregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

**Figure 3.** Downregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). Bar graphs show the number of downregulated genes in each GO term cluster. Clusters that showed less than 10 DEGs (BP and CC for Aki, BF and BL and MF for Aki) or 20 DEGs (MF for BF and BL) were included under the term “other”, indicating in parenthesis the number of clusters that represent. Venn diagrams show the total downregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

**Figure 4.** REVIGO graphs of upregulated GO term clusters (regulation of defense response and stress response) in cv. Garnacha Blanca grapevine leaves included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with **Table 3**.

**Figure 5.** REVIGO graphs of downregulated GO term clusters (regulation of defense response and stress response) in cv. Garnacha Blanca grapevine leaves included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with **Table 6**.

**Figure 6.** Expression levels of twenty-seven genes selected for validation of the RNA-Seq data by RT-qPCR. The gene expression was analysed after treatments with Akivi (A) and lyophilized *Bacillus* UdG. RNA-seq (stripped bars) and RT-qPCR (black bars) analysis. Gene functions are indicated in Table 1. RT-qPCR data are shown as the mean of  $\text{Log}_2(\text{FC})$  of three biological replicates, where FC is the fold-change value and was calculated as  $2^{-\Delta\Delta\text{Ct}}$  using non-treated control (NTC) samples as the calibrator and UBQ gene for data normalization. Error bars mean confidence interval of three biological replicates.

**Figure 7.** Transcriptional pattern of selected DEGs after treatments of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi (A) or lyophilized *Bacillus* UdG (B). The fold change was assessed by the  $\Delta\Delta\text{Ct}$  method. The UBQ gene was used as the internal control for data normalization. The  $\Delta\text{Ct}$  of the non-treated control (NTC) samples was defined as the calibrator. Three independent biological replicated were performed. Gene functions are indicated in **Table 1**.

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LOX, LipOxygenase; AOS, Allene Oxide Synthases; AOC, Allene Oxide Cyclase; OPR, OPDA Reductase; ACX, Acetyl-CoA oXidase; EDS1/ NPR1, Enhanced Disease Susceptibility/ Non-expressor of Pathogen Related genes 1; NIM1, Non-Inducible Immunity 1; SAR, Systemic Acquired Resistance; JAZ, JAsonate-Zim domain; PR, Pathogenesis Related proteins; BHLH TFs, Helix Loop Helix TFs, WRKY TFs, Transcription Factors with domain WRKY.

ICS/SID2, IsoChorismate Synthase; PAL, Phenylalanine Ammonia Lyase; PAD4, PhytoAlexin Deficient 4; FS, Flavonoid Synthase; LAR, LeucoAnthocyanin Dioxygenase; GSTs, Glutathione-S-Transferase; ISR, Induced Systemic Resistance; STS, STilbene Synthase; MyB TF, MyB Transcription Factors.

ACS; 1-Aminocyclopropane-1-Carboxylate Synthase; ACO, 1- Aminocyclopropane-1-Carboxylate Oxidase; ERF TF, (AP2)/ERF TF/AP2TF; Ethylene Response Factors Transcription Factors; PR, Pathogenesis Related proteins.

NCED, 9-Cis-Epoxycarotenoid Dioxygenase. MAPKs, Mitogen Activated Protein Kinases; CDPKs, Ca<sup>2+</sup> DePendent Kinases; CaM, CalModulin; RBOHF, Respiratory Burst Oxidase Homologue protein F; PO, PerOxidases; HS TF, Heat Shock Transcription Factors.

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*The concentrations are expressed in ng/g of dried peach peel and in ng/g of peach fresh mass. Means above 10 ng/g of fresh mass are colored in orange.*

*a: ng/g of dried peach peel; b: ng/g of peach fresh mass.*

### Chapter 3. Characterization of the Plant Response to Treatment with Biocontrol Products

**Table 1.** Differentially Expressed Genes (DEGs) and endogenous genes primer sequences used in the present study. The optimized primer concentrations for qPCR analysis are also shown.

**Table 2.** Gene Ontology (GO) terms influenced by treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG compared to the non-treated control (NTC) on cv. Garnacha Blanca grapevine leaves. UP: upregulated, DOWN: downregulated. Criteria:  $\text{Log}_2(\text{FC}) \geq |1.4|$  (FDR significant) and four or more genes per GO term.

**Table 3.** Representative groups (clusters) of upregulated GO terms of biological processes obtained with REVIGO and associated to plant defence responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

**Table 4.** Upregulated genes included in the GO terms that belong to regulation and stress response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

**Table 5.** Representative groups (clusters) of downregulated GO terms of biological processes obtained with REVIGO and associated to plant defence responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

**Table 6.** Downregulated genes included in the GO terms that belong to stress-related response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

**Table 7.** KEGG pathways influenced by Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments compared to the non-treated control (NTC).

**Table 8.** Selected Differentially Expressed Genes (DEGs) on cv. Garnacha Blanca grapevine leaves after treatment with Akivi (Aki) or lyophilized (BL) *Bacillus* UdG.

**Table 9.** Expression levels in fold change of the selected DEGs influenced by treatments of cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi (Aki) and lyophilized *Bacillus* UdG (BL). Data correspond to RT-qPCR. The relative expression level of each gene was calculated by the comparative critical threshold ( $2^{-\Delta\Delta\text{Ct}}$ ) method using the non-treated control samples (NTC) as the calibrator and UQB gene as internal control for data normalization. Data mean the fold change ( $2^{-\Delta\Delta\text{Ct}}$ ) and significant differences are represented by \*.

**Table 10.** Phytohormone, organic acids, and total phenolic contents in leaves of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo treated with Akivi (Aki) and lyophilized *Bacillus* UdG (BL) and water (NTC). The following concentrations correspond to phytohormone (ng/g FW), organic acid (mg/g FW, except for oxoglutaric acid in µg/g FW), and total phenolic (µg gallic acid equivalent/g PF). Results are means (n=3 biological replicates). Significant differences between treatment (Aki or BL) and NTC are represented by asterisks (\*).

## PALVIP PROJECT

This thesis is part of PALVIP project (local Mediterranean crops' alternative protection), that is associating universities and technical structures **to develop new biocontrol products (BPs) for organic farming and to develop new tools in order to develop biopesticides approval processes**. The purposes are 1) to characterize BPs adapted to Mediterranean crops (wine, fruits and vegetables growing), 2) to give advice to farmers about the use of these products, and 3) to enhance the development of biocontrol industry and sustainable agriculture. To reach that goal, BPs developed by the local small and medium size businesses associated with the project were studied in the experiments conducted i) on grapevine against downy mildew, powdery mildew and gray mold; ii) on peach tree against brown rot; iii) on apricot tree against flowers' drying up caused by *Monilia laxa*; iv) on cherry tree against flies; and v) on lettuce against weeds. Field efficacy study of the developed BPs were evaluated by the Chambre d'Ariculture 66 (CA66, France) and the INCAVI (Spain) respectively coordinated by Julien Thierry and Xoan Lois Elorduy Vidal. Samples from the field experiments were used to study the environmental impact of BPs, that was evaluated by the Université de Perpignan Via Domitia (UPVD), the Universitat de Girona (UdG), and Futureco Bioscience (FBIO) respectively coordinated by Cédric Bertrand and Marie-Virginie Salvia, Emilio Montesinos, and Carolina Fernandez. Samples from field experiments and from greenhouse controlled conditions experiments were conducted to study the effects of BPs on treated plants, that was evaluated by the UdG and the Universitat Autònoma de Barcelona (UAB) respectively coordinated by Emilio Montesinos and Mercè Llugany. Eventually, the PALVIP project was coordinated by Sophie Gabolde from the CA66.

PALVIP project has been 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain France Andorra programme ([POCTEFA 2014-2020](#)). POCTEFA aims to reinforce the economic and social integration of the French-Spanish-Andorran borders. Its support is focused on developing economic, social and environmental cross-border activities through joint strategies favoring sustainable territorial development.





## INTRODUCTION

France and Spain are the top two pesticides consumers in European Union (EU), together representing 40% of EU pesticides sales with 150 000 tons sold in 2018 (European Commission, 2020). Principal categories of pesticides sold are ‘fungicides and bactericides’ (45%), ‘herbicides, haulm destructors and moss killers’ (32%) and ‘insecticides and acaricides’ (11%). Pesticides are everywhere in the environment: in the air, soils, water, food; and they can highly impact the environment. Indeed, the ecosystems contaminated can be deeply disturbed and may never recover their initial state or in a very long time (Inra – Cemagref, 2011). For example, it can cause the reduction of prey’s populations and behavior trouble on predator’s population or favor a specie at the expense of another specie.

That is why a big effort is made to look for solutions to complement chemical pesticides use like: (i) rational chemical control by reducing the frequency and the dose of the treatment or choosing products and treatment period with lower environmental impact; (ii) crop selection by developing new varieties resistant or tolerant to targeted pests; (iii) physical control by attacking directly the pests using thermic, electromagnetic, mechanic or pneumatic struggle; (iv) cultural control to prevent pests development by modifying crops’ rotation, undercropping or intercropping, modifying dates of harvest, fertilization, irrigation and drainage; (v) genetic engineering by creating genetically-modified organism varieties fully resistant to targeted pests; (vi) biocontrol products using living organisms and natural extracts to prevent or reduce pests’ damages (Aubertot *et al.*, 2005).

Those options are promoted by the European Union through the directive 2009/128/CE (European Parliament and Council Of The European Union, 2009) whose objective is to establish a “*framework to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of integrated pest management and of alternative approaches or techniques such as non-chemical alternatives to pesticides*”. The directive gives four lines to focus on: (i) training, sales of pesticides, information, and awareness-raising, (ii) pesticide application equipment, (iii) specific practices and uses and (iv) indicators, reporting and information exchange. With this basis, every European country have finalized national action plans to reach the EU expectations. In France, the Ecophyto II+ plan was set up in 2018 (following the Ecophyto I initiated in 2008 and the Ecophyto II initiated in 2015). Its major goal is to reduce the use of plant protection products (50% by 2025) through pesticides uses’ restrictions in public spaces and initiatives encouragement by giving certificates of ‘pesticides use’s saving’. However, the objective is far from being reached. In Spain, the national action plan foresees quite similar measures objectives for pesticides use’ reduction. Its major goal is information to the public

(farmers have to advise the neighbors before treatment) and specific measures to protect farming workers within treated zones. In addition, both French and Spanish governments promote the use of biocontrol products: the global market recorded a growth rate of around +20% in a year (2019) (IBMA Global, 2021). Nevertheless, references regarding technical efficacy and ecotoxicological data are lacking.

To fill that gap, **the present PhD work focus on the characterization of BPs effect on treated plants, while studying their residues fate and the plant response to the treatment** in order to better understand the mechanisms involved in BPs efficacy and how they interact with the plant and the environment. For that, the thesis is divided into two parts: (i) the study of the BPs' residues dissipation that has been performed in the University of Perpignan Via Domitia (UPVD) using metabolomics, (ii) the study of the effect of the treatment in the treated plant using transcriptomics done at the Universitat de Girona (UdG) and using metabolomics at the Universitat Autònoma de Barcelona (UAB).

This work will be firstly contextualized, giving the frame of the project. Then, the field experiments will be detailed. Afterwards, the research work done about residues' fate as well as the investigations carried out on plant response to the treatments will be described. Lastly, the results will be discussed in relation to the current knowledge in the field, and the conclusions of this thesis outlined.

## **Chapter 1.**

**Context of the Study:  
Biocontrol Products Applied to  
Mediterranean Crops,  
Potential Environmental Impacts and  
Modes of Action.**



# 1. BIOCONTROL PRODUCTS

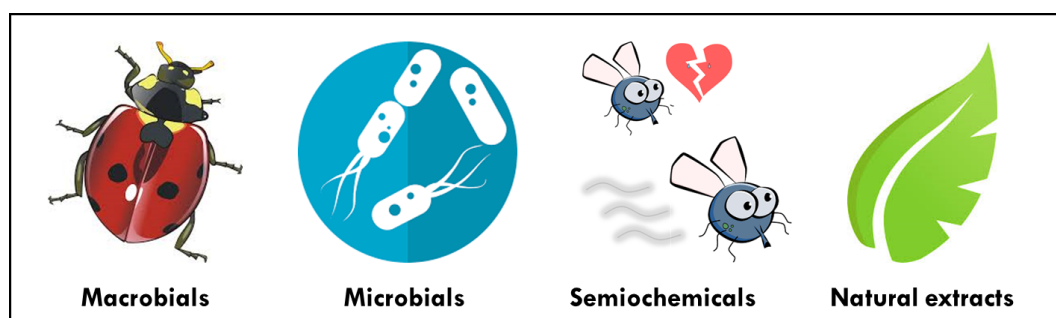
## 1.1. Definition

Biocontrol Products (BPs) are part of the Plant Protection Products (PPPs) that are defined by the Food and Agriculture Organization of the United Nations (FAO, 2006) as:

*“Plant protection product means a pesticide product intended for preventing, destroying or controlling any pest causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products.”*

Four types of BPs can be defined (**Figure 1**):

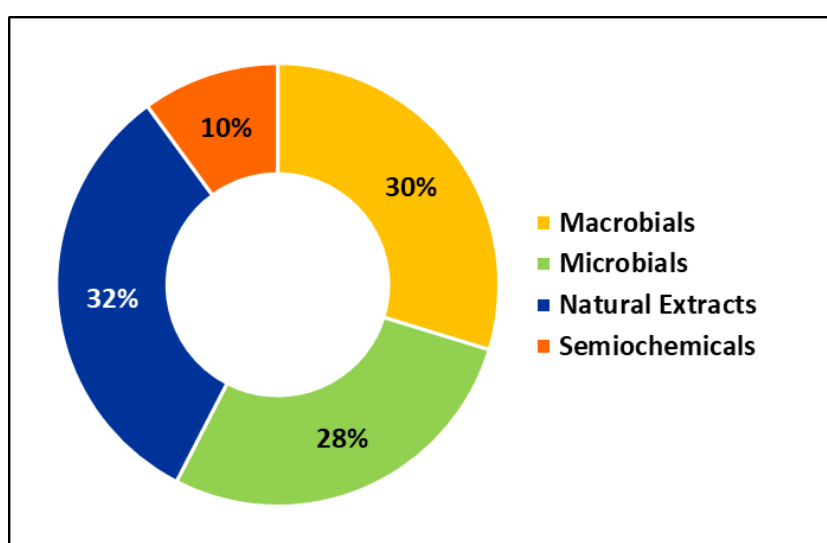
- Beneficial **macrobiols** used to protect crops from pests. It includes (i) insects like the ladybug (*Adalia bipunctata*) that eats aphids, (ii) acarids like *Typhlodromus pyri* that is a natural predator of phytophagous acarids and thrips or (iii) nematodes like *Steinernema carpocapsae*, natural parasite of several insects' larvae (crane fly, maybug, weevil...) (Herth, 2010).
- Beneficial **microbiols** used to protect crops from pests and diseases or to stimulate plants' natural defenses. It includes (i) fungi like *Coniothyrium minitans* that is a parasite of cereals pathogenic fungi of the genus *Sclerotinia* and prevents it from producing mycelium, (ii) bacteria like *Bacillus thuringiensis* whose toxins kill mosquitoes after being ingested and (iii) virus like *Cydia pomonella* granulovirus that kills codling moth larvae after being ingested (Herth, 2010).
- **Semiochemicals** used to follow insects' movements, to trap them or to regulate insect populations by sexual confusion method. It includes (i) pheromones that are molecules used for intraspecies communication, sexual pheromones are mainly use for BP; and (ii) allelochemicals that are molecules implied in interspecies interaction, for example kairomones **that** are a type of allelochemical with a negative impact on the emitter species (i.e. attracting that species parasites) (Herth, 2010).
- **Natural extracts** derived from plant, animal, or mineral sources. For example: pyrethrins extracted from *Chrysanthemum cinerariaefolium* are used as insecticide, it attacks the nervous system of all insects (Herth, 2010).



**Figure 1.** The four types of biocontrol products

## 1.2. Key Figures on Biocontrol Products' Market

European Union (EU) governments are promoting the use of BPs as one of the options for a sustainable complement to chemical PPPs through the directive 2009/128/EC (European Parliament and Council of the European Union, 2009a), that explains why BPs have a promising future with good economic indicators. In fact, European biocontrol market presents an accelerating growth from 2016 to 2019, with a global growth rate of 20% in one year (2019). In 2019, it recorded a market size of 1.2bn € representing around 10% of the global PPPs market, so there is plenty of development possibilities (IBMA Global, 2021). As shown in **Figure 2**, European biocontrol market is dominated by natural extracts (32%), macrobials (30%) and microbials (28%). Focusing on French BPs market, the major segments are bioinsecticides (37%) and slug killers (26%) (IBMA France, 2021). It is worth to mention that global PPPs market share is dominated by fungicides and herbicides. The difference between PPPs and BPs major uses segments can be explained by the fact that the main BPs sales are bioinsecticides based on *Bacillus thuringiensis* (Bt) microbial.



**Figure 2.** Biocontrol products types repartition in 2019, European market sales (adapted from (IBMA Global, 2021))

### 1.3. Regulation of Biocontrol Products

#### 1.3.1. Macrobiales

They are not considered as PPPs, so they don't need market authorization. However, to preserve local ecosystems, the introduction of non-natives species is regulated by each state of the EU. For example in France, the 2012-140 decree (République française, 2012) foresees two types of authorization demand for two different uses: (i) "the territory entrance" demand which is limited within a confined space for research for example and (ii) "the environmental introduction" demand which is not limited and leads to the dispersion of the organism. The demand file must contain the description of the product, the macrobiales taxonomy, its biology and ecology, its origins and repartition, its use and the targeted organisms. In addition, the demand contains information about the confinement in case of "territory entrance" demand and environmental risks evaluation in case of "environmental introduction" demand. The evaluation of the demands relies on phytosanitary and environmental risks analysis as well as the efficacy and the profit brought by the product. This evaluation is made by the EFSA at European Union level (European Food Safety Authority), once the macrobial is authorized by the EFSA, it can be submitted by country. The evaluation of macrobiales products is made by the ANSES in France (Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail), and by the INIA in Spain (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria). Main of macrobiales are registered in the organic farming authorized products' list excepted for genetically-modified organisms.

#### 1.3.2. Microbiales, Semiochemicals and Natural Extracts

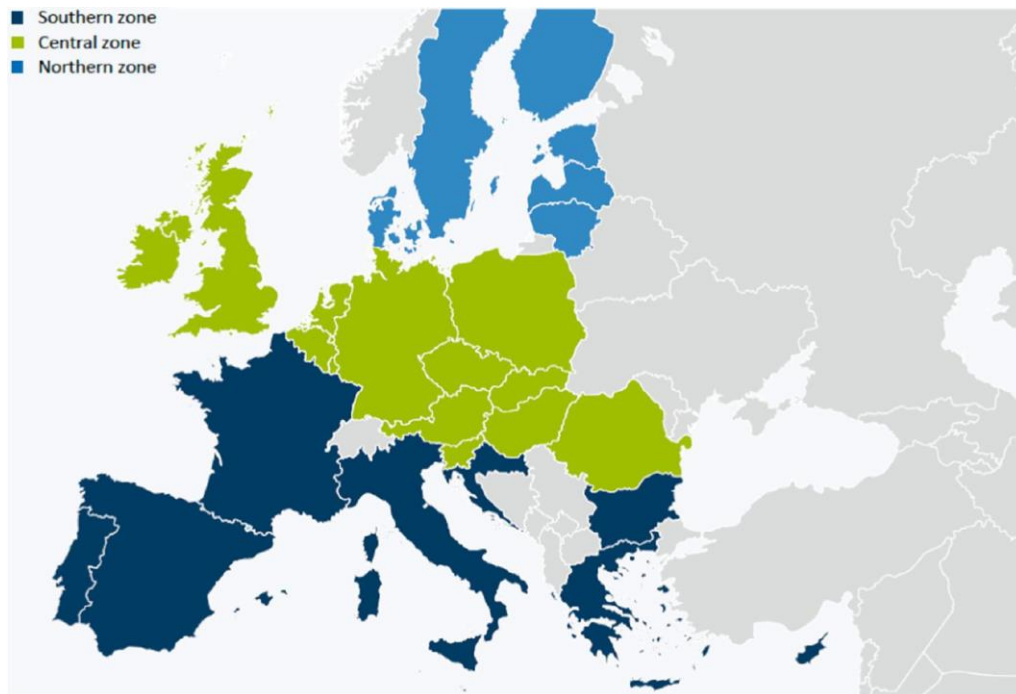
Concerning the microbiales, semiochemicals and natural extracts, the new regulation for PPPs substances approval by the regulation (EC) 1107/2009 (European Parliament and Council of the European Union, 2009b) and modified by the Commission regulation (EU) 2017/1432 (European Commission, 2017a) distinguishes three types of substances: (i) basic substances, (ii) low risk active substances and (iii) active substances.

A basic substance is a substance mainly non-used as a PPP and in several cases used as a food ingredient, but with plant protection activity, and is not able to impact the endocrine system, neuronal system or have immunotoxicity effects. For instance, unprocessed natural extracts like Nettle (*Urtica* spp.) (European Commission, 2017c) or Horsetail (*Equisetum arvense*) (European Commission, 2017b) extracts are approved by EU as basic substances. In the basic substances case, the approval demand is reduced. There is no time limit of the approval, there is no need for market authorization and the demand is valid in all European countries. Association between basic substances is considered as a basic substance, for



example within the PALVIP project an association between Nettle and Horsetail was tested as fungicide in grapevine.

A low risk substance is an active substance presenting low risks impact on human health and on the environment. An active substance can be approved as a low risk substance if it meets the criteria described by the Commission regulation (EU) 2017/1432 (European Commission, 2017a). It is worth to mention that the main part of microbials BPs can be considered as low risk substances. In the cases of low risk active substances and active substances, the approval demand is followed in the classic way through the request of a market authorization. The market authorization demand consists in studying the safety of the active substance or the product for users as well as consumers, living organisms and the environment; its agronomic profits are studied too. This evaluation is made for active substances by the EFSA at EU level; once the active substance is authorized and registered by EU, companies can submit products using the active substance to national market authorization. This evaluation is made by the ANSES in France and by the INIA in Spain. Once a product obtains a national market authorization, it can be submitted for the same purposes with a lighter procedure for market authorization in all the European countries of the same zone (**Figure 3**) presenting climatic, agronomic, and environmental similarities (ITAB and ONEMA, 2013).



**Figure 3.** Map of the pesticides authorization zones divided within the European Union: Southern zone in dark blue, Central zone in green, and Northern zone in blue (Kudsk and Mathiassen, 2020).

Main of microbials are registered in the organic farming authorized products' list excepted for genetically-modified organisms. Regarding the other active substances used in organic farming the authorized list is available online (European Commission, 2021c).

It is worth to mention that some mixtures and microorganisms very similar to natural extracts BPs and microbials BPs are used as biostimulants, according to the regulation (EU) 2019/1009 (European Parliament and Council of the European Union, 2019). Biostimulants are defined as products that aim “*solely at improving the plants' nutrient use efficiency, tolerance to abiotic stress, quality traits or increasing the availability of confined nutrients in the soil or rhizosphere, they are by nature more similar to fertilizing products than to most categories of plant protection products. They act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates*”. That is to say, if the product widely reinforces the treated plant it is considered as a biostimulant product, like some plant growth promoting microorganisms (Vasseur-Coronado *et al.*, 2021). However, if one or more claims of the product's functions is directed against a pest or a pathogen, the product is beyond the scope of the regulation (EC) 1107/2009 (European Parliament and Council of the European Union, 2009b) and it is considered as a PPP.

### 1.3.3. Selected BPs Developed by the Partners of PALVIP Project

Several PALVIP project partners are developing BPs tested within the project, like the botanical extracts Akivi (AkiNaO) and Bestcure® (Futureco Bioscience) or the beneficial microbials *Bacillus* UdG and *Lactobacillus* UdG (UdG). Among them this PhD thesis focuses on two products:

**Akivi** (AkiNaO, France): prototype of a formulated botanical extract from *Dittrichia viscosa* with a high content of polyphenols and terpenes (Tamm *et al.*, 2017). It presents a contact fungicide activity mainly due to its terpenes content. Akivi is already tested in field efficacy studies with good results against Downy mildew, Brown rot, Powdery mildew, and Scab.

***Bacillus* UdG** (UdG, Spain): composed of the bacteria *Bacillus amyloliquefaciens*, the strain recently renamed as *Bacillus velezensis*. It is used for biological control of bacterial and fungal diseases in a wide range of host crops (Montesinos *et al.*, 2018). The active compounds produced by *Bacillus* UdG are cyclolipopeptides and other antimicrobial secondary metabolites (including surfactin, iturin, bacillomycin, fengycin and several antibiotics). Also, due to the presence and development of the bacteria, competition for nutrients and space plays an important role in the pathogen/disease biocontrol process.



## 2. LOCAL MEDITERRANEAN AGRICULTURE

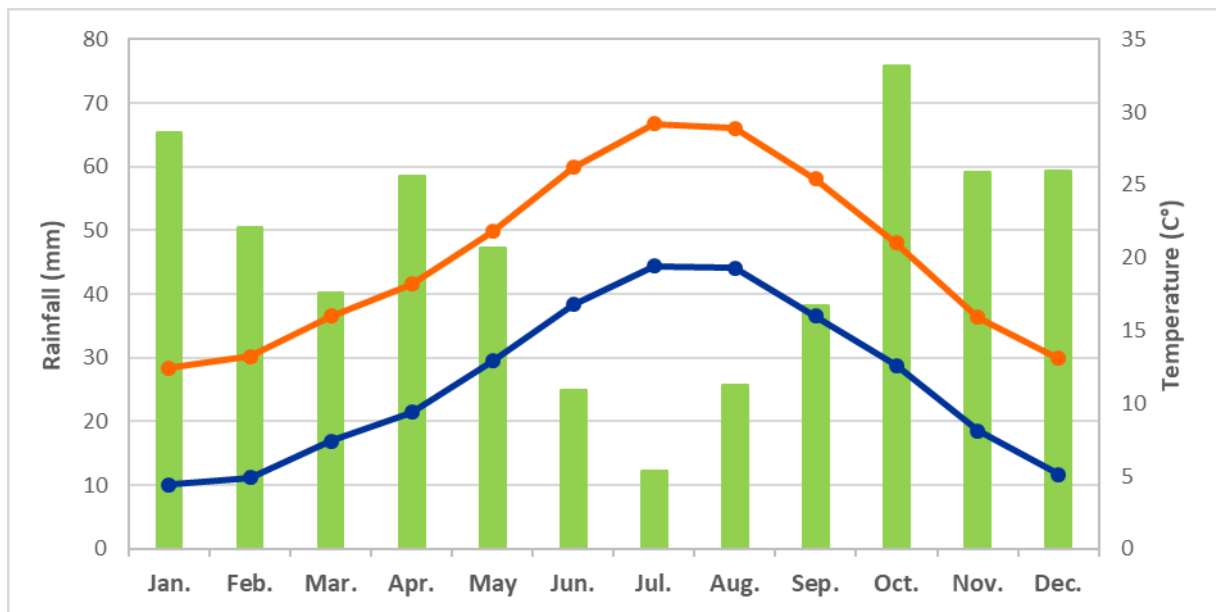
### 2.1. Climate and Main Crops

PALVIP project is located in the Mediterranean border between France and Spain; in the Roussillon, near Perpignan; and the Catalunya, near Girona and Barcelona (**Figure 4**).



**Figure 4.** Map of PALVIP project localization

Mediterranean climate is a temperate zone characterized by a very mild winter, allowing the persistence of several fruit trees, and a very hot and dry summer. In fact, the annual mean temperature (1981-2010) is 16°C with a minimum of 5°C (mean for January) (**Figure 5**). Moreover, annual mean rainfall is very low: 558mm (**Figure 5**). For comparison, the annual mean rainfall in Biarritz, located in the west coast of the Pyrenees and under Oceanic climate influence, is 1451mm (République française, 2021a). In addition, sudden and heavy rainfall uses to occur in summer in the Mediterranean region. Consequently, the water doesn't get into the soils, it runs on the surface and doesn't benefit the plants.



**Figure 5.** Annual mean rainfall (bar chart), minimum (blue line) and maximum (orange line) temperatures, in Perpignan (France), (République française, 2021b)

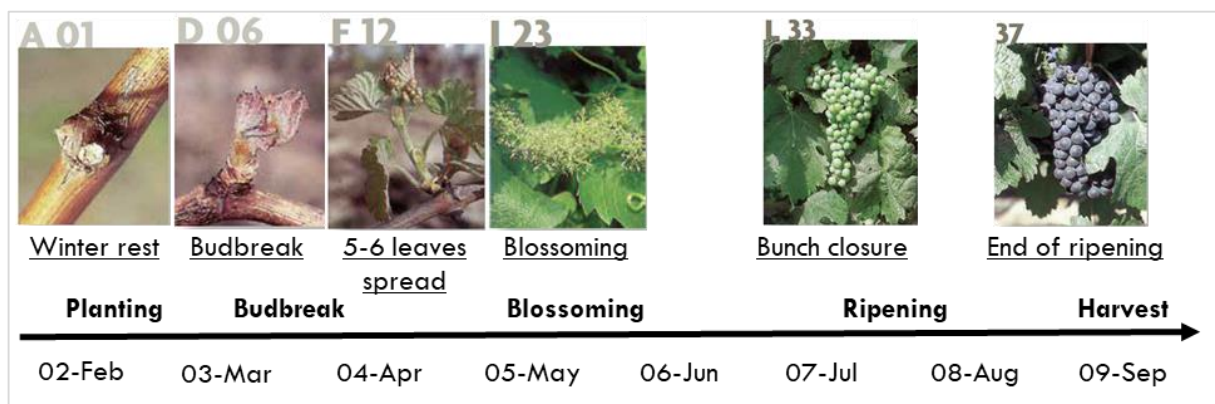
This climate, with relatively high temperatures and little rain, is propitious for vine growing which is the main crop in the Mediterranean region as well as arboriculture, and market gardening.

## 2.2. Selected Crops Used for PALVIP Project Field Experiments

Crops and pests selected in PALVIP project were chosen regarding the products developed by the partners and the local needs. The experiments were done (i) on grapevines treated against downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*) and gray mold (*Botrytis cinerea*); (ii) on peach trees treated against brown rot (*Monilinia fructigena*); (iii) on apricot trees treated against flowers' drying up caused by *Monilinia laxa*; (iv) on cherry trees treated against flies (*Rhagoletis cerasi*); and (v) on lettuce for soil maintenance and treated against weed development. This thesis work was integrated into PALVIP project and focused on some parts described above: grapevines treated against powdery mildew (*Erysiphe necator*) and peach trees treated against brown rot (*Monilinia fructigena*). In this section, the general characteristics of the selected crops are described. Their growing cycles are presented on **Figures 6** and **7** but it is all completely variable depending on each year's weather and especially the rainfalls.

### 2.2.1. Grapevine

Grapevine (*Vitis vinifera*) is a perennial plant that rests during winter before bud breaking that occurs in March. Leaves spread along the new branch to achieve photosynthesis and bunches of flowers that are appearing in May, it is called blossoming. The bunches of flowers turn into bunches of grapes at the end of spring and ripening is occurring during summer. Grapes are ready for harvest in September. This growing cycle is illustrated on **Figure 6**. However, as mentioned previously, it depends on the cultivar and weather conditions. Grapevine growing cycle can be disrupted by several diseases impacting the harvest; mainly downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), and gray mold (*Botrytis cinerea*) (Chambre d'Agriculture Rhône-Méditerranée *et al.*, 2014).



**Figure 6.** Grapevine phenological stages

(adapted from (Chambre d'Agriculture Rhône-Méditerranée *et al.*, 2014))

### 2.2.2. Peach Tree

Peach trees (*Prunus persica*) also rest during winter before budbreaking that occurs in February prior blossoming which takes place at the end of February. During spring, leaves spread, branches grow, and fruits are formed. The ripening and harvest are occurring during summer but the exact time of harvest is depending on varieties. Peach growing cycle (**Figure 7**) can be impacted by several pests and diseases: mainly brown rot (*Monilinia fructigena*), leaf curl (*Taphrina deformans*), oriental fruit moth (*Grapholita molesta*), aphids (mainly *Myzus persicae*), and sharka (*Plum pox virus*) (Chambre d'Agriculture Rhône-Alpes, 2011).



**Figure 7.** Peach phenological stages

## 2.3. Selected Diseases and Current Treatments in Conventional and Organic Agriculture

In this section, the general characteristics of the selected diseases are described but it is all completely variable depending on the weather and the rain.

### 2.3.1. Powdery Mildew on Grapevine

Grapevine powdery mildew is a fungal disease caused by the fungi *Erysiphe necator* (**Figure 8**). It attacks in spring during blossoming but may attack earlier, during new leaves spreading for instance, it depends on the susceptibility of both the cultivar and the agricultural plot. Usually, the product used for the treatment is the sulfur (organic) or fungicides containing spiroxamine (conventional). The first treatment is mainly made at the stage '6 leaves spread' but can be made earlier or later according to the observations. From this stage to blossoming, some of the treatments can be removed from the treatment campaign depending on the weather and the disease pressure. However, between blossoming and bunch closure, the vine is highly sensitive, and the fungi must be contained in order to have a good harvest in terms of quantity and quality (Chambre d'Agriculture Rhône-Méditerranée *et al.*, 2014).





**Figure 8.** Comparison between a healthy grapevine leaf (left) and a leaf infected by powdery mildew (right) (adapted from (Agurto *et al.*, 2017))

### 2.3.2. Brown Rot on Peach Tree

Peach tree brown rot is a fungal disease caused by the fungus *Monilinia fructigena* in Europe (**Figure 9**). It attacks in summer when the peach is mature; it is a preservation disease. Two conventional treatment strategies are mainly used: (i) a fungicide containing anilinopyrimidins and phenylpyrrols, three treatments are made before harvest; or (ii) three treatments with three different fungicides. The first treatment contains pyraclostrobin and boscalid, the second treatment contains fenbuconazole, and the third treatment contains fluopyram and tebuconazole. There is no solution in organic farming (Chambre d'Agriculture Rhône-Alpes, 2011).



**Figure 9.** Comparison between healthy peaches (left) and a peach infected by brown rot (right) (adapted from (Hu *et al.*, 2011))

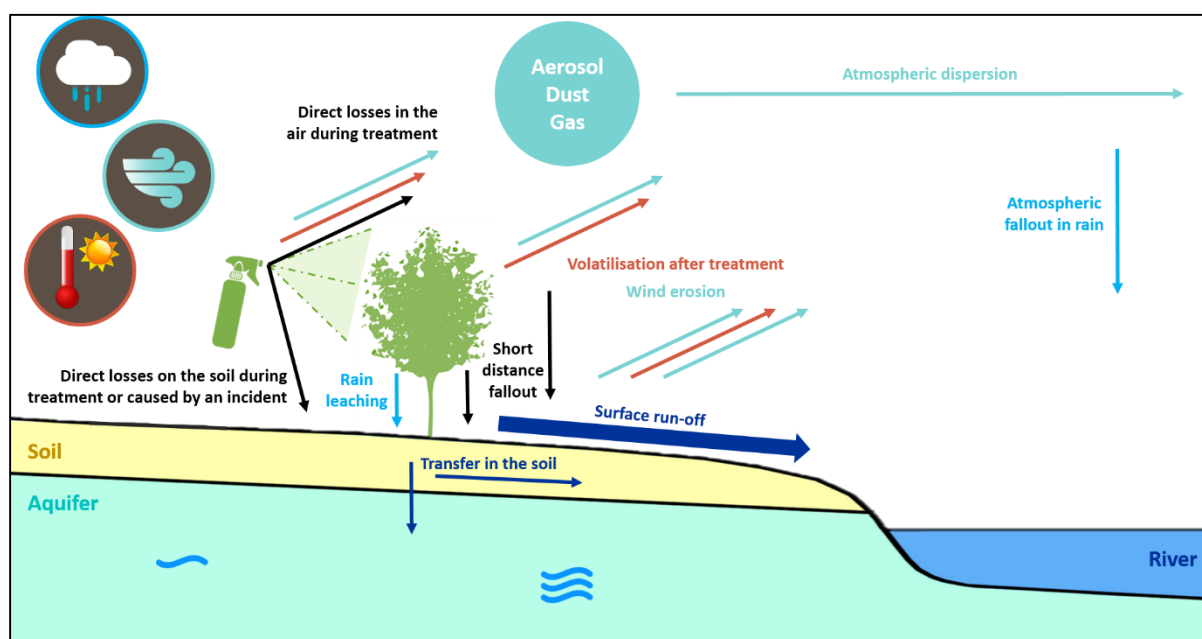


### 3. PLANT PROTECTION PRODUCTS WITHIN THE ENVIRONMENT

#### 3.1. Environmental Fate and Impact of Plant Protection Products

##### 3.1.1. Contamination and Transfer within the Biosphere

All the environmental compartments are contaminated by PPPs: air, water, and soil. Several PPPs application techniques are used depending on the product formulation and the pest target but most of the products are applied as sprays, that is why spraying application was chosen here to illustrate environmental contamination by PPPs. The contamination takes place during the treatment by direct losses on soil or in the air. For this reason, sprayers are constantly evolving in order to reduce losses. On the other hand, the contamination can occur after the treatment by transfer (**Figure 10**) between the different compartments through rain (leaching, atmospheric fallout or run-off), wind (erosion), and heat effects (volatilization) (Alix *et al.*, 2005). For all these reasons, it is recommended (i) to spray in light breeze conditions (3 to 7 km/h); (ii) to avoid the heat of midday during summer treatments; and (iii) to avoid spraying before rain.



**Figure 10.** Transfer of PPPs in the environment after spraying  
(adapted from (Alix *et al.*, 2005))

### 3.1.2. Impacts of Plant Protection Products on the Environment

Once released in the environment, PPPs have a highly complex behavior depending on various parameters like chemical-physical properties of both the product and the contaminated substrate. Evidence of PPPs impact on all the environmental compartments have been highlighted. For instance, it was mentioned that in the air, insecticides and fungicides mixtures affect CO<sub>2</sub> assimilation of apple trees; causing 6 to 9% photosynthesis inhibition and an increase of plant dark respiration up to 72% (Untiedt and Blanke, 2004). In soils, some fungicides can have an impact on earthworm behavior; indeed, they flee from treated plots to lower soil layers (Christensen and Mather, 2004). Finally, in water, herbicides can disturb aquatic communities by reducing sensitive populations that lead to proliferation of other species. An example are green algae that are replaced by diatom species after simazine and terbutryn treatments (Gurney and Robinson, 1989). Also, PPPs impact all living beings from microbials to mammals including beneficial insects. The impact can be direct like insecticides on pollinating insects (Kevan, 1999). But it can also be indirect. It is the case of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) that are suffering from secondary poisoning due to consumption of poisoned prey (Berny *et al.*, 1997). Indeed, treatments based on bromadiolone rodenticide are done against field vole (*Arvicola terrestris*) and coypu (*Myocastor coypus*) that are preys of foxes and buzzards following the treatment with bromadiolone rodenticide (Berny *et al.*, 1997). That is why it is essential to study environmental risks for every PPP.

Environmental risk is regulated by the Commission directive 93/67/EEC (European Commission, 1993), the Council regulation (EEC) 793/93 (Council of the European Union, 1993), and the Commission regulation (EC) 1488/94 (European Commission, 1994). Environmental risk relies on two parameters: the substance toxicity, and the exposure of the environment to the substance. For instance, a substance presenting high toxicity but very low exposure has a low environmental risk compared to a substance presenting moderate toxicity but high exposure that represents a high environmental risk. To calculate these parameters, indicators are defined for each substance:

- First, the PNEC (Predicted No Effect Concentration), is the highest concentration of the substance with no environmental risk. It mainly relies on LC50 values (median lethal concentration, the concentration that causes the death of 50% of the model organisms during the exposure period). PNEC represents the toxicity of the substance.
- Second, the PEC (Predicted Environmental Concentration), permits to predict the substance concentration within the environment. It mainly depends on its use, its behavior in the environment (in order to evaluate its degradation), its chemical-

physical properties (to evaluate its environmental distribution *via* its solubility, volatility...) as well as the treatment conditions. PEC represents the substance exposure to the different compartments of the environment.

For each compartment (air, soil, and water), PEC and PNEC values are evaluated and a Risk Characterization Ratio (RCR) is calculated as follows:  $RCR = PEC / PNEC$  (Pellizzato, 2014). The RCR must be equal or lower than 1 or the authorization is compromised because a RCR value  $>1$  means that the environmental risk is not under control and risk reduction measures have to be taken. That is why PPPs residues are deeply studied for marketing authorization procedures.

### 3.2. Detection of Plant Protection Products' Residues

PPP are composed of (i) an Active Substance (AS) including one or several molecules that concentrate the activity of the product against the pest; (ii) co-formulants (like solvents or carriers) ensuring the homogenization and stability of the PPP; and (iii) adjuvants (like adhesive for seed treatment products) in order to optimize PPP efficacy. For marketing authorization processes, adjuvants need an approval as well as ASs with a complete residue investigation (European Commission, 2013). Concerning co-formulants, they are not fully investigated, except for some toxicity tests, but European Union regulation is currently evolving to better frame this class of components. In fact, some co-formulants are forbidden according to the list in the Commission regulation (EU) 2021/383 (European Commission, 2021b).

This part will focus on PPP residues in terms of approval processes, that is to say the AS remaining after the treatment. It also includes AS's by-products that are coming from the degradation processes that occur after the treatment with PPP. Those degradation processes mainly include: (i) biotic degradation primarily caused by microbials and (ii) abiotic degradation including photolysis, hydrolysis, oxidation, and reduction. The dominant degradation pathway depends on various parameters like the chemical-physical properties of the molecule, the weather (light, humidity), or the type of application used for the treatment (Alix *et al.*, 2005). For example, aerial plant parts treatments are more sensible to photodegradation.

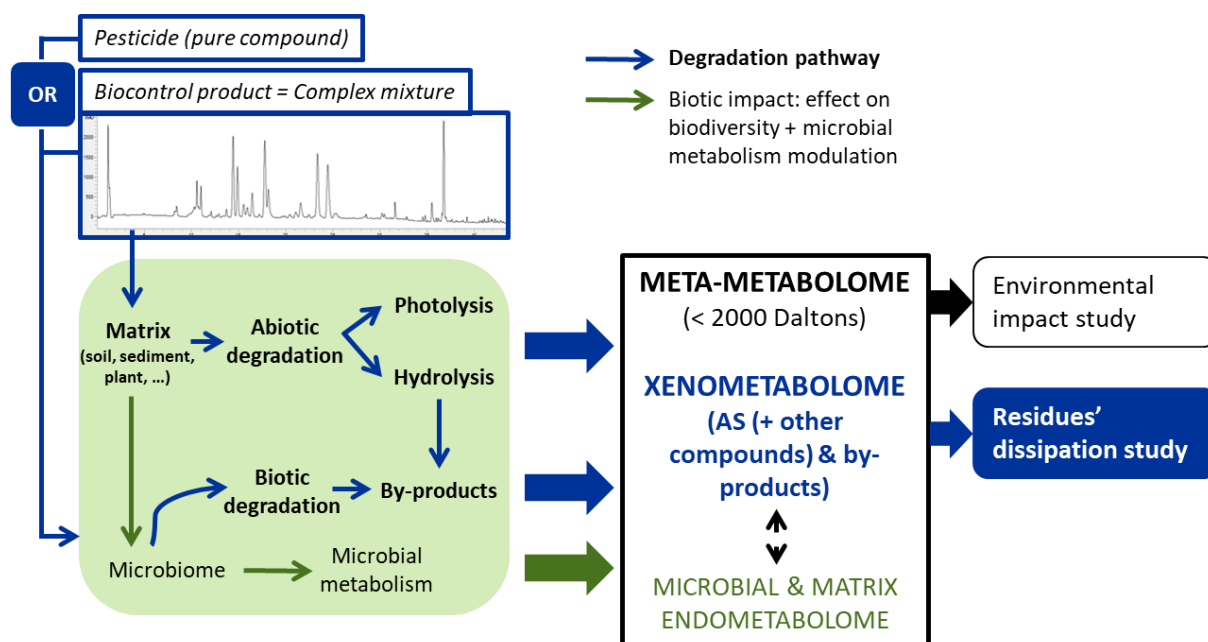
For approval processes, ASs must be well characterized in terms of structure, chemical-physical properties, and mode of action (European Commission, 2013). Then AS behavior on treated plants is monitored through isotopic labelling (OECD, 2007b). First, knowing the AS structure, all the significant parts are identified and radiolabeled using the appropriate radioisotopes ( $^{14}\text{C}$ ,  $^{32}\text{P}$ , or  $^{35}\text{S}$ ) depending on the moieties' chemical nature. Then, the studied

crop is grown in laboratory-controlled conditions and treated with radiolabeled AS. The crop is harvested and the maximum of radioactive labelled molecules are extracted from the samples, 90% of the Total Radioactive Residues (TRR) must be identified, it represents the AS and its major by-products (OECD, 2007b). After the identification of the AS and its major by-products, a dissipation study of these molecules is conducted within the crop and in soils. For each molecule, two specific values are targeted: (i): the half-life or DT50, that corresponds to the time needed to dissipate 50% of the substance from the system, and (ii): the 90% dissipation time, DT90, time to dissipate 90% of the substance (European Commission, 2000). Their values, expressed in days, may lead to further investigations. For example, if the DT90 in soils is higher than 100 days, complementary study on next rotation culture may be necessary (European Commission, 2000).

### 3.3. Environmental Metabolic Footprinting (EMF)

As mentioned above, the half-life or DT50 was often used in order to study the fate of PPPs in environmental matrices. However, this value alone gives restricted information as it doesn't give any information regarding the formation of by-products and the effect on biodiversity. Moreover, focusing on BPs, they usually are complex mixtures and they yield chromatograms that are extremely difficult to interpret, which precludes the DT50 from being considered as a viable tool. Consequently, an innovative approach based on metabolomics (LC-MS), the Environmental Metabolic Footprinting (EMF), was recently developed in the laboratory (Patil et al., 2016; Salvia et al., 2018). Metabolomics is a relatively recent development in the "omics" approach, which enables the detection of thousands of metabolites in a single sample without an "a priori" knowledge of metabolite profiles (Heyman and Meyer, 2012). In the EMF metabolomics approach, the matrix is considered as a living system able to transform pollutants and produce its own metabolome that could be disrupted in the presence of contaminants. Extractible meta-metabolome that corresponds to xenometabolome (presence of the ASs + by products coming from both abiotic and biotic degradation) and the studied matrix endometabolome (matrix and its associated microbiome) is investigated (**Figure 11**). Changes in the matrix meta-metabolome after PPPs treatments are investigated at various time intervals and compared with the control. This approach gives rise to a new integrative proxy, the resilience time, i.e. when the matrix returns to its initial state ("control"). Indeed, the resilience time corresponds to the time needed for the compound dissipation and its effects on the matrix. It has the potential to evaluate all the post-application effects of the BPs. Moreover, the EMF can be optimized in order to determine the "dissipation interval" that corresponds to the time needed to have complete residues dissipation, i.e. we don't have any more residues differences between the treated sample and

the control. In this case, only the xenometabolome will be investigated. The optimization of the EMF approach in order to target residues will be the subject of a part of this thesis work.



**Figure 11.** Schematic representation of Environmental Metabolic Footprinting approach

## 4. PLANT RESPONSE TO BIOCONTROL PRODUCTS TREATMENT

### 4.1. Biocontrol Products Mode of Action

In order to protect the plant from pests and diseases, biocontrol products (BPs) present various modes of actions depending on BP type described in section [Chapter 1. - 1.1.]. The main mode of actions will be described in this section.

#### 4.1.1. Macrobiotics

Beneficial macrobiotics present direct action against pests through predation, parasitism, or competition. Predation is a classic “+/- *interaction that benefits one side, the predator, and harms the other side, the prey*” (Jeschke *et al.*, 2021). Usually, the prey is killed and consumed by the predator, like the ladybug that eats aphids. Parasitism is also a “+/- *interaction*” that benefits one side, the parasite, and harms the other side, the host; but the parasite does not usually kill its host. However, for BPs use, parasites that kill the host are targeted like the nematode *Steinernema carpocapsae*, a natural parasite of several insects’ larvae (crane fly, maybug, weevil...) (Herth, 2010). The difference between predation and parasitism lies on the high quantity of prey consumed by predators along their lives against the few hosts infested by a parasite along its life. Beneficial macrobiotics can also compete with pests and pathogens for space and nutrient resources.

#### 4.1.2. Microbiotics

Beneficial microbiotics used as BPs present various mode of actions (Köhl *et al.*, 2019), each microbial BP covers one or several mechanisms (Montesinos and Bonaterra, 2019), the main ones will be presented in this section. They are grouped on three main mode of actions: (i) direct action against the pathogen, (ii) indirect interaction to the pathogen, and (iii) plant resistance induction. Firstly, microbiotics can directly act against the pathogen by hyperparasitism. That is parasitism as described in section [Chapter 1. - 4.1.1.] but in cases where the host is also a parasite like a plant pathogen. It is often observed between fungi like *Coniothyrium minitans* that is a parasite of cereals pathogenic fungi of the genus *Sclerotinia* and prevents it from producing mycelium (Herth, 2010). On another hand, microbiotics can also directly act against the pathogen by antibiosis through antimicrobial metabolites production. In fact, a wide range of antibiotics are known to be produced by microbiotics: 8700 different antibiotics are produced by actinomycetes, mainly *Streptomyces* sp.; 2900 are produced by bacteria, mainly *Bacillus* spp.; and 4900 are produced by fungi, mainly *Penicillium* spp. and *Aspergillus* spp. (Bérdy, 2005). For instance, *Bacillus* lipopeptides are widely investigated as BP (Mora *et al.*, 2015; Vilà *et al.*, 2016) like *Bacillus subtilis* that produce iturins and fengycins

involved in the bacteria antagonism towards *Podosphaera fusca* infecting melon leaves (Ongena and Jacques, 2008). Secondly, microbials can indirectly act against the pathogen by nutrient and space competition that is considered as the major mechanism of microbial BPs (Bonaterra *et al.*, 2012). In fact, some non-biotrophic pathogens are dependent on exogenous nutrients during their life cycle. Thus, microbials that present high competition potential may be good candidates for biological control of pathogens. In order to prevent pathogen infection, BP microbials' candidates must be able to occupy potential infection niches like wound or senescent flowers; they also must survive and consume rapidly sugars, pollens and others plant exudates that are essential nutrient sources for pathogens development. Eventually, the outcompeted pathogen population will decline without being able to infect the host. This mode of action needs more detailed knowledge to identify pathogen life cycle stages where its development will be impacted by the lack of space and nutrients. For instance, a fast-colonizing yeast *Aureobasidium pullulans* can protect wounded apples from *Penicillium expansum* infection by rapidly consuming carbohydrates and colonizing the wound (Spadaro and Droby, 2016). Another indirect action mechanism has recently emerged; it is the case of bacteria that can interfere with pathogen signals. These beneficial bacteria seem to be able to degrade pathogen bacteria chemical signal messengers that are needed to start the infection process of the host plant (Molina *et al.*, 2003). Thirdly, BP microbials can interact with plant metabolism in order to induce plant resistance against the pathogen. This mode of action is highly complex and will be described in next sections [Chapter 1. - 4.2. and Chapter 1. - 4.3.]. Briefly, beneficial microbials are able to produce molecules called Microbe-Associated Molecular Patterns (MAMPs) that trigger the plant defense response like getting the cuticles thicker (Köhl *et al.*, 2019).

### 4.1.3. Semiochemicals

Semiochemicals are molecules produced by an organism and able to impact another organism's behavior; for BPs purposes they are used to control insect pests. Two types of semiochemicals are used as BPs: (i) pheromones, that represent the most used type of semiochemicals and act between individuals from the same species; and (ii) allelochemicals, that act between individuals from different species. First, pheromones are semiochemicals produced for sexual purposes (sexual pheromones) allowing males and females to meet and mate. It mainly concerns *Lepidoptera* spp. like Oriental fruit moths or European grapevine moths. Sexual pheromones are used for mating disruption techniques that consists of spreading sexual pheromones everywhere in the agricultural plot, preventing the males from being able to find the females. Another type of pheromone exists called aggregation pheromones, they are produced to bring food supply location or mating location to others' individuals' attention. It mainly concerns beetles. The two types of pheromones are used for

mass trapping; pheromones attract insects into the trap, it is highly used in greenhouses against *Tuta absoluta* (Herth, 2010). On another hand, allelochemicals are semiochemicals produced by plants and able to attract some insects. Several allelochemicals types exist like food attractants or repellents. Kairomones are also allelochemicals; they have a negative impact on the emitter species like attracting species parasites (Herth, 2010). Food attractants and kairomones can be used for mass trapping purposes. Pheromones, food attractants and kairomones can also be used as “Attract and kill” traps. Those traps attract the pest insects inside the trap where they are put in contact with an insecticide.

#### 4.1.4. Natural extracts

Natural extracts are BPs mainly coming from plants but also from animal, or mineral sources. In fact, plants can produce several molecules that have a wide range of potential activities among which BPs candidates (Ogunnupebi *et al.*, 2020). Natural extracts used as BPs present various modes of actions; the main ones will be presented in this section. They rely on three mechanisms: (i) direct action against the pathogen; (ii) mechanical action; and (iii) plant defense elicitation.

Firstly, natural extracts can act directly against the pathogen by contact like the extracts of thyme (*Thymus vulgaris*) or lemongrass (*Cymbopogon flexuosus*) that inhibit *Rhizoctonia solani* mycelial growth (Persaud *et al.*, 2019). An example with a BP from animal source is milk that is known to have a contact action against grapevine powdery mildew (*Erysiphe necator*); however, this action is highly dependent on natural light amount (Crisp *et al.*, 2006). Natural extracts can also directly have an action against the pathogen by ingestion like the cysteine protease contained in the latex of Papaya tree (*Carica papaya*), called papain, that inhibits growth of herbivorous insect larvae like *Samia ricini* (Konno *et al.*, 2004).

Secondly, natural extracts can mechanically act against the pathogen or other aggressions. For instance, kaolin clay is a biocontrol product from mineral sources formulated as a wettable powder easily dispersible in water. Once sprayed on the aerial plant part, water evaporates, and a protective particle film stays on the plant surface. This particle film protects the plant from abiotic stresses like radiations, ultraviolet, solar injuries and alter insects or pathogens interaction with the plant (Brito *et al.*, 2019).

Thirdly, natural extracts BP can interact with plant metabolism to induce plant defense response. This mode of action is highly complex and will be described in next section [Chapter 1. - 4.2.]. Briefly, some natural extracts contain molecules imitating the pathogens infection signals like Pathogen-Associated Molecular Patterns (PAMPs), Herbivore-



Associated Molecular Patterns (HAMPs) or Damage-Associated Molecular Patterns (DAMPs). Those molecules can trigger plant defense responses.

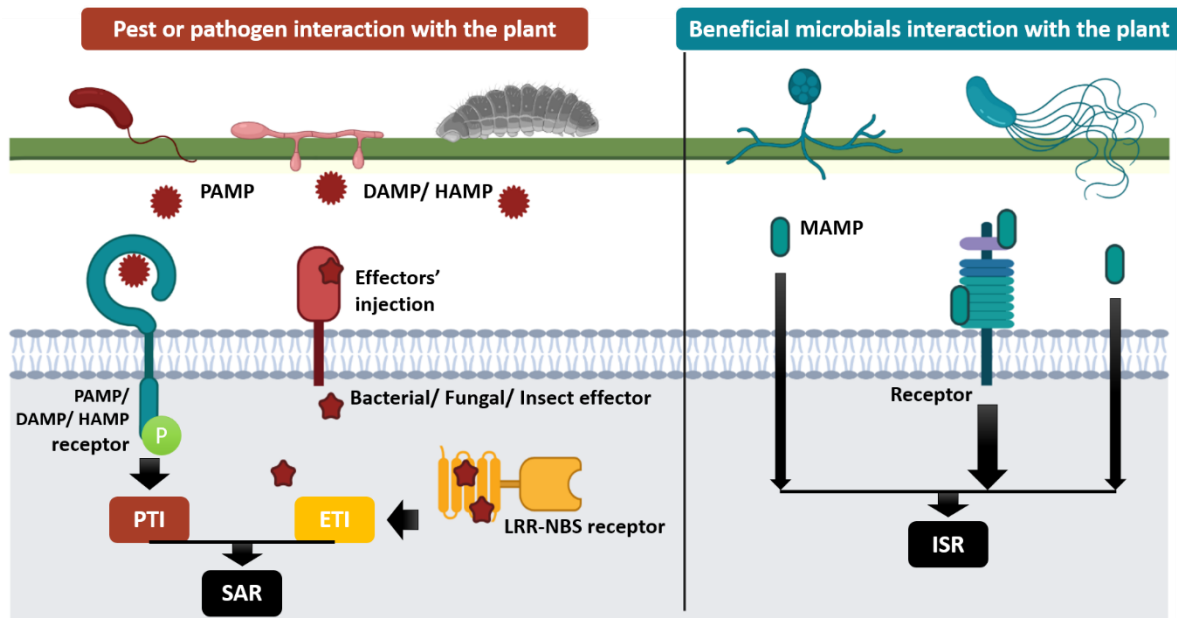
## 4.2. Plant Defense System

Firstly, plants present a passive defense system against pathogen attack and herbivory which consist on physical barrier like waxy cuticles, rigid cell wall or thorns (Nishad *et al.*, 2020); but also antimicrobial secondary metabolites (Chassagne *et al.*, 2021).

Secondly, plant response to biotic and abiotic stresses consists of different levels of recognition. After an infection, like penetration of plant cells by a fungus or a bacterium, the plant recognizes molecules secreted by the pathogen. If the molecular pattern belongs to a symbiotic partner, the host response occurs to set up the symbiosis. If the molecules belong to a pathogen, a plant innate immune response occurs also called PAMP-Triggered Immunity (PTI) (Jones and Dangl, 2006). PTI relies on Pattern Recognition Receptors (PRRs) that can specifically perceive and recognize PAMPs, HAMPs and DAMPs. This recognition triggers Mitogen-Activated Protein Kinases (MAPKs) signaling cascade involved in the activation of transcriptional regulators and defense-related genes for Systemic Acquired Resistance (SAR) (**Figure 12**). Depending on the plant susceptibility to the pathogen and on the pathogen pressure, the pathogen can also inject effectors inside the plant cells in order to stop PTI response (Jones and Dangl, 2006). However, the plant is able to recognize bacterial, fungal, or insect effectors by Resistance (R) proteins like Nucleotide Binding Site and Leucine Rich Repeat (NBS-LRR) proteins (**Figure 12**). It is called Effector-Triggered Immunity (ETI) and also lead to SAR stimulation. Long-distance SAR signaling is conducted by peptides, lipids metabolites and phytohormones like Salicylic Acid (SA), Jasmonic Acid (JA), Ethylene (ET), and Abscisic Acid (ABA). SAR is triggered by SA accumulation that causes Pathogenesis-Related (PR) proteins production like chitinases or hydrolytic enzymes, induces phytoalexins synthesis, it also regulates Hypersensitive Response (HR) and Reactive Oxygen Species (ROS) production that is involved in HR process through oxygen burst leading to plant cell death (Vlot *et al.*, 2008; Stael *et al.*, 2015; Abdul Malik *et al.*, 2020). SA signaling pathway is more specific against biotrophic pathogens whereas ET/JA pathways are more specific against necrotrophic pathogens and herbivorous pests. For example, JA induces the production of proteins that interfere with herbivore digestion (Abdul Malik *et al.*, 2020).

Thirdly, beneficial microbes also produce molecules that are recognized by the host plant called Microbial-Associated Molecular Patterns (MAMPs) that trigger Induced Systemic Resistance (ISR) (**Figure 12**). ISR is a defense system that relies on priming the plant immune system without directly activating costly defenses; whereas SAR activates immediate

defensive measures in the plant like the accumulation of SA and PR proteins (Choudhary *et al.*, 2007). In fact, ISR priming is characterized by an enhanced sensitivity of the whole plant to JA and ET phytohormones that leads to a faster and stronger activation of defenses in case of a pathogen attack (Pieterse *et al.*, 2014).



**Figure 12.** Plant defense system (adapted from (Abdul Malik *et al.*, 2020))

As previously mentioned, JA, SA, ET, and ABA are the main phytohormones that intervene in the regulation of defense genes expression (Rienth *et al.*, 2019). Their biosynthesis involving several intermediates and enzymes, can be triggered by different stresses or molecular patterns recognition (Xiong and Zhu, 2003; Broekaert *et al.*, 2006; Guerreiro *et al.*, 2016; Larrieu and Vernoux, 2016; Wang *et al.*, 2016; Hao *et al.*, 2017; Abdul Malik *et al.*, 2020; Lefevre *et al.*, 2020). Excepted from its biosynthesis, various modulators intervene in SA accumulation like Enhanced Disease Susceptibility 1 (EDS1), Nonexpressor of Pathogenesis-Related genes 1 (NPR1), or Non-Inducible immunity 1 (NIM1). These modulators are produced in cross-talk between SA and JA pathways (Rustérucchi *et al.*, 2001; Zhu *et al.*, 2011; Chen *et al.*, 2021).

Once biosynthesized, phytohormones are able to regulate plant defense response such as EDS1, NPR1 and NIM1 proteins upregulated by SA and JA pathways that are involved in SAR modulation; these proteins also are essential for the expression of PR proteins related genes, themselves related to transcription factors regulation (Ochsenbein *et al.*, 2006; Kazan and Manners, 2012; Pajerowska-Mukhtar *et al.*, 2013; Guerreiro *et al.*, 2016; Backer *et al.*, 2019). Another example is SA able to upregulate several genes like Glutathione-S-Transferase (GST) family (Gullner *et al.*, 2018) that are implied in detoxifying cytotoxic compounds, the process involve transmembrane transport (Burdziej *et al.*, 2021). ET pathway trigger a

transcription factors activation cascade such as ethylene response factors (ERF TF, (AP2)/ERF TF, AP2 TF). They are key regulators of JA, ET, and ABA pathways in response to biotic and abiotic stresses, like activating PR genes such as osmotins (PR-5), chitinases (PR-3) y  $\beta$ -1,3-glucanases (PR-2) (Mizoi *et al.*, 2012; Bahieldin *et al.*, 2016; Rienth *et al.*, 2019). Moreover, plant defense response leads to the accumulation of defense metabolites through phenylpropanoids pathway like anthocyanins, flavonoids, phytoalexins, and stilbenes (Campos *et al.*, 2003; Schnee *et al.*, 2008; Ahuja *et al.*, 2012; Höll *et al.*, 2013; Piasecka *et al.*, 2015; Jiao *et al.*, 2017; Yonekura-Sakakibara *et al.*, 2019) in close relation with phytohormones pathways (Dubrovina and Kiselev, 2017). Particularly, JA and ET strongly trigger stilbene biosynthesis (Belhadj *et al.*, 2008; Gutiérrez-Gamboa *et al.*, 2019; Rienth *et al.*, 2019).

### 4.3. Plant Defense Elicitation

As described in section [Chapter 1. - 4.1], some BPs from natural sources or microbials BPs are able to interact with the treated plant to trigger plant defense response. Several molecules are used along the plant defense response to induce SAR as well as ISR mechanisms.

#### 4.3.1. Systemic Acquired Resistance Elicitation

Analogue molecules or imitating SAR phytohormones are studied like ethylene-inducing xylanase, a fungal elicitor that induces ethylene synthesis. It leads to electrolytes leakage, to the expression of PR-proteins, and sometimes to HR (Abdul Malik *et al.*, 2020). Another study on grapevine used Methyl Jasmonate (MeJA) and benzothiadiazole an analogue of SA to confer resistance to downy mildew (*Plasmopara viticola*) (Burdziej *et al.*, 2021). MeJA triggered the JA pathway leading to stilbene synthesis stimulation. Benzothiadiazole triggered SA pathway leading to polyphenols accumulation, PR-proteins genes overexpression and cell wall reinforcement. Molecules imitating pathogen attack are widely investigated like plant defense elicitor peptides that are endogenous molecules able to induce and amplify the plant defense against bacteria, fungi, and herbivores' attacks (Boller and Felix, 2009). Chitin and chitosan are used and studied for several years as fungal elicitor. In fact, chitin is a component of most pathogenic fungi cell walls and chitosan is a derivative of chitin that is very concentrated in crustacean shells. Crustacean chitosan was used on greenhouse tomato culture to induce resistance against *Fusarium oxysporum* (Benhamou and Picard, 2005). It indeed triggered some defense responses such as increasing cell wall thickness, cell wall reinforcement by lignins, and phenolic compounds accumulation. Chitin was also tested in rice-inducing resistance to rice blast by ion efflux induction, accumulation of chitinases and phytoalexins, ROS production, as well as HR in infected cells (Abdul Malik

*et al.*, 2020). Concerning bacteria elicitors, hairpin protein isolated from *Erwinia amylovora* is able to trigger HR response but also to activate SAR (Wei *et al.*, 1992; Dong *et al.*, 1999). Eventually, insect elicitors are also investigated like inceptin isolated from fall armyworm (*Spodoptera frugiperda*) feeding on cowpea (*Vigna unguiculata*). This elicitor induces JA, ET, and SA accumulation as well as it increases the concentration of defensive compounds in cowpea (Wu and Baldwin, 2009).

### 4.3.2. Induced Systemic Resistance Priming

MAMPs from beneficial microbes are able to prime ISR in plants, like beneficial bacteria of *Pseudomonas* and *Bacillus* spp. that are able to protect the tomato from *Phytophthora infestans* through biosurfactants (rhamnolipids, fengycins, and surfactins) production that triggered ISR (Abdul Malik *et al.*, 2020). ISR trigger various range of defense mechanisms in case of pathogen or herbivory attack from enhancing defense gene expression to increasing structural barriers. For instance, ISR-primed *Arabidopsis thaliana* by Plant Growth Promoting Rhizobacteria (PGPR) revealed an enhanced expression of the JA-responsive gene *VSP* when the plants were infected by the bacterial pathogen *Pseudomonas syringae*. Similarly, ISR-primed *A. thaliana* by PGPR revealed an enhanced expression pattern of JA/ET-responsive genes *PDF1.2* and *HEL* when the plants were under the generalist insect herbivore *Spodoptera exigua* attack (Pieterse *et al.*, 2014). The priming can also increase the plant structural barriers like *Pseudomonas fluorescens* inducing cell wall apposition on pea infected by *Pythium ultimum* or inducing callose deposition on *A. thaliana* infected by *Hyaloperonospora arabidopsidis* (Pieterse *et al.*, 2014). ISR can also be primed by fungi like *Trichoderma* spp. that is able to induce resistance in a wide range of crops such as bean, cotton, cucumber, lettuce, maize, rice, tobacco, and tomato against various pathogens from bacteria to fungi to oomycetes to virus. For instance, *T. virens* produce peptaibols and Sm1 protein that are able to trigger ISR in maize leaves infected by *Cochliobolus heterostrophus* and *Colletotrichum graminicola* (Hossain *et al.*, 2017). The link between the plant and its beneficial microbes can be more complex like *A. thaliana* leaves infection by *P. syringae* resulting in the attraction of *Bacillus subtilis* to the plant root system that trigger ISR protecting the uninfected parts of the plant against the pathogen attack (Pieterse *et al.*, 2014). It is worth to mention that some molecules are shared by pathogenic and beneficial microbes like flagellin recognized by PRRs in plants that initiate the immune response by triggering ROS and ET production, and activating defense-related genes (Abdul Malik *et al.*, 2020).

Plant response to BPs treatment is complex, it relies on distinct mechanisms, and it may imply plant defense elicitation depending on the crop, the BP treatment and the pathogen. The plant response also depends on weather and crop's developmental stage. The study in greenhouse condition of 1-year-old grapevine stocks response to 2 types of BP without pathogen infection will be the subject of a part of this thesis work.

## 5. OBJECTIVES AND SCIENTIFIC CONTRIBUTION

The objective of the present work is to characterize new Biocontrol Products' (BPs) effect on treated plants: (1) in terms of residue's fate monitoring and (2) in plant response to the treatment. Two BPs were studied along this work's investigations, a natural extract BP (Akivi, Akinao) and a microbial BP (*Bacillus* UdG, UdG). These two products are complex and not fully described. Thus, holistic approaches like omics sciences were chosen in order to conduct the experiments, particularly (i): untargeted metabolomics for residues' fate monitoring and (ii): the combination of transcriptomics and targeted metabolomics for plant response to the BP treatments.

### 5.1. Omics Sciences Chosen Approaches

Omics sciences were defined by Vailati-Riboni *et al.* (Vailati-Riboni *et al.*, 2017) as follow “*the objective of omics sciences is to identify, characterize, and quantify all biological molecules that are involved in the structure, function, and dynamics of a cell, tissue, or organism*”. Omics sciences investigate all biological molecules at a given time in a given matrix. Different types of omics sciences exist depending on the type of biological molecules studied. Omics studies are primarily aimed to be holistic approaches carried out in a non-targeted and non-biased manner. They present the benefit of being able to study the whole biological sample acquiring and analyzing all the available data to define a hypothesis. These kinds of approaches are suitable for holistic studies and give first insights in an investigation to uncover the underlying links and to detect new emergent properties that may arise from studying the global matrix components. Targeted omics sciences also exist to validate the hypothesis and prove the connection between the many faces of a complex biological process (Vailati-Riboni *et al.*, 2017).

#### 5.1.1. Transcriptomics

Transcriptomics is the study of the set of all messenger RNA molecules within the extracted matrix (cell, tissue, or organism). It is carried out using mass sequencing, the RNA molecules are quantified and identified thanks to mapping on the matrix reference genome (Vailati-Riboni *et al.*, 2017). Transcriptomics is an extremely powerful and highly automated approach allowing the massive screening of all the genome at once. That is why, transcriptomics studies success depends on the availability of studied species-specific reference genome sequences. This kind of studies are expensive thus biological replicates are not always included, that is why Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) is used to confirm the observed transcript levels (Carpentier *et al.*, 2008). In our study, we decided to include biological replicates in the transcriptomics experiment but

also to use RT-qPCR technique in order to validate some selected genes' expression response to then pursue with additional studies with these validated genes.

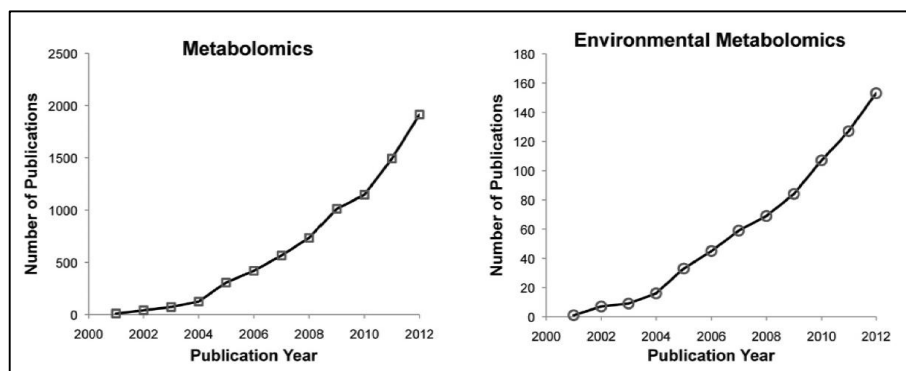
Functions of most of the genes within crops are still not experimentally determined as reference genome sequences are recently available. For instance, grapevine first genome sequencing was achieved in 2007, but improvements are still conducted, the latest ones were published in 2017 (Canaguier *et al.*, 2017). Post-genomics approaches are needed in order to clarify molecular mechanisms like transcriptomics to have insights on gene expression patterns involved in the studied biological processes. The results of transcriptomics studies must be discussed cautiously because gene overexpression does not always lead to its effector synthesis like protein or other metabolites; the link is more complex and further studies have to be conducted. In fact, transcriptomics is often linked with other post-genomics approaches like proteomics or metabolomics in order to identify all the mechanisms involved in the studied biological process. Indeed, gene expression is regulated by a metabolite accumulation pattern and metabolite synthesis is regulated by gene expression (Hirai and Saito, 2004).

### 5.1.2. Metabolomics

Metabolomics is the study of all the metabolites within the extracted matrix; these metabolites collection is called metabolome and represents the end products of cellular processes. Thus, metabolomics is the science that studies the chemical fingerprints left by these specific cellular processes; *“it is the study of all small molecule metabolite profiles”* (Vailati-Riboni *et al.*, 2017). In fact, metabolomics study metabolites from 30 Da to a maximum range of 3000 Da, usually metabolites with a molecular weight lower than 1500 Da are studied (Ibarra-Estrada *et al.*, 2016). Metabolites are an essential part of the behavior of the individual as, as mentioned previously, it represents the final products of the regulatory processes of the cell. They show the response of biological system to environmental or genetic changes. Metabolome evolves with plant developmental stages, tissue differentiation, and stresses. In fact, metabolic response to stress is one of the main objectives of most metabolomics studies. Metabolomics is a sensitive tool that can detect subtle molecular changes as well as detect stress biomarkers.

Metabolomics was firstly developed and is nowadays widely used in fields such as basic biology, medicine, clinical pharmacology and toxicology and nutrition. Its employment in environmental sciences has only emerged in the past few years. The field of environmental metabolomics is now progressively growing (**Figure 13**) and some metabolomics based methodologies were developed or are under development. Nevertheless, challenges are still remaining and many developments are needed. In particular, appropriate statistical tools for

the analysis of large metabolomics datasets must be more deeply considered. Indeed, all these developments are necessary in order to ensure a correct interpretation of the data. For instance, metabolomics is more and more used in order to study xenobiotic effects on organisms as processes involved in xenobiotic metabolism as well as response to toxic effect generated by xenobiotic presence (Rodríguez *et al.*, 2020).



**Figure 13.** Comparison of the publications per year for the keywords metabolomics and environmental metabolomics in Scopus for the years 2001-2012 (adapted from (Lankadurai *et al.*, 2013))

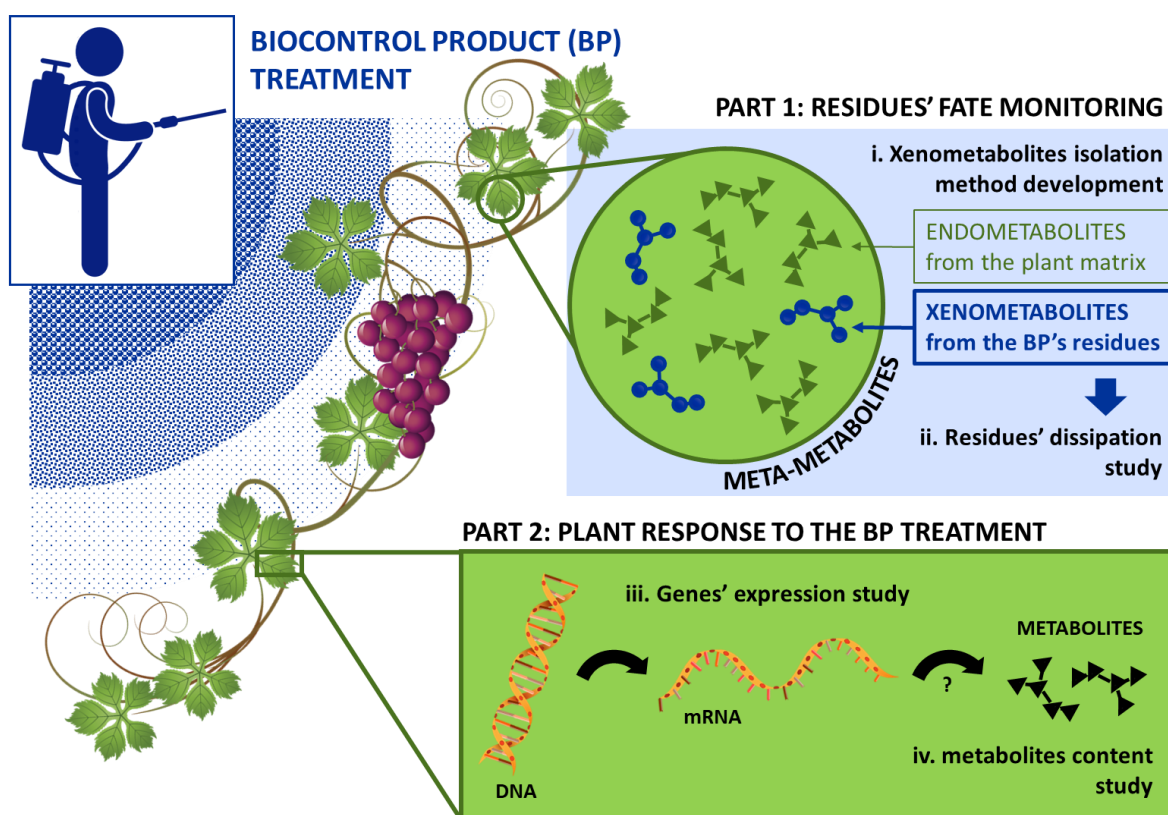
Two types of metabolomics studies exist; untargeted metabolomics representing a non-biased analysis of all metabolites found in the sample, and targeted metabolomics representing the analysis of metabolites from a molecular group targeted within the sample (Lankadurai *et al.*, 2013). The main benefit of untargeted metabolomics approach is to be able to study, without prior knowledge of involved processes, the behavior of metabolites composition of the sample with low probability of missing key metabolites (Ibarra-Estrada *et al.*, 2016). Targeted metabolomics study is based on prior knowledge and is focused on specific pathways to validate hypothesis.



## 5.2. Specific Objectives

In this work, we aim to develop an innovative approach in order to target and monitor residue's fate within treated crops; Akivi (Akinao) was chosen as model BP in order to set up this approach. Another objective of this PhD. work was to evaluate the plant response to new BPs treatment to have insights on BPs' interaction with the crop and maybe identify the studied BPs' mode of action. In this second part, Akivi (botanical extract) was used and compared to another type of BP, *Bacillus* UdG (bacteria). As described in **Figure 14**, the objectives of this work are to:

- i. develop an innovative method based on untargeted metabolomics for the isolation and monitoring of BPs' residues (xenometabolites)
- ii. apply the residues' fate monitoring approach to peach (fruits) and grapevine (leaves) in order to study residues' dissipation in plant matrices
- iii. investigate grapevine genes' expression response after BP treatment using transcriptomics
- iv. investigate grapevine metabolites content after BP treatment using targeted metabolomics



**Figure 14.** Objectives of the PhD

## **Chapter 2.**

# **Characterization of Biocontrol Products' Residues Fate on Treated Plants**



This first part laboratory investigations were performed during the 18 months I spent at the Université de Perpignan Via Domitia (UPVD). As previously mentioned, the main objective of the present PhD work is to characterize new BPs in terms of residue's fate monitoring and plant response to the treatment. More specifically in this Chapter 2, we aim to develop an innovative approach in order to target and monitor BPs residue's fate within treated crops. In fact, current dissipation kinetics studies of PPP residues in plants are conducted through half-life measurement of targeted active compounds of the product (Fantke and Juraske, 2013) that is not adapted for BPs monitoring. Thus, EMF approach was optimized to be used on plant matrices for PPP residues dissipation monitoring, especially BP residues monitoring. In fact, EMF was developed in CRIOBE laboratory for PPPs monitoring in soil and sediment matrices as previously described [Chapter 1. - 3.3.]. Various aspects influence PPPs dissipation in plants like chemical and physical properties of the product; treated plant properties; and environmental conditions (Fantke and Juraske, 2013). For that, a botanical extract formulated prototype BP candidate (Akivi, AkiNaO) was chosen as model BP for this study and a plant matrix was selected (peach fruit) to adapt and optimize EMF approach. Samples from PALVIP project field experiments were used, Akivi was evaluated in peach orchard (*P. persica*) against brown rot (*M. fructigena*) as previously described [Chapter 1. - 2.2.]. Extractions and metabolomics analysis were optimized for the selected matrices. Akivi residues targeting and monitoring was conducted on peach matrix, that will be presented in this Chapter 2.

# UNTARGETED METABOLOMICS AS A TOOL TO MONITOR BIOCONTROL PRODUCT RESIDUES' FATE ON FIELD-TREATED *PRUNUS PERSICA*.

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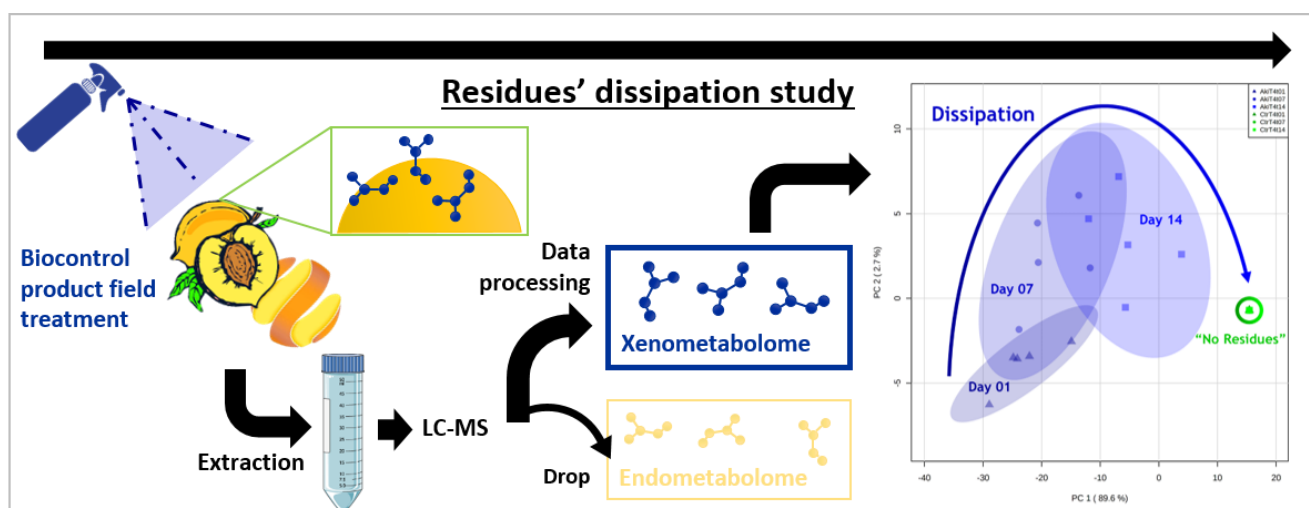
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## Graphical abstract:



## Highlights:

- Innovative untargeted metabolomics approach to study PPPs residue on fruits.
- 3 PPPs' residues were monitored on *Prunus persica* in field conditions.
- "Dissipation interval" for the 3 products were investigated.
- Kinetic patterns of product compounds and degradation by-products were highlighted.
- The approach was proven reliable. Nonetheless, field experiments must be improved.

## ABSTRACT

Evidence of chemical plant protection products' (PPPs) long-term impact has been found in all environmental compartments. Therefore, other types of PPPs are developed to complement chemical PPPs like PPPs from natural sources, namely biocontrol products (BPs). Little is known about those new BPs, and it is important to assess their potential long-term environmental impact. Recently, the Environmental Metabolic Footprinting (EMF) approach was developed. It permits studying sample's entire meta-metabolome (endometabolome and xenometabolome) through a kinetics tracking of metabolomes of treated and untreated samples. Those metabolomes are compared time-by-time to estimate the "resilience time" of the samples after treatment. The current study aims to investigate BP residues' dissipation on peach fruits (*Prunus persica*). For that, an untargeted Liquid Chromatography-Mass Spectrometry metabolomics approach based on the EMF was optimised to separate the xenometabolome of the PPP from the endometabolome of the fruits. This "new version" of the EMF approach is able to target the BP treatment residues' (xenometabolome) dissipation exclusively. Thus, it is able to determine the time needed to have no more residues in the studied matrix: the "dissipation interval". Field experiment was conducted on peach tree orchard against brown rot treated with (i) a plant extract BP (Akivi); (ii) a reference mineral extract BP (Armicarb®); and (iii) a Chemical reference treatment campaign. Formulated Akivi and its by-products' dissipation was monitored, a degradation kinetics appeared but the sampling did not last long enough to allow the determination of the "dissipation interval". Armicarb® and the Chemical reference's residues and by-products showed a persistence pattern along the sampling kinetics. These results indicate that the EMF approach, formerly developed on soil and sediment, is applicable for fruit matrices and can be used to investigate the fate of complex BP treatment on the matrix through the xenometabolome tracking on treated fruits.

**Keywords:** Biocontrol Products; Residues Monitoring; Pesticide Dissipation; Metabolomics; Liquid Chromatography-Mass Spectrometry.

## 1. INTRODUCTION

Plant protection products (PPPs) are products used in agriculture to prevent, to destroy, or to control any pest or disease that harm or interfere with the agricultural production (FAO, 2006). Chemical PPPs present various issues in terms of environmental and health impact. Therefore, other types of PPPs are developed to complement chemical PPPs, *e.g.* biocontrol products (BPs) that are increasingly being promoted by several governments (European Parliament and Council Of The European Union, 2009; Ministère de l'Agriculture et de l'Alimentation, 2015). BPs are PPPs from natural sources, *i.e.* molecules or organisms that already exist as it is within nature, like botanical extracts or beneficial bacteria. The development of these new BPs requires the development of new methodologies in order to monitor their residues' dissipation, which is a compulsory step to put any PPP on the market.

At present, the existing methodologies are only adapted for chemical PPPs. For instance, some of them are described by the Organisation for Economic Co-operation and Development (OECD) in the international guidelines for the testing of chemicals (OECD, 2007a), currently used by several institutions delivering marketing authorisations (*e.g.*: the French agency "Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES)"; the Spanish agency "Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)"; the European Union agency "European Food Safety Authority (EFSA)"). The section describing methodologies to monitor PPPs residues' metabolism in crops (OECD, 2007b) will be explained as follows: The component of a PPP that works against the pathogen is called Active Substance (AS). For approval processes, that AS must be well characterised in terms of structure, chemical-physical properties, and mode of action. Moreover, PPP residue monitoring in treated plants can be conducted through isotopic labelling of the AS (OECD, 2007b). The different moieties of the molecule are radiolabelled using  $^{14}\text{C}$ ,  $^{32}\text{P}$ , or  $^{35}\text{S}$  radioisotopes so that all significant parts can be tracked. Crop grown in laboratory-controlled conditions are treated with radiolabelled AS and its behaviour within the plant is studied. Radioactive labelled molecules are extracted and 90% of the Total Radioactive Residues (TRR) must be identified representing the AS and its major by-products (OECD, 2007b). Degradation of PPP are determined by various processes that can be classified in 2 types: (1) biotic degradation and (2) abiotic degradation, among which hydrolysis (acid, alkaline, or enzymatic), oxidation, reduction, or photolysis. The domination of a degradation pathway depends on various parameters like the chemical-physical properties of the molecule, the weather (*e.g.* light, pH), or the type of application used for the treatment. For example, aerial plant parts treatments are more subject to photodegradation. Once identified, the dissipation of the AS and its major by-products is measured within the crop and in soils. The dissipation times of 50% of the AS's initial amount "DT50" and of 90% of the AS's initial

amount “DT90” are studied particularly (European Commission – Directorate General for Agriculture, 2000). Their values, expressed in days, may lead to further investigations. For example, if the DT90 in soils is higher than 100 days, complementary study on next rotation culture is necessary (European Commission – Directorate General for Agriculture, 2000).

However, guidelines reporting monitoring methods for BPs are neither available for crops, nor for soils and sediments. The previously described methodologies for chemical PPPs are not suitable for BPs as the ASs of BPs are very rich and complex mixtures, with a relatively large number of unidentified components. There are 3 types of BPs affected by marketing authorisation processes (ITAB and ONEMA, 2013): (1) living or part of microorganisms: fungi, bacteria or virus; (2) extracts from natural sources: mineral, botanical or animal sources; and (3) semiochemicals: pheromones and kairomones. In addition, BPs activities are often the result of an interaction between several of its components. Moreover, the components responsible for the main activity of the product are usually unknown and the most abundant components are not always the most active against the pathogen. Thus, it is impossible to radiolabel such complex ASs and to determine their DT50 or DT90.

Hence, as classic residues monitoring methodologies are neither fitting to BPs, nor in crops, nor in soils or sediments, and as all or part of the components of the ASs are not identified, an untargeted approach seems to be a potential solution. Therefore, an innovative approach relying on untargeted metabolic profiling was recently developed; the Environmental Metabolic Footprinting (EMF) (Patil *et al.*, 2016; Salvia *et al.*, 2018). EMF concept relies on the meta-metabolome study of a treated environmental matrix *versus* an untreated environmental matrix along a kinetics study. This approach aims to monitor the evolution of the differences between the metabolic profiles of the treated and the untreated control matrices through time. The meta-metabolome represents the combination of the endometabolome from the original matrix and the xenometabolome from the treatment, *i.e.* the PPP residues composed of the ASs and the formulation ingredients of the product and their transformation by-products. On one hand, the EMF gives rise to a new integrative proxy: the “resilience time” (Patil *et al.*, 2016; Salvia *et al.*, 2018). It corresponds to the time needed for the xenometabolome (PPP residues) to dissipate, and for the PPP’s impact on the matrix to disappear (*i.e.* the endometabolome of the treated samples to re-establish the same profile as that of the endometabolome of the untreated control samples at a given time point). On the other hand, the EMF is potentially useful for determining the “dissipation interval” that corresponds to the time needed to have no difference between the residues profiles of the treated sample and the profile of the control samples that must not contain residues. This investigation can be done by selecting and monitoring the xenometabolome exclusively.



The aim of the work described in this article is (i) to optimise the existing EMF approach in order to target, exclusively, the treatment residues (xenometabolome) on the fruit matrix, and (ii) to investigate BP residues' dissipation on the treated fruit matrix. In the current work, the peach carposphere was selected as a typical matrix to be studied in such a context. For that, the EMF approach formerly developed on soil (Patil *et al.*, 2016) and sediment (Salvia *et al.*, 2018) laboratory microcosm experiments will be adapted in the current work to peach peels from a field experiment. This study will focus on the xenometabolome selection part of the EMF, which is a challenging part. In fact, the xenometabolome isolation from the meta-metabolome must be optimised. The experiments were conducted in field conditions with a botanical extract BP; the "Akivi" (Tamm *et al.*, 2017). This product presents direct antifungal activity due to a high content on polyphenols and terpenes. Akivi was compared in field conditions (peach orchards) with a reference BP; "Armicarb®" based on the potassium hydrogen carbonate mineral compound, and a chemical reference treatment campaign; based on a mix of 5 synthetic organic compounds (Boscalid, Fenbuconazole, Fluopyram, Pyraclostrobin and Tebuconazole). The 3 treatments modalities were used against brown rot (*Monilia fructigena*) with interesting efficacy results during this field experiment. This fungus is one of the main diseases affecting peach fruits and the agricultural sector needs new products to protect the crops due to the lack of solutions in organic farming against this disease.

## 2. MATERIAL AND METHODS

### 2.1. Experimental Design

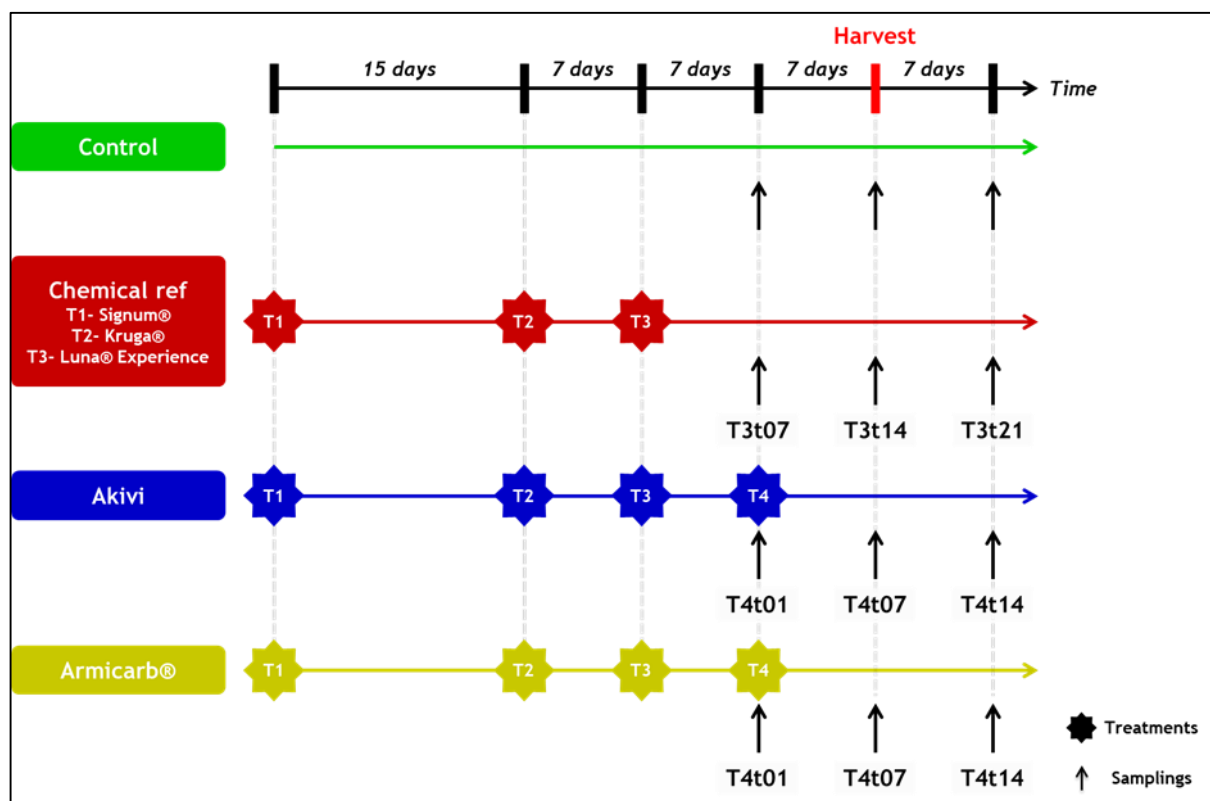
#### 2.1.1 Field Experiments

Field experiments were conducted in collaboration with the “Centre Expérimental des Fruits et Légumes du Roussillon” (Sica CENTREX). They were performed in their agricultural domain in Torreilles (France) [GPS: (DMS) 42°45'14.221"N 2°58'35.712" E] on peach trees orchard *Prunus persica* ‘CORINDON®’ treated against brown rot (*Monilia fructigena*). Brown rot is a post-harvest disease affecting the fruit. Thus, peaches are the main target of the treatment, so peach peel matrix was selected for this study. Different groups of trees were treated separately with 3 products. A first group called “Aki” was treated with 0.75 Kg/ha “Akivi” formulated plant extract BP (S.A.S. AkiNaO). A second group called “Arm” was treated with 5 Kg/ha “Armicarb®” formulated mineral extract BP (De Sangosse) with Potassium Hydrogen Carbonate as AS. A third group called “Chi” was treated with a Chemical reference treatment campaign usually used against brown rot: first treatment with 0.75 g/ha “Signum®” (AS: Boscalid and Pyraclostrobine, BASF), second treatment with 3 L/ha “Krugatm” (AS: Fenbuconazole, Dow AgroSciences), and third treatment with 0.5 L/ha “Luna® Experience” (AS: Fluopyram and Tebuconazole, Bayer). The chemical structure of the AS of the Chemical reference modality are presented in **Figure S1**. “Arm” and “Aki” treated trees were distributed in Fisher blocks of 3 replicates of 2 trees (6 trees in total); “Chi” treated trees and untreated controls “Ctr” trees were distributed in 3 replicates of 3 trees (9 trees in total) (**Figure S2**). The treatment campaign was made in August 2018 with 4 treatments. The first and second treatments were spaced 15 days apart and then trees were treated every 7 days (**Figure 1**).

#### 2.1.2 Plant Material & Sampling Method

For the metabolomics approach, peach samplings (**Figure 1**) were made according to a kinetics beginning after the last BPs treatment (T4): one day after T4 (T4t01); 7 days after T4 (T4t07), corresponding to the harvest; and 14 days after T4 (T4t14). These kinetics points correspond to 7 days after T3 (T3t07), 14 days after T3 (T3t14) and 21 days after T3 (T3t21) respectively. T3 corresponding to the last “Chemical” treatment. Peaches were sampled in the area at the middle of the trees identified by the trees’ trunks, in order to avoid the part of the branches that can be contaminated by the next treatment. Peaches were sampled at different positions on the tree: two peaches were sampled on each side of the rank and one peach inside the foliage (**Figure S3**). For each modality of time and treatment, 5 peaches were sampled in each of the 3 replicate blocks. The 15 peaches were randomly mixed and separated in 5 biological replicates of 3 fruits. The peaches were then peeled, and peels of each of the samples

were stored separately in freezer-safe bags at  $-32\text{ }^{\circ}\text{C}$  until the analyses (the extractions and analyses were done at once after the end of the kinetic experiment, *i.e.* after the collection and storage of all the samples).



**Figure 1.** Peach field-sampling campaign after the 4 different treatments modalities: (i) the untreated Control (green); (ii) first treatment (T1) with Signum®, second treatment (T2) with Kruga®, and third treatment (T3) with Luna® Experience for the Chemical reference (red); (iii) 4 treatments with a plant extract BP Akivi (blue); and (iv) 4 treatments with a mineral extract BP Armicarb® (yellow).

## 2.2. Chemical Analysis

### 2.2.1. Chemicals

For sample preparation, Acetonitrile HPLC grade and Methanol HPLC grade were purchased from VWR (Fontenay-sous-Bois, France). For UHPLC-HRMS analysis, water LC-MS grade was purchased from VWR (Fontenay-sous-Bois, France), and Methanol LC-MS grade was purchased from Carlo Erba (Val de Reuil, France). Formic acid 99% (for analysis) was obtained from Acros Organics (Geel, Belgium). Boscalid, Diclofenac, Fenbuconazole, Fluopyram, Progesterone, Pyraclostrobin, and Tebuconazole analytical standards were obtained from Sigma-Aldrich (Saint Quentin-Fallavier, France).

### 2.2.2. Sample Preparation

Before extraction, samples were put in the freezer ( $-32\text{ }^{\circ}\text{C}$ ) overnight prior to freeze-drying (Heto, FD3) that lasted 48h. The peach peel content of each freezer-safe bag, corresponding to one laboratory repetition, was then grinded. 4.50 g ( $\pm 0.05\text{ g}$ ) of the dry peach

peel powder were transferred into a 50 mL tubes (Fisher Scientific) in order to perform the extraction with 40 mL of acetonitrile. Acetonitrile was chosen as a classic extraction solvent used for PPPs residues' studies (Rajski *et al.*, 2014; Rizzetti *et al.*, 2016; Rutkowska *et al.*, 2018). A one-step-based extraction protocol was set in order to reduce sample manipulation-linked biases and uncertainties. The protocol was as follows: all the tubes were manually shaken, swirled for 1 min on Vortex shaker (Heidolph, Hei-MIX Multi Reax), and then put on an agitation table (Benchmark Scientific, BV1010) for 20 min at 500 RPM. After, a centrifugation is performed for 10 min at 4500 RPM and room temperature (~20 °C). Then, the supernatant was transferred into vials after filtration through 0.22 µm PTFE filters. The final extract is diluted by a dilution factor of 2 in methanol. An internal standard composed of a mix of Diclofenac and Progesterone is added to the sample at a concentration of 5 µg/mL for each of the two molecules.

### 2.2.3. UHPLC-HRMS Analysis

Metabolic profiling analyses of the extracts of peach peels were achieved by Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) using a Vanquish™ Flex UHPLC hyphenated with a QExactive™ Plus Heated Electrospray-Quadrupole/C-Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). Metabolites were separated on a Luna® Omega 1.6 µm Polar C18 100 Å, 100 × 2.1 mm column (Phenomenex) put in an oven set at 30 °C. 5 µL of extract were injected. A gradient-based separation was applied with the following mobile phases: water/methanol 65:35 v/v + 0.1 % formic acid (v/v) (A), and methanol + 0.1% formic acid (v/v) (B). The mobile phase flow was maintained at 0.35 mL/min. The gradient program was the following: initially 2 min with 0% (B), then from 0% to 70% (B) in 3 min, from 70% to 100% (B) in 11 min, 6 min at 100% (B), and from 100% to 0% (B) in 1 min back to initial conditions that were maintained for 2 min with 0% (B). Each run lasted for 25 min in total. For the HRMS conditions, the acquired RT range was between 2 and 23 min (in Full MS). The Heated Electrospray (HESI) was operated in positive mode (ESI+). Sheath gas (N<sub>2</sub>) flow rate was set to 35 arbitrary units (a.u.); auxiliary gas (N<sub>2</sub>) flow rate was set to 10 a.u.; sweep gas (N<sub>2</sub>) flow rate was equal to 0 a.u.; capillary temperature was equal to 320 °C; auxiliary gas temperature was 200 °C; spray voltage was set to 3.2 kV; and the S-lens RF level was 50.0. The mass spectra were acquired in a scanning range of 200-1500 *m/z* in "Profile" acquisition mode. The resolution was set to 35000 at a *m/z* equal to 200; the Automatic Gain Control Target of the C-Trap was set to 3e6 charges, the Maximum Injection Time to the Orbitrap was equal to 200 ms. Samples of all time points and treatment modalities were prepared and analysed at once in a random order. Blank extraction samples were injected at the beginning of each of the two analytical batches. The blank extraction samples correspond to acetonitrile that underwent all extraction steps without

peach peel sample addition. Three different Quality Control (QC) pool samples –each is specific to one treated group (“Aki”, “Arm”, “Chi”)– were injected every 8 samples in order to assess the analytical variations during data acquisition. Each QC pertaining to a treatment group was prepared by mixing an equal volume from 3 out of 5 treated samples of the group for each time point.

#### 2.2.4. Quantification of Chemical Reference ASs

The 5 chemical product ASs (Boscalid, Pyraclostrobin, Fenbuconazole, Fluopyram and Tebuconazole) were quantified in some peach peel samples using the standard addition method. The quantification was carried out within 3/5 repetitions of both contaminated control “Ctr” samples and treated “Chi” samples at the last sampling point: 21 days after the third treatment. The ranges of spiking concentrations, that comprised 4 points, were different for the “Ctr” and “Chi” samples. For the “Ctr” samples, the calibration curve was made from 0 (no addition) to 20 ng/mL. For the “Chi” samples, the calibration curve was made from 0 to 200 ng/mL.

### 2.3. Software and Data Processing

LC piloting, LC-MS hyphenation, analytical sequence piloting and UHPLC-HRMS data acquisitions were performed using Xcalibur 4.1.31.9 (Thermo Fisher Scientific). The Mass Spectrometer and the HESI source were configured using Q Exactive Plus – Orbitrap MS 2.9 build 2926 software (Thermo Fisher Scientific). Data were acquired in RAW format. They were then converted to “.mzML” using the MSConvertGUI software (ProteoWizard) (Chambers *et al.*, 2012) in order to upload and process them using Galaxy Workflow4Metabolomics platform (Giacomoni *et al.*, 2015; Guitton *et al.*, 2017). Data of the three different PPP treatments modalities were processed using the same workflow but separately (*i.e.* “Aki” *vs.* “Ctr”; “Arm” *vs.* “Ctr”; “Chi” *vs.* “Ctr”). The pre-processing workflow and all its parameters are published on the Galaxy Workflow4Metabolomics platform (Ramos, 2021). The “XCMS” algorithm-based pre-processing (Smith *et al.*, 2006) consisted of a “centWave” peak piking (Tautenhahn *et al.*, 2008), “PeakDensity” peak grouping, loess/non-linear “PeakGroups” retention time adjustment (degree of smoothing: 0.8), peak filling and “CAMERA” peak annotation (Kuhl *et al.*, 2012). For the retention time adjustment, the “PeakGroups” algorithm used the chromatographic peaks corresponding to the internal standards (among others). Indeed, these reference peaks are present in all samples, pools and blank extractions and are used in order to correct the retention times of the chromatographic peaks of the compounds. The considered signal value for ion features was the chromatographic peak area. The first three “raw” matrices obtained for each of the three treatments contained an important number of features (16058 for “Aki”, 11717 for “Arm”, and 11310 for “Chi”). Such

large numbers of variables render difficult the data handling and the statistical analyses. Hence, matrices clean-up should be performed. Therefore, a first clean-up was performed in order to eliminate all features that are significantly detected in blanks (based on p-Values and t-Stat outputs generated by the “CAMERA” step). Then, as analytical drifts could occur in LC-MS sequences, an “inter/intra-batch” signal correction was applied using the “Batch correction” function with a “loess” regression model (span = 0.8) (van der Kloet *et al.*, 2009). “Loess” regression model was chosen because it better fits the variation of the peak intensities over the analytical sequence than a “linear” regression model (span = 0). A span lower than 1 was selected (span = 0.8) in order to avoid the overestimation of the outliers. This step was followed by a second clean-up according to feature’s CV in pool QC injections (all features with area RSD upper than 30 % through pool QC injections were eliminated from the dataset) (Thévenot *et al.*, 2015). A third clean-up was then applied in order to eliminate ion redundancies as much as possible (the ion with the highest intensity was selected as the representative ion). This elimination was done using the Analytic Correlation Filtration approach developed by Monnerie *et al.* (Monnerie *et al.*, 2019). After generating those “intermediate” data matrices, significant features were filtered in order to select xenometabolites exclusively, as the current work is focused on BPs residues. This filtration was performed following two main steps: 1) features showing significant intensity folds between the treated and the untreated samples were selected (p-Value  $\leq 0.05$  and Fold Change  $\geq 5$  with a higher intensity in the treated samples), and 2) features detected in the untreated control samples were eliminated after a manual investigation of their EICs was carried out using Xcalibur 4.1.31.9. After the mentioned pre-processing, clean-ups and filtration were achieved, three different “final” matrices pertaining to the three investigated products were obtained, with 382 features for the “Aki” xenometabolome data matrix, 14 features for the “Arm” xenometabolome data matrix, and 17 features for the “Chi” xenometabolome data matrix. Statistical analyses were then performed on those final matrices.

## 2.4. Statistical Analysis

Statistical analysis was made using the R-based MetaboAnalyst platform (Pang *et al.*, 2021). Pareto scaling was conducted to normalise the data prior to make Principal Component Analysis (PCA), Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA), and boxplots of the features. For the Heatmaps, data scaling and normalisation were not suitable, as this type of analysis was conducted in order to visualise the dissipation of molecular traces in samples through the time. Thus, only a  $\text{Log}_{10}$  transformation was applied before this analysis in order to minimise the “size effect” of the peaks with high intensity (that

can hide the other peaks with low but non-null intensity). The  $-\infty$  values (issued from the  $\text{Log}_{10}$  transformation of intensities originally equal to 0) were converted to 0 in order to adjust the intensity scale and to allow null intensities to be observable.

#### 2.4.1. Principal Component Analysis

PCA is a descriptive unsupervised multivariate statistical model. It relies on linear combinations of the correlating variations associated to variables in the dataset. The PCA aims to simplify the variations by combining them and then to identify the combinations giving the best explanation of the systematic variations in the dataset. Those combinations are the principal components (PCs); they are associated with a value in per cent representing the ability of each PC to explain a variation in the dataset. Usually, the PCs with the highest percentages of variance explanation are selected to project the samples in a 2D-graph. Then, the samples are projected on the graph and can be grouped or clustered according to the degree of similarity of their variables profiles, *i.e.*, their metabolic profiles when it comes to metabolomics.

#### 2.4.2. Orthogonal Projections to Latent Structures Discriminant Analysis

OPLS-DA is an explicative supervised multivariate statistical model (Trygg and Wold, 2002). It relies on the linear combinations of the correlating variations associated to variables like in the PCA, but it identifies the combinations giving the best explanation of the data variance correlating to a defined experimental factor. Moreover, it separates the predictive variation (horizontal axis) representing the variation that is correlated with the defined factor (*i.e.* the predictive component “p”), from the orthogonal variations (vertical axis) representing some systematic variations that are uncorrelated (orthogonal) to the defined factor (*i.e.* the orthogonal components “o<sub>(n)</sub>”). This multivariate analysis is a model that needs to be validated. Therefore, a Cross-Validation (CV) test should be performed. It provides different scores for each of the components that are needed for the assessment of the model: the R2X representing the percentage of the variation explained by the component; the R2Y representing the correlation coefficient of the samples' discrimination to the component; and the Q2 representing the predictivity of the component. For the “p” component, the model performance is given by the R2Y<sub>(p)</sub> value that has to be close to 1; the model predictivity is given by the Q2<sub>(p)</sub> value that has to be > 0.5 in metabolomics studies; the R2Y<sub>(p)</sub> should be higher than the Q2<sub>(p)</sub>; and R2Y<sub>(p)</sub> – Q2<sub>(p)</sub> should be lower than 0.3 (Wiklund, 2008). For the “o” component, the R2Y<sub>(o)</sub> and Q2<sub>(o)</sub> values should be as low as possible; if the values are > 0.5 it compromises the validation of the model (Wiklund, 2008).

To ensure that the difference explained by the OPLS-DA model is the result of a real effect caused by the defined factor, and not due to a random effect, a permutation test must be performed. The “original” model represents the model that has been constructed after sorting samples according to the defined factor. The samples are then mixed up by the permutation test in new random groups for several times (the test randomly permutes samples in between the different groups). For each random distribution, an OPLS-DA model is then constructed and for each model, as well as for the “original” model, a CV test is performed. After, the permutation test calculates a p-Value defined as:

$$p\text{-Value} = \frac{\text{(number of permutations giving better model than the “original” model)}}{\text{(total number of permutations)}}$$

The total number of permutations is set here to 1000. p-Values lower than 0.05 must be obtained, *i.e.* there is less than 5% of chance that the mixed model is better than the “original” one. Thus, there is less than 5% of probability that the discrimination between samples is due to a random effect instead of being caused by an effect related to the defined factor.

### 2.4.3. Heatmaps

A Heatmap is a 3D visualisation technique combining a vertical axis, a horizontal axis and a colour scale within the map. The vertical axis represents the features classified by similarities between each other thanks to a Dendrogram-based hierarchical clustering (Distance Measure: Euclidean; Clustering Algorithm: Ward). The horizontal axis represents the samples classified by treatment modality and by kinetics sampling points. The colour scale represents the intensity of the features from 0 in dark blue to the most intense in dark red.



### 3. RESULTS

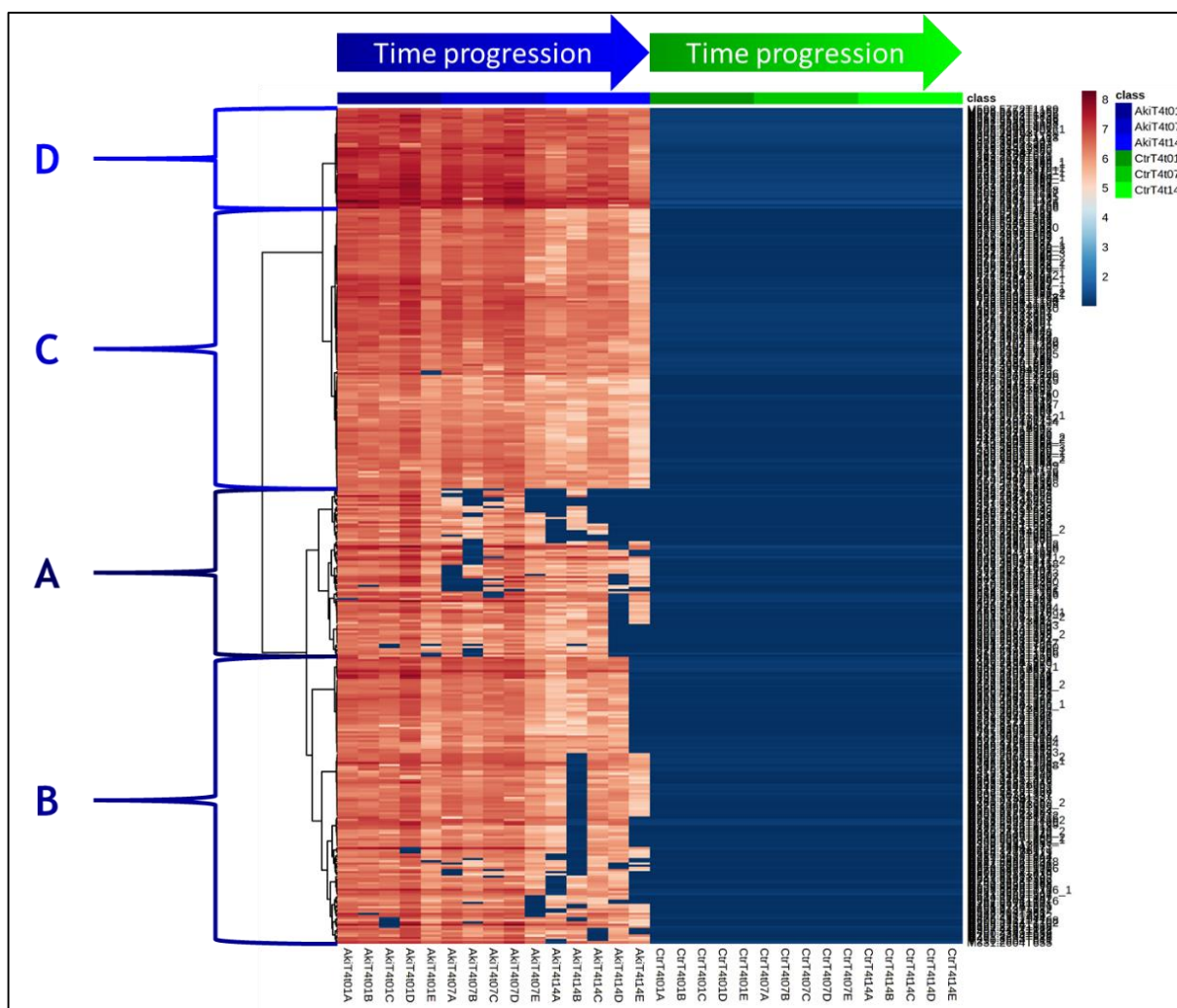
After generating the “final” data matrices, xenometabolome of each studied product *vs.* the untreated control are analysed separately (in separated datasets): 1) Akivi; the botanical extract BP, 2) Armicarb®; the mineral extract BP used as BP reference on the field experiment, and 3) the Chemical reference composed of 3 treatments with 5 chlorinated compounds (**Figure S1**). Due to the exclusive selection and filtering of the xenometabolome in the data matrices, untreated control samples are all at a total relative intensity level equal to 0 (except for the data matrix of the Chemical reference; the reason will be explained subsequently). The untreated control samples profiles thus represent the “No Residues” point that must be reached in order to determine the “dissipation interval”.

#### 3.1. Akivi

Akivi final xenometabolome data matrix is visualised on a Heatmap after a  $\text{Log}_{10}$ -transformation and a conversion of  $-\infty$  values to 0 were applied (**Figure 2**). 382 xenometabolite features are detected. The Akivi treated samples “Aki” are put in column on the left side (in blue) and the “Ctr” untreated control samples are put on the right side (in green). Within the 2 modalities, the samples are arranged by time sampling from the left (T4t01) to the right (T4t14). On the ordinate axis, the features are represented and sorted following the Euclidean Distances through samples. Inside the Heatmap, the features are coloured according to their relative intensity from 0 in dark blue to the highest intensity in dark red (on a  $\text{Log}_{10}$  scale).

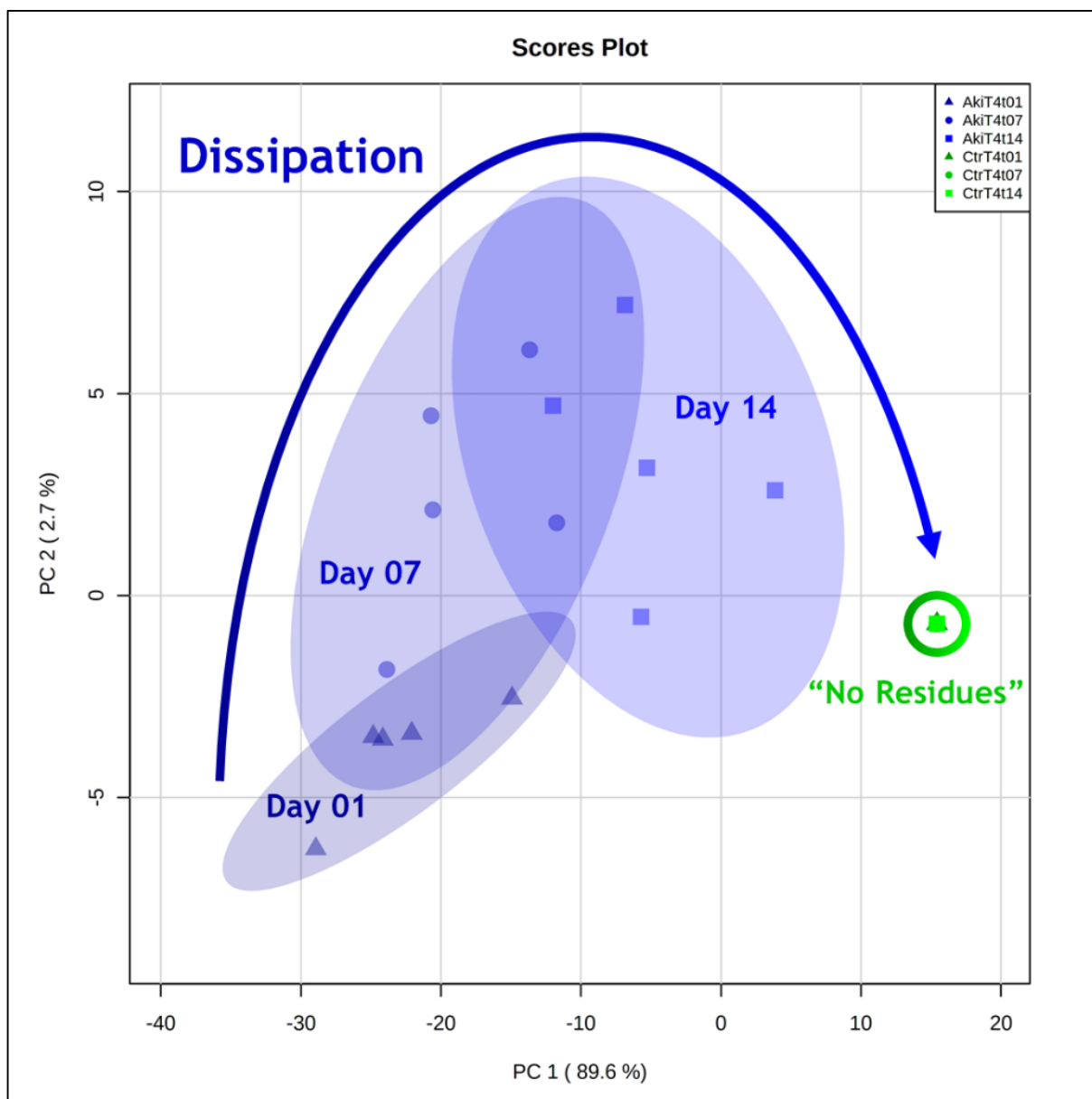
A global dissipation pattern for the Akivi treated samples along time is observed. In fact, relative intensities of features seem to be decreasing from T4t01 to T4t14, and some of the features have completely disappeared 14 days after T4 (T4t14). However, the “No Residues” point is not reached. In order to investigate closely the features behaviour, boxplots of the features along the time samplings are observed and their behaviour can be grouped into 4 blocks from A to D, respectively from the less persistent to the most persistent features. In fact, boxplots representing block A pattern (**Figure S4A**) show a quick dissipation kinetics with total disappearance 14 days after T4 (T4t14). Boxplots representing block B pattern (**Figure S4B**) show a certain persistence between T4t01 and T4t07 but quick dissipation between T4t07 and T4t14 and nearly reaching disappearance 14 days after T4 (T4t14). On the contrary, boxplots representing block C pattern (**Figure S4C**) show quick dissipation between T4t01 and T4t07 but persistence at low intensity level between T4t07 and T4t14. Eventually, boxplots representing block D pattern (**Figure S4D**) show persistence at high intensity between T4t01 and T4t07 but certain dissipation between T4t07 and T4t14 with persistence at low intensity 14 days after T4 (T4t14). Therefore, the Heatmap visualisation is

able to show a global dissipation of the features that must represent the molecules belonging to the “Aki” extract within the treated samples. However, this model is not able to underline any by-product appearance patterns. Thus, PCA is used in order to search for such patterns, by projecting the “Aki” xenometabolome data matrix after Log<sub>10</sub>-transformation and Pareto scaling were applied (**Figure 3**).



**Figure 2.** Heatmap of Akivi xenometabolites abundance (the darker is the red, the higher is the intensity).

“Aki” treated samples from 1 day (dark blue) to 14 days (light blue) after treatment, vs. “Ctr” untreated control samples from 1 day (dark green) to 14 days (light green) after treatment. (A, B, C, D): Blocks of features’ dissipation patterns from the less persistent (A) to the most persistent (D).

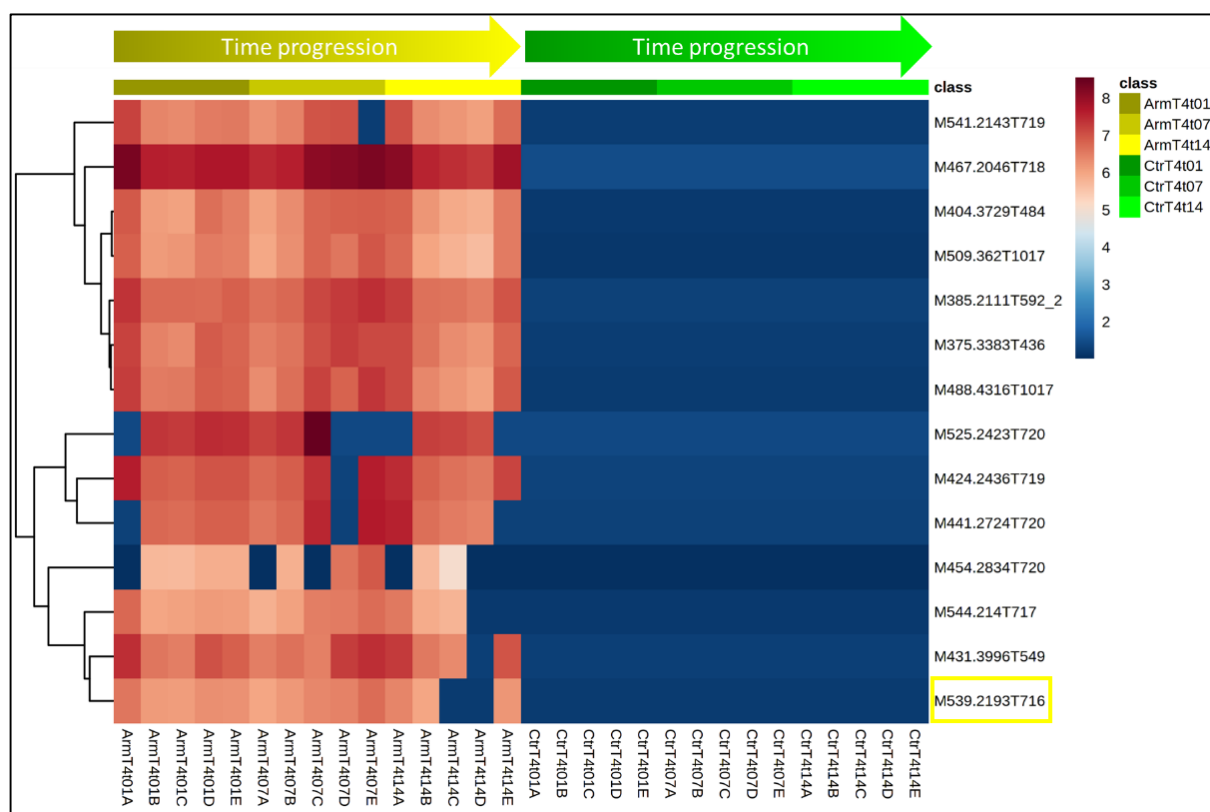


**Figure 3.** PCA of Akivi xenometabolites degradation kinetics: 1 day after the fourth treatment (T4) (T4t01), 7 days after T4 (T4t07), 14 days after T4 (T4t14) (from dark blue to light blue, respectively), and the “No Residues” point in green, assembling all “Ctr” samples.

The samples are projected on the 2 most relevant principal components: PC1 and PC2. The PC1 explains 89.6% of the variations. It discriminates the Akivi treated samples “Aki” (in blue) from the “Ctr” untreated control samples (in green –all grouped in one point representing the “No Residues” point (the “0” point)–). PC1 also discriminates the “Aki” treated samples T4t01 from the group T4t14 that heads to the “No Residues” point. On the other hand, the PC2 explaining 2.7% of the variations discriminates the “Aki” treated samples T4t01 from the groups T4t07 and T4t14. All these observations could be explained by the disappearance of features from the original BP applied on the peach peels –characteristic of the T4t01 group–, and with the appearance of by-products' features at T4t07 and their disappearance within T4t07 and T4t14. To verify this hypothesis, the loading plots of the PCA are observed (**Figure S5**). The features at the bottom of the loadings plot are most intense within the “Aki” treated samples at T4t01. The boxplots of these features represented in **Figure S6A** show a quick dissipation patterns with total dissipation 14 days after T4 (T4t14). Whereas, the features at the top of the loadings plot (**Figure S3**) present the highest intensities within the “Aki” treated samples at T4t07. The boxplots of these features represented in **Figure S6B** show by-product evolution patterns. That is to say, a higher intensity at T4t07 than at T4t01 and a dissipation between T4t07 and T4t14. On another hand, the “Aki” treated samples *vs.* the “Ctr” untreated control samples from the Akivi xenometabolome data matrix are compared for each sampling time point using the OPLS-DA after a  $\text{Log}_{10}$ -transformation and a Pareto scaling were applied (**Table S1**). The OPLS-DA model is validated for every time sampling ( $R^2Y > R^2X$ ,  $R^2Y > Q^2$ ,  $R^2Y - Q^2 \leq 30\%$ , and  $Q^2 > 50\%$  (Wiklund, 2008)) but the values decrease from 1 day after T4 ( $R^2Y$ : 97.20%,  $Q^2$ : 96.70%) to 14 days after T4 ( $R^2Y$ : 89.90%,  $Q^2$ : 87.80%). These results support the interpretation claiming that the Akivi xenometabolome is dissipating as discussed above.

### 3.2. Armicarb®

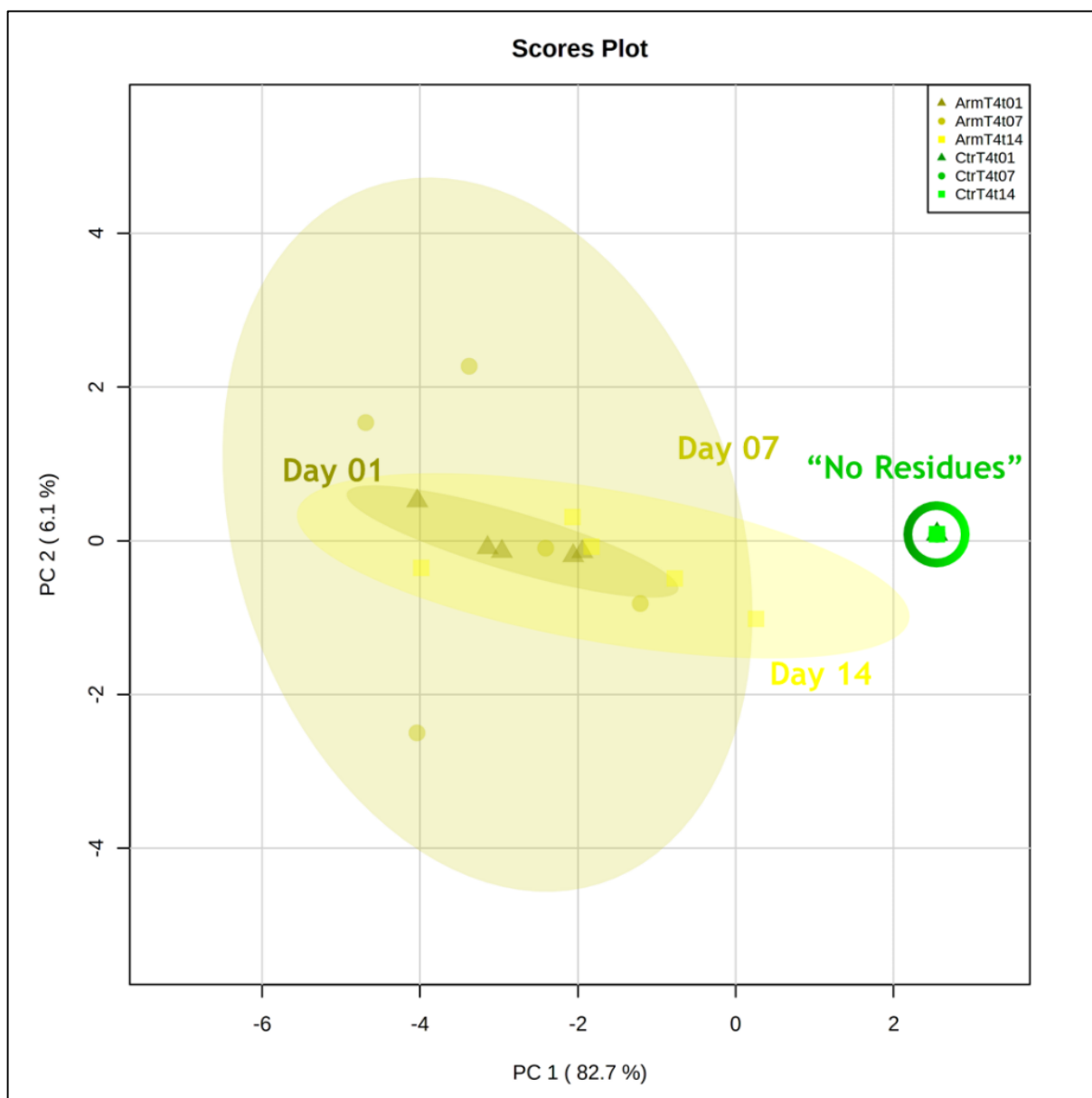
Armicarb® is a mineral extract and its AS is Potassium Hydrogen Carbonate salt ( $\text{KHCO}_3$ ). This compound has a high solubility in water, which renders difficult its retention on the C18 column. Thus, the analytical method is not able to detect the AS but it should be able to detect some of the co-formulants and adjuvants. In fact, 14 xenometabolites features are detected. Armicarb's® final xenometabolome data matrix is visualised on a Heatmap after a  $\text{Log}_{10}$ -transformation and a conversion of  $-\infty$  values to 0 were applied (**Figure 4**). The Armicarb® treated samples "Arm" are put in column on the left side (in yellow) and the "Ctr" untreated control samples are put on the right side (in green). Within the 2 modalities, the samples are arranged by time sampling from the left (T4t01) to the right (T4t14). On the ordinate axis, the features are represented and sorted following the Euclidean Distances through samples. Inside the Heatmap, the features are coloured according to their relative intensity from 0 in dark blue to the highest intensity in dark red (on a  $\text{Log}_{10}$  scale).



**Figure 4.** Heatmap of Armicarb® xenometabolites abundance (the darker is the red, the higher is the intensity).

“Arm” treated samples from 1 day (dark yellow) to 14 days (light yellow) after treatment, vs. “Ctr” untreated control samples from 1 day (dark green) to 14 days (light green) after treatment.

No specific patterns can be observed. Heatmap visualisation of “Arm” xenometabolome data matrix shows persistence of the detected xenometabolites of the product. To investigate these data further, PCA is used to analyse “Arm” xenometabolome data matrix after a Log<sub>10</sub>-transformation and a Pareto scaling were applied (**Figure 5**). The samples are projected on the 2 most relevant principal components: PC1 and PC2. PC1, explaining 82.7% of the variations, is discriminating the Armicarb® treated samples “Arm” in yellow from the “Ctr” untreated control samples in green all grouped in the “No Residues” point.



**Figure 5.** PCA of Armicarb® xenometabolites: 1 day after the fourth treatment (T4) (T4t01), 7 days after T4 (T4t07), 14 days after T4 (T4t14) (from dark yellow to light yellow, respectively), and the “No Residues” point in green, assembling all “Ctr” samples.

The PCA is not able to discriminate the Armicarb® treated samples by time sampling even if it shows a tendency of the day 14 after T4 (T4t14) samples to head to the “No Residues” point compared with the other samples. In fact, boxplots of one of those features are shown in (Figure S7). They show a degradation tendency pattern and an almost disappearance 14 days after T4. Moreover, the Armicarb® treated samples *vs.* the untreated control samples are compared for each time sampling using OPLS-DA after a Log<sub>10</sub>-transformation and a Pareto scaling were applied to the Armicarb® xenometabolome data matrix (Table S2). The OPLS-DA model is validated for every time sampling (R<sup>2</sup><sub>Y</sub> > R<sup>2</sup><sub>X</sub>, R<sup>2</sup><sub>Y</sub> > Q<sup>2</sup>, R<sup>2</sup><sub>Y</sub> – Q<sup>2</sup> ≤ 30 %, and Q<sup>2</sup> > 50 % (Wiklund, 2008)) but the values decrease from 1 day after T4 (R<sup>2</sup><sub>Y</sub>: 97.20%, Q<sup>2</sup>: 97.20%) to 14 days after T4 (R<sup>2</sup><sub>Y</sub>: 84.20%, Q<sup>2</sup>: 79.10%). These results are concordant with the results previously observed with the PCA (Figure 5) showing a global persistence of the detected Armicarb® xenometabolites with a dissipation tendency observed 14 days after T4.

### 3.3. Chemical Reference

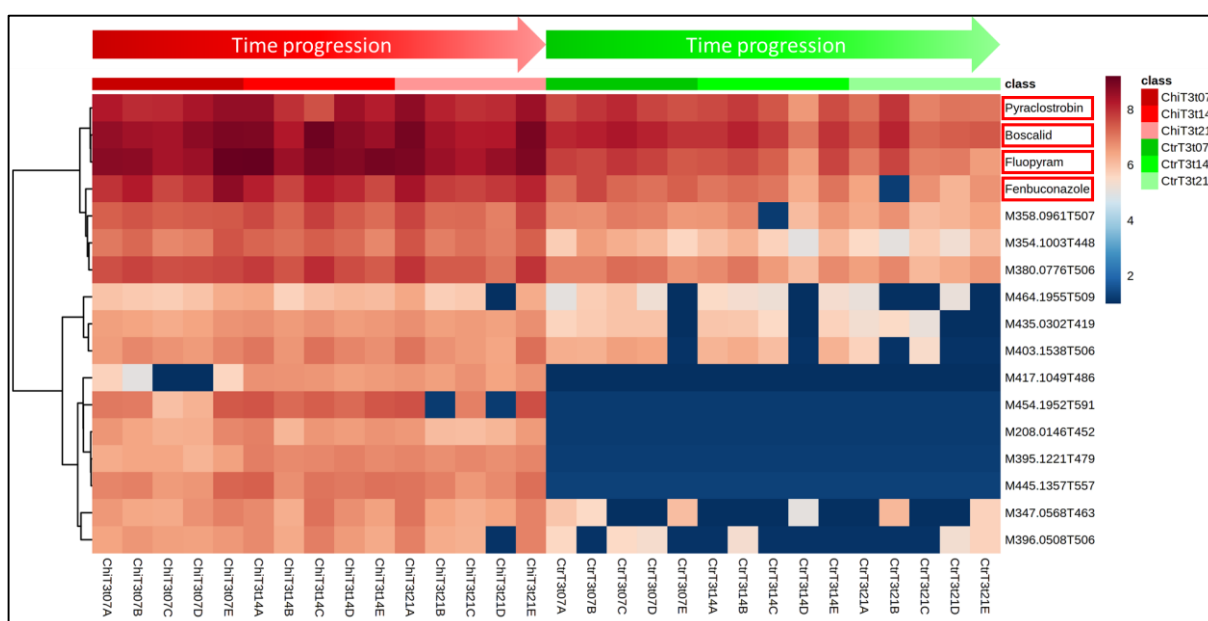
The Chemical reference treatment campaign is composed of 3 different treatments with 3 different products as described on Figure 1. The AS of the 3 different products (5 different molecules in total) are well-known, their chemical structures are presented Figure S1 and their exact masses are summarised in Table 1.

**Table 1.** Exact masses of the active substances of the chemical reference treatment campaign.

Application order	Product	Active Substance (AS)	CAS number	Exact monoisotopic mass [M] (g/mol)	[M+H] <sup>+</sup> (m/z)
1 <sup>st</sup>	Signum® (BASF)	Boscalid	188425-85-6	342.0327	343.0399
		Pyraclostrobin	175013-18-0	387.0986	388.1059
2 <sup>nd</sup>	Kruga® (Dow)	Fenbuconazole	114369-43-6	336.1142	337.1215
3 <sup>rd</sup>	Luna® Experience (Bayer)	Tebuconazole	107534-96-3	307.1451	308.1524
		Fluopyram	658066-35-4	396.0464	397.0537

Considering that the ASs are all chlorinated (Figure S1) and their exact masses are known, they were detected and identified within the xenometabolome data matrix “Chi”, except the Tebuconazole that was detected and identified within the global data matrix only (the matrix acquired before the clean-up process). As the features of those compounds were identified, their presence in the untreated control samples “Ctr” was noticed. These compounds were thus contaminating the “Ctr” samples with a relatively low rate (their intensities are 5-times higher in “Chi” than in “Ctr” (Fold Change (FC) > 5), except for the Tebuconazole (Figure S8) –which explains its disappearance from the data matrix after

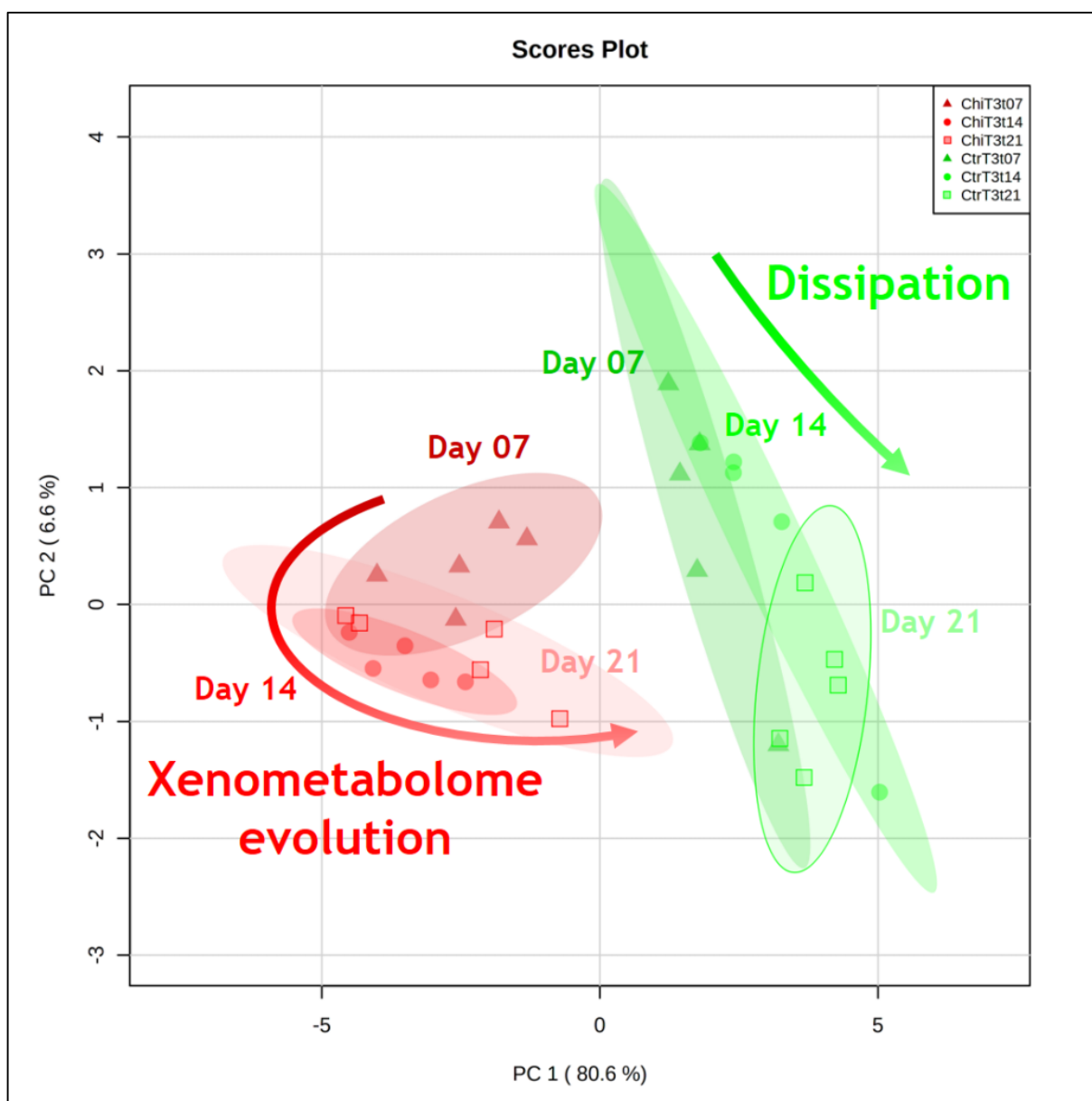
filtering features with  $FC < 5$ ). In order to avoid introducing any bias during the data analysis, the subsequent investigations (statistical analyses) are pursued on the final xenometabolome data matrix “Chi” containing the 4 compounds only (Boscalid, Pyraclostrobin, Fenbuconazole and Fluopyram). Moreover, all the compounds exclusively detected within the chemically treated samples and showing Chlorine isotope Mass Spectrometry patterns with a  $FC > 5$  were considered in the “Chi” xenometabolome data matrix. Therefore, in this part of the results, the “No Residues” point is not an overall point of untreated control samples as it considers the presence of the contaminations. It will be thus referred as “contaminated untreated control samples” (“Ctr”).



**Figure 6.** Heatmap of Chemical reference xenometabolites abundance (the darker is the red, the higher is the intensity): “Chi” treated samples from 7 days (dark red) to 21 days (light red) after treatment, vs. “Ctr” untreated control samples from 7 days (dark green) to 21 days (light green) after treatment. Identified ASs’ molecular traces are circled in red.



All MS spectra with Chlorine isotopic patterns belonging to the products' xenometabolome are summarised in **Figure S9**. The  $m/z$  peaks of the principal ions “[M+H]<sup>+</sup><sub>35</sub>Cl” and their “[M+H]<sup>+</sup><sub>37</sub>Cl” and “[M+H]<sup>+</sup><sub>37</sub>Cl<sub>2</sub>”  $m/z$  peaks are circled in red. Chemical final xenometabolome data matrix is visualised on a Heatmap after a Log<sub>10</sub>-transformation and a conversion of  $-\infty$  values to 0 were applied (**Figure 6**). The chemically treated samples “Chi” are put in column on the left side (in red) and the untreated control samples “Ctr” are put on the right side (in green). Within the 2 modalities, the samples are arranged by time sampling from the left (7 days after T3) to the right (21 days after T3). On the ordinate axis, the features are represented and sorted following the Euclidean Distances through samples. Inside the Heatmap, the features are coloured according to their relative intensity from 0 in dark blue to the highest intensity in dark red (on a Log<sub>10</sub> scale). Concerning the 4 identified compounds of the ASs, they are circled in red on the Heatmap and they present a persistent pattern along the kinetics, with a higher intensity level within the chemically treated samples “Chi”, as well as within the contaminated untreated control samples “Ctr”, if compared to the other features. Thus, Heatmap visualisation of “Chi” xenometabolome data matrix shows a persistence of the chemical xenometabolites. To investigate the data further, PCA is used to analyse “Chi” xenometabolome data matrix after a Log<sub>10</sub>-transformation and a Pareto scaling were applied (**Figure 7**). The samples are projected on the 2 most relevant principal components: PC1 and PC2. PC1 explaining 80.6% of the variations is discriminating the chemically treated samples “Chi” in red from the contaminated untreated control samples “Ctr” in green. PC2 explaining 6.6% of the variations discriminates the heterogeneous contamination within the untreated control samples “Ctr”. It is also discriminating the chemically treated samples 7 days after T3 (T3t07) from the samples 14 days and 21 days after T3 (T3t14 and T3t21, respectively).



**Figure 7.** PCA of the Chemical reference xenometabolites degradation kinetics: 7 days after the third treatment (T3) (T3t07), 14 days after T3 (T3t14), 21 days after T3 (T3t21) (from dark red to light red, respectively), and the contaminated untreated control (corresponding to T3t07, T3t14 and T3t21, from dark green to light green, respectively).

To investigate and understand the discriminations on the PC2, the Biplot of the PCA is observed (**Figure S10**). The Biplot mainly highlights 4 features: 2 on the top of the Biplot correlating with the contaminated control samples 7 days and 14 days after T3, and 2 on the bottom of the Biplot correlating with the contaminated control samples 21 days after T3 and also the chemically treated samples 14 days and 21 days after T3. Boxplots of these 4 features are shown in **Figure S10**. Boxplots “A” and “B” of the features from the top of the Biplot (**Figure S10**) present, on one hand, high relative intensity levels within the treated samples “Chi” that persist through the time. However, within the contaminated untreated control samples “Ctr”, they generally show lower intensity levels when compared to the “Chi” treated

samples at all the time points. Moreover, a dissipation pattern is observed through the time in those control samples, with a nearly complete dissipation 21 days after T3. On the other hand, boxplots “C” and “D” of the features from the bottom of the Biplot (**Figure S10**) show significantly low levels of contamination in the untreated control samples “Ctr” (nearly at the limit of the background noise or with intensities equal to 0). Concerning the chemically treated samples “Chi”, boxplots show a persistence along all the kinetics tracking for the feature “M347.0568T463” (D) (**Figure S10**). For the other feature “M417.1049T486” (C), boxplots (**Figure S10**) show an appearance kinetics from 7 days to 14 days after T3 and a persistence from 14 days to 21 days after T3. These results showed that PCA is a tool that permit monitoring features through the kinetics. PCA is able to reveal a dissipation tendency of the contaminant xenometabolite features within the untreated control samples from 7 days after T3 to 21 days after T3. Moreover, PCA is able to discriminate the chemically treated samples 7 days after T3 from the 14 days and 21 days after T3, which can be explained by the appearance of some by-product patterns 14 days after T3 and persisting at the day 21 after T3 (with a slight tendency to head to the contaminated untreated control samples). These results are concordant with the OPLS-DA comparing the chemically treated samples *vs.* the contaminated untreated control samples after a Log<sub>10</sub>-transformation and a Pareto scaling were applied on the datasets (**Table S3**). The OPLS-DA is validated for every time sampling ( $R^2Y > R^2X$ ,  $R^2Y > Q^2$ ,  $R^2Y - Q^2 \leq 30\%$ , and  $Q^2 > 50\%$  (Wiklund, 2008)). The values increase from 7 days ( $R^2Y$ : 88.20%,  $Q^2$ : 85.10%) to 14 days after T3 ( $R^2Y$ : 91.00%,  $Q^2$ : 89.90%) and slightly decreases from 14 days to 21 days after T3 ( $R^2Y$ : 91.00%,  $Q^2$ : 87.70%).

In order to quantify the 5 ASs (Boscalid, Pyraclostrobin, Fenbuconazole, Fluopyram and Tebuconazole) within the samples (“Ctr” and “Chi”) 21 days after the last Chemical reference treatment, standard addition method was applied. Calibration curves were drawn for each of the 5 chlorinated ASs integrating the areas of the “[M+H]<sup>+</sup><sub>-35Cl</sub>” ion and comparing them with those of the “[M+H]<sup>+</sup><sub>-37Cl</sub>” ion. The results were consistent for the 2 types of ions and all calibration curves had a calculated  $R^2 > 0.99$  (**Figure S11**). The results are summarized in **Table 2**. 21 days after the last treatment, all the 5 compounds could be quantified. The values were obtained per gram of peach peel and converted to per gram of peach (fresh mass) (**Formula S1**). This “conversion” was done in order to compare the results with the thresholds considered by the E.U. regulation authorities as limit of quantification, and No Residue threshold (European Parliament and Council Of The European Union, 2005). This limit is defined as 10 ng/g of fresh mass. Most of the measured concentrations were upper than this threshold within the “Chi” samples (**Table 2**). Thus, 21 days after their application, ASs show persistence as they could be quantified within all the samples. 3 of the ASs (*i.e.* Pyraclostrobin, Boscalid, and Fluopyram) showed a high persistence within the peach peels of

the treated samples, with concentrations between 2-times and 10-times higher than the No Residue threshold of 10 ng/g of fresh mass. The results obtained for the quantification are in agreement with the observations made previously with the statistical analyses: the 4 ASs (Boscalid, Pyraclostrobin, Fenbuconazole and Fluopyram) are more concentrated within the “Chi” samples than in the “Ctr” samples. Within “Ctr” samples, most of the ASs are lower than the No Residue threshold, except Boscalid that is higher with 23.68 ng/g of fresh mass. For Tebuconazole, the same concentrations were more or less obtained for control and treated samples (taking into account the SD). Its peach fresh mass concentration is lower than the No Residue threshold settled by regulation authorities (10ng/g of fresh mass): 0.52 ng/g for “Ctr” samples and 0.18 ng/g for treated “Chi” samples. However, it should be mentioned that an important field samples variability could be underlined by the relatively high SD values (**Table 2**).

**Table 2.** Concentration of Chemical reference active substances measured within the untreated control samples “Ctr” and the Chemical reference treated samples “Chi” 21 days after the last treatment (means between 3 biological replicates).

*The concentrations are expressed in ng/g of dried peach peel and in ng/g of peach fresh mass. Means above 10 ng/g of fresh mass are colored in orange.*

*a: ng/g of dried peach peel; b: ng/g of peach fresh mass.*

Compound	Residues "Ctr" (ng/g) <sup>a</sup>	SD (ng/g) <sup>a</sup>	Residues "Chi" (ng/g) <sup>a</sup>	SD (ng/g) <sup>a</sup>	Residues "Ctr" (ng/g) <sup>b</sup>	Residues "Chi" (ng/g) <sup>b</sup>
<i>Pyraclostrobin</i>	138.25	90.51	681.48	185.04	4.60	22.69
<i>Boscalid</i>	711.23	470.29	3072.83	1550.44	23.68	102.33
<i>Fenbuconazole</i>	29.63	20.53	266.67	153.96	0.98	8.88
<i>Fluopyram</i>	118.52	59.29	1481.48	1068.33	3.95	49.33
<i>Tebuconazole</i>	15.76	9.06	5.27	0.74	0.52	0.18

## 4. DISCUSSION

According to the results described above, EMF approach applied to peach peel matrix seems to be suitable to study the fate of botanical extracts like Akivi. In fact, Akivi's xenometabolites were detected, separated from the peach endometabolites, and then tracked through time without the need for their identification at this stage of the study. Akivi's xenometabolites evolution showed a clear dissipation kinetics along the samplings time points. Moreover, the statistical analyses allowed the observation of different xenometabolites patterns: features from the original Akivi BP more or less persistent, and degradation by-products. Hence, the EMF seems to be a reliable approach to study the fate of complex BPs with a partially or completely unknown biochemical composition. It also allows for the post-analysis filtration of the xenometabolome from the entire complex meta-metabolome, in order to provide a clear fate tracking by using different statistical approaches.

In the case of Armicarb® BP mineral extract, the analytical method used in this study (particularly, the use of the C18 Reverse-Phase LC (RPLC) column) is not adequate to detect its mineral AS ( $\text{KHCO}_3$ ) due to its high solubility in water. Probably, the potential development of some relatively adapted analytical methods in the future (*e.g.* those based on Ion Chromatography) may allow such an untargeted approach to study the fate of such compound families. Nonetheless, despite being unable to detect the AS *per se*, the EMF was able to discriminate between the treated and the untreated samples by detecting certain of Armicarb®'s xenometabolites. Most probably, those xenometabolites are the co-formulants and adjuvants of the formulated product that represent 15% (m/m) of its composition. They were persistent all over the 14 days of the kinetics study. Therefore, the ability of the EMF to assess the fate of PPP formulation compounds in the crop or in the environment represents an important plus-value that might allow identifying a treated/polluted group of samples *via* the detection of PPPs' formulation ingredients, especially that those compounds usually represent a significant percentage of the total composition of the formulated product.

Concerning the Chemical reference treatment, the EMF approach was able to detect the 4 molecules pertaining to the ASs' of the 3 Chemical reference PPPs and some by-products features. It was thus able to discriminate between the treated and the untreated samples, despite the contamination of the untreated control samples by the applied PPPs (this contamination was identified because chlorinated compounds are not reported in peach endometabolome so far). The discrimination was feasible thanks to comparative semi-quantitative analysis of the EMF that takes in consideration the difference of PPP's components quantities between the two compared samples (treated *vs.* untreated). In the current case, the abundances of the PPP's AS were significantly higher in the treated samples.

Furthermore, results analysis was able to reveal a potential dose-effect on the degradation kinetics. In fact, for the treated samples, where the quantity of the AS is significantly higher, a persistence pattern through time was observed for AS's compounds. On the other hand, in the contaminated control samples, where the quantity of the AS's compounds is relatively low, degradation patterns through time could be observed. Nevertheless, the contamination of control samples by the PPPs is still a significant issue for the EMF-based studies. In fact, the untreated control samples are taken as a basis to select the xenometabolome. In addition, the untreated control samples represent the "No Residues" point that must be reached in order to determine the "dissipation interval".

From all the described results, we can note that at the pre-harvest interval (PHI) of the 3 products that is set to 3 days, the residues dissipation is not reached neither for AS and co-formulants nor for by-products. However, it is worth to mention that the analyses were conducted using a high-resolution mass spectrometer that is able to detect molecular features with relatively high selectivity and sensitivity. This system allowed detecting the persistence of xenometabolites features at the last sampling point. These xenometabolites are detected with high Signal-to-Noise (S/N) over the Limit Of Quantification (LOQ:  $S/N > 10$ ), so the concentration of the features could be considered measurable. For instance, the 4 chlorinated compounds of the 3 Chemical reference products are still detected 21 days after the last treatment with a  $S/N > 10000$ , *i.e.* significantly higher than the LOQ. These compounds were all quantified and three of them (Pyraclostrobin, Boscalid, Fluopyram) presented concentrations above the No Residue threshold settled by regulation authorities (10ng/g of fresh mass) (European Parliament and Council Of The European Union, 2005). This quantification study confirms that at  $t = 21$  days after the last treatment, the xenometabolome was not dissipated. The quantification results are in agreement with those obtained with our developed untargeted metabolomics approach. The methodology optimised in this work is highly sensitive and seems to be suitable to monitor the xenometabolome fate after the treatment of fruit matrix.

The novelty of this work is that it was conducted in the field, in contrast to the previous studies that were previously carried out in laboratory microcosms (Patil *et al.*, 2016; Salvia *et al.*, 2018). The current study was therefore confronted with some more difficulties that are important and must be taken into account. The first point, already mentioned, was the contamination of the untreated samples. This contamination issue could be hypothetically explained as the following: during the field experiments, the BPs and the Chemical reference were manually sprayed on the peach trees. Even if it was cautiously conducted, the spraying was directed to the top of the tree and some spray drift cannot be totally avoided. The cautious sampling method (**Figure S2**) was not sufficient to prevent the fruits from being

contaminated. For further studies, a better protection of the untreated control trees must be discussed, as isolating some untreated control trees on a corner of the orchard to decrease spray drift risks and take fruits from those control trees for residue monitoring. Spray drift may have occurred for all the studied treatments. For the chemical reference, the contamination of the samples (and in particular the untreated control samples “Ctr”) was underlined thanks to the known chlorinated Chemical reference AS and the MS isotopic patterns of the chlorinated compounds (as no chlorinated compound are produced by the peach itself). However, a contamination by the Akivi cannot be verified as it is a natural extract and it is difficult to discriminate between its metabolites and the metabolites produced by the peach itself. Thus, this study was able to highlight that the spray drift is still an important phenomenon that can occur in field condition and must be taken into account in the future studies, especially as it can cause a serious problem for the untargeted metabolomics-based EMF approach.

Besides, working with biological samples always induces variability due to the multi-factor differences between plants, trees, leaves or fruits. In field conditions, the variability increases significantly because the soil is slightly different within the plot. Moreover, the trees receive a heterogeneous quantity of light, rain and wind compared to experiments in controlled conditions. In addition, focusing on this study, the fruits received a heterogeneous quantity of treatment because leaves around the fruit may hide part of the fruit and only the parts of the fruit exposed to the outside of the tree were treated. To reduce variability during the sampling, every sample is composed of the peel of 3 peaches and 5 repetitions are made for each time sampling. However, when studying the xenometabolome, an important variability appeared among the repetitions, which may mask some information. It appeared in particular on the Heatmaps (**Figure 2**, **Figure 4**, and **Figure 6**) where some features had already disappeared in some repetition on a sampling time point and the same features were detected in the next sampling time point. It can be explained by the heterogeneous exposure of the fruits to the treatment but also to light, wind, and rain that could cause a differential dissipation of the compounds between the repetitions of samples. For future field experiment, it would be interesting to collect more samples repetitions and include more fruits in the repetitions in order to limit variability between the biological repetitions.

All these points are highly important and it is interesting to consider them. They must be in-depth investigated in order to improve the field experimentations.

## **5. CONCLUSION**

The current study aimed to adapt the EMF approach to fruit matrices and to target the xenometabolome in order to investigate the fate of BPs and the dissipation of their residues within treated crops. The EMF allowed to isolate post-analytically the xenometabolome from the total complex meta-metabolome and proved its ability to monitor the concentration evolution of the different components of the formulated PPPs (BPs and chemical PPP as well) within the studied matrix over the time. To conclude, no complete residues dissipation was reached for all the 3 studied treatments during the experiments that were carried out. The approach was proven reliable. Nonetheless, the experimental design should be improved for the future studies in order to avoid the contamination of the untreated control samples by spray drift during field treatment. Moreover, the sampling strategy should be improved in order to bypass the field-linked physical and biochemical variations.



## **AUTHOR CONTRIBUTIONS**

**Mélina Ramos:** Conceptualisation, Investigation, Data processing, Visualisation, Writing – Original draft, **Hikmat Ghosson:** Investigation, Data processing, Visualisation, Writing – Original draft, **Delphine Raviglione:** Resources, **Cédric Bertrand:** Conceptualisation, Writing – Reviewing and Editing, **Marie-Virginie Salvia:** Conceptualisation, Writing – Reviewing and Editing.

## **DECLARATION OF COMPETING INTEREST**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## **ACKNOWLEDGEMENTS**

We are grateful towards Aude Lusetti from the Sica CENTREX who helped us for peach samplings and storage of the samples. We are thankful towards Vanessa Andreu from S.A.S. AkiNaO who helped us on the early stage of analysis optimisation with her expertise on Akivi BP chemical characteristics. The LC-Q/Orbitrap method development and analyses were performed using the Biodiversité et Biotechnologies Marines (Bio2Mar) facilities – Métabolites Secondaires Xénobiotiques Métabolomique Environnementale (MSXM) platform at the Université de Perpignan Via Domitia (<http://bio2mar.obs-banyuls.fr/>). This work was supported by PALVIP project that has been 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain France Andorra programme ([POCTEFA 2014-2020](#)). POCTEFA aims to reinforce the economic and social integration of the French-Spanish-Andorran borders. Its support is focused on developing economic, social and environmental cross-border activities through joint strategies favouring sustainable territorial development.

Appendix A. Supplementary data for:

**UNTARGETED METABOLOMICS AS A TOOL TO MONITOR  
BIOCONTROL PRODUCT RESIDUES' FATE ON FIELD-  
TREATED *PRUNUS PERSICA*.**

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**Table S1.** OPLSDA cross-validation and permutation test results for each sampling time point comparing Akivi treated samples “Aki” *versus* Untreated Control samples “Ctr”.

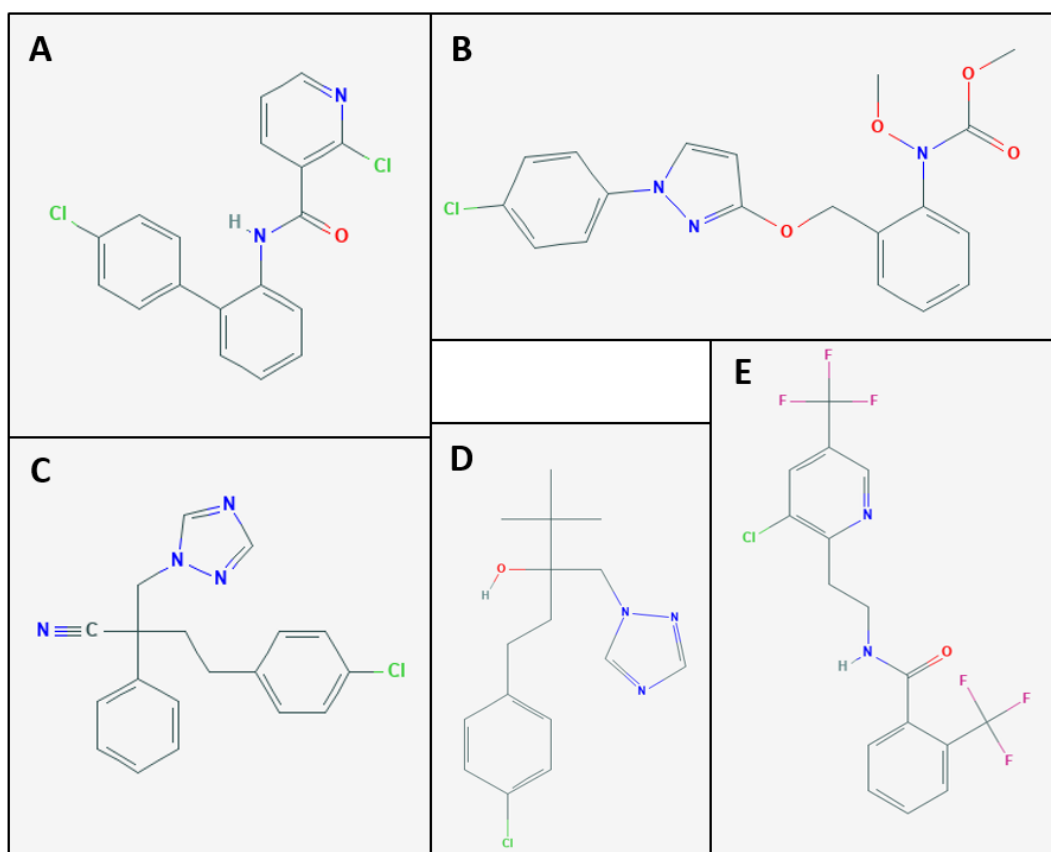
	p1			o1			Permutation test	
	R2X	R2Y	Q2	R2X	R2Y	Q2	R2Y	Q2
t01	94.0%	97.2%	96.7%	3.8%	2.4%	1.6%	1.3%	1.3%
t07	87.7%	96.5%	95.6%	5.8%	3.4%	1.1%	0.4%	0.4%
t14	69.0%	89.9%	87.8%	12.1%	9.6%	2.0%	0.7%	0.7%

**Table S2.** OPLSDA cross-validation and permutation test results for each sampling time point comparing Armicarb® treated samples “Arm” *versus* Untreated Control samples “Ctr”.

	p1			o1			Permutation test	
	R2X	R2Y	Q2	R2X	R2Y	Q2	R2Y	Q2
t01	85.3%	97.2%	97.2%	13.3%	1.6%	(-)1.4%	0.6%	0.6%
t07	80.3%	91.8%	91.3%	10.2%	1.5%	(-)17.5%	1.0%	1.0%
t14	65.6%	84.2%	79.1%	21.3%	11.8%	9.6%	0.8%	0.8%

**Table S3.** OPLSDA cross-validation and permutation test results for each sampling time point comparing Chemical reference treated samples “Chi” *versus* Untreated Control samples “Ctr”.

	p1			o1			Permutation test	
	R2X	R2Y	Q2	R2X	R2Y	Q2	R2Y	Q2
t07	71.7%	88.2%	85.1%	14.8%	8.2%	7.5%	0.7%	0.7%
t14	81.0%	91.0%	89.9%	14.1%	7.5%	7.6%	1.0%	1.0%
t21	78.8%	91.0%	87.7%	11.7%	6.9%	6.9%	0.6%	0.6%

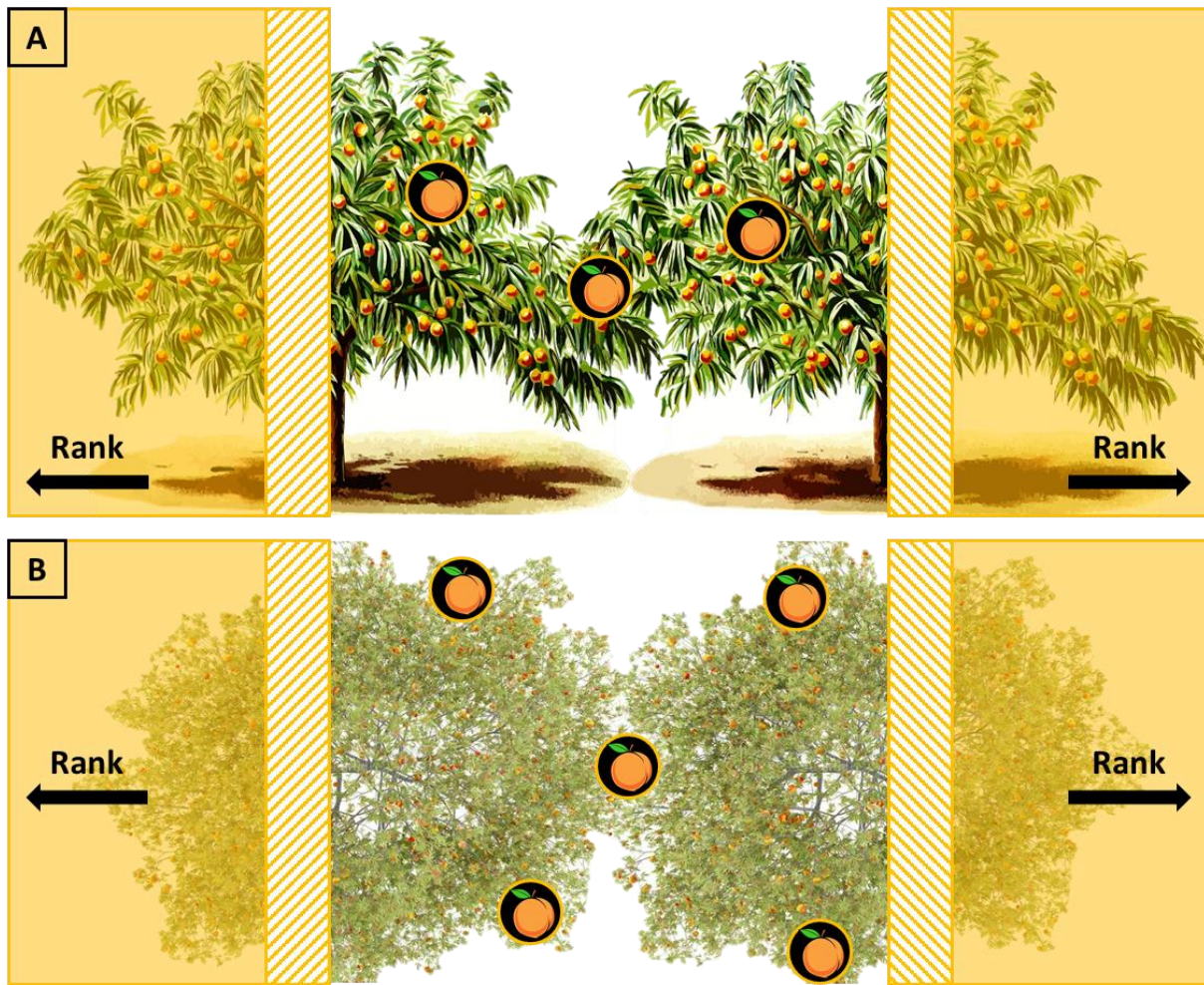


**Figure S1.** Chemical structure of the Chemical reference AS. **A:** Boscalid; **B:** Pyraclostrobin; **C:** Fenbuconazole; **D:** Tebuconazole; **E:** Fluopyram.

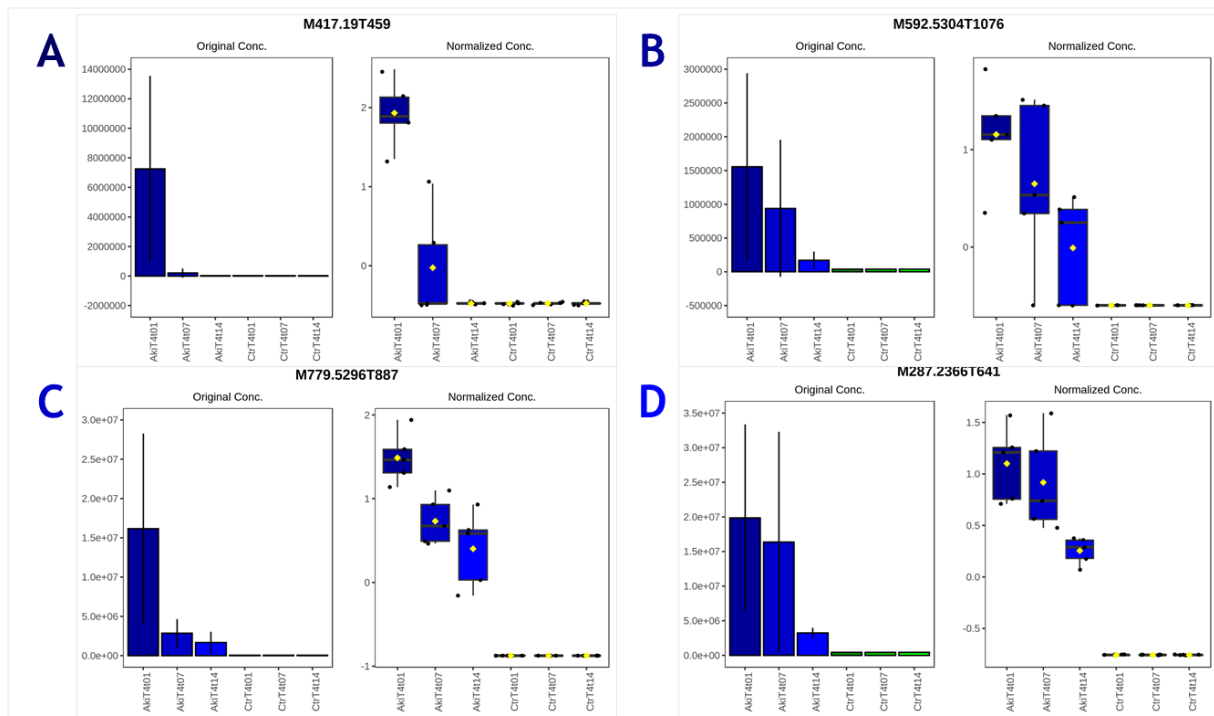
(**A&B**) are the AS of the first treatment (T1) with Signum®; (**C**) is the AS of the second treatment (T2) with Kruga®; and (**D&E**) are the AS of the third treatment (T3) with Luna® Experience. for the Chemical reference.

			X		X		X
Grey zone	X		X		X		X
	X	"Aki"_2	X		X		X
	X		X		X	"Arm"_3	X
	X		X		X		X
	/		X	"Arm"_2	X		X
	/		X		X		X
	X		X		X		X
	X		X		X		X
	X		X		X	"Aki"_3	X
	X		X	"Chi"_2	X		X
	X		X		X		X
	X	"Ctr"_1	X		X		X
	"Aki"_1	X		X		X	
	O		X		X		X
"Arm"_1	X		X	"Ctr"_2	X		X
	X		X		X		X
	X		X		X		X
	X		X		X		X
Grey zone	X		X		X	"Ctr"_3	X
	O	"Chi"_1	X		X		O
	/		X		X		X
	X		X		X		X
	X		X		X	"Chi"_3	X
	X		X		X		X
	X		X		X		X

**Figure S2.** Peach orchard field experiment. Trees that can be used for the experiment are marked by an 'x', absent trees are marked by an '/', and young trees are marked by an 'o'. Grey zones are untreated; green zones represents the 3 "Ctr" replicates of 3 trees; red zones represents the 3 "Chi" replicates of 3 trees; yellow zones represent the 3 "Arm" replicates of 2 trees; blue zones represent the "Aki" replicates of 2 trees; and purples zones represent the trees treated with additional treatments unstudied in this work.



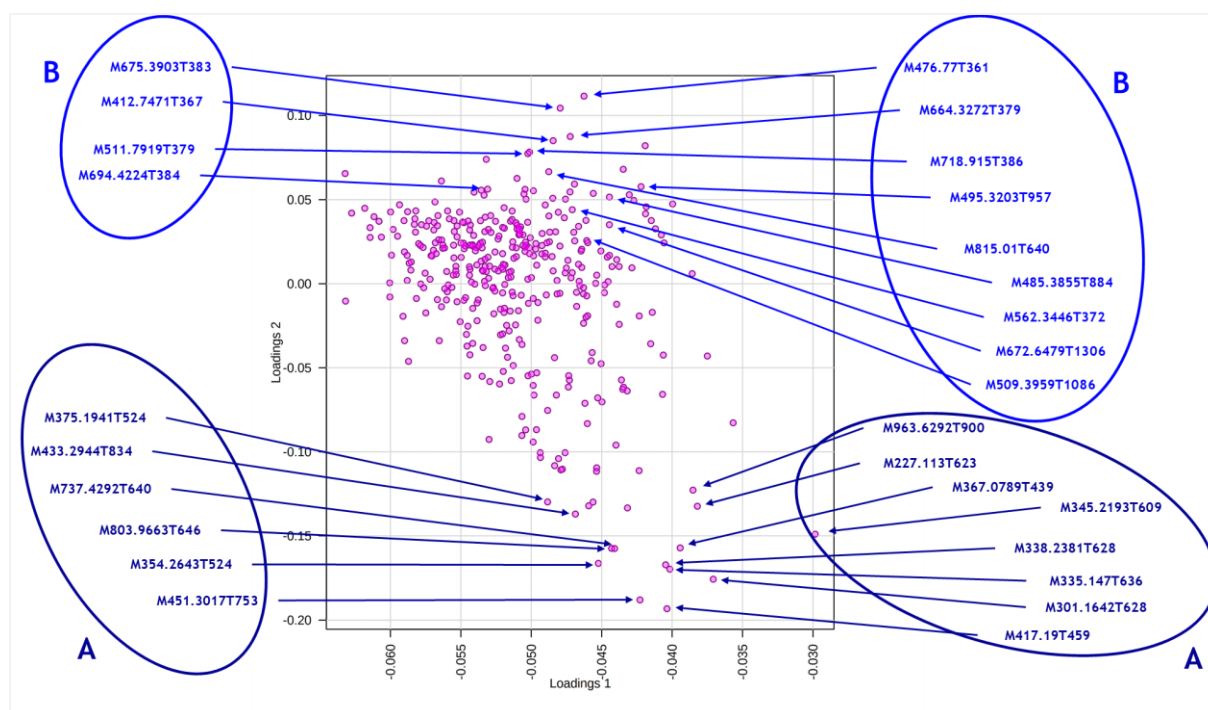
**Figure S3.** Field sampling method, on lateral view (A), and on aerial view (B). Sampling is made between the peach trees trunks marked by the yellow strips. The yellow boxes marks the peach tree parts that are not sampled. 5 peaches are sampled by treatment repetition modality, 2 peaches on each side of the rank and 1 peach inside the foliage to represent all the position possibilities.



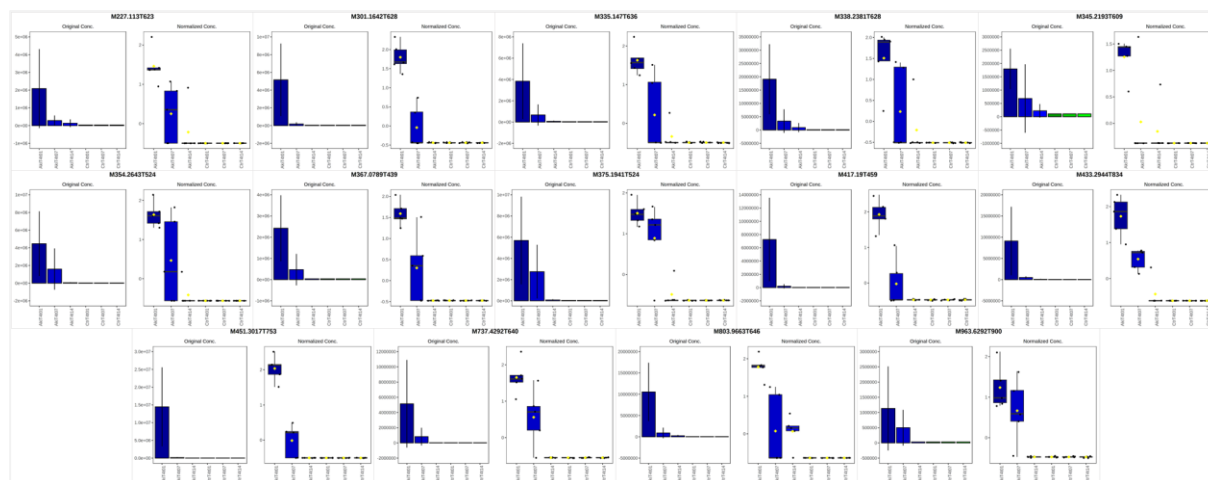
**Figure S4.** Boxplots of the 4 Akivi xenometabolites features representing the 4 different degradation kinetics patterns (A, B, C, D) identified on the heatmap (**Fig. 2**).

For each feature, boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the “Aki” treated samples collected 1 day after the fourth treatment (T4), 7 days after T4, 14 days after T4 are represented from dark blue to light blue, and boxplots of the “Ctr” samples are represented in green, they are all at an intensity equal to 0 (“No Residue” point).





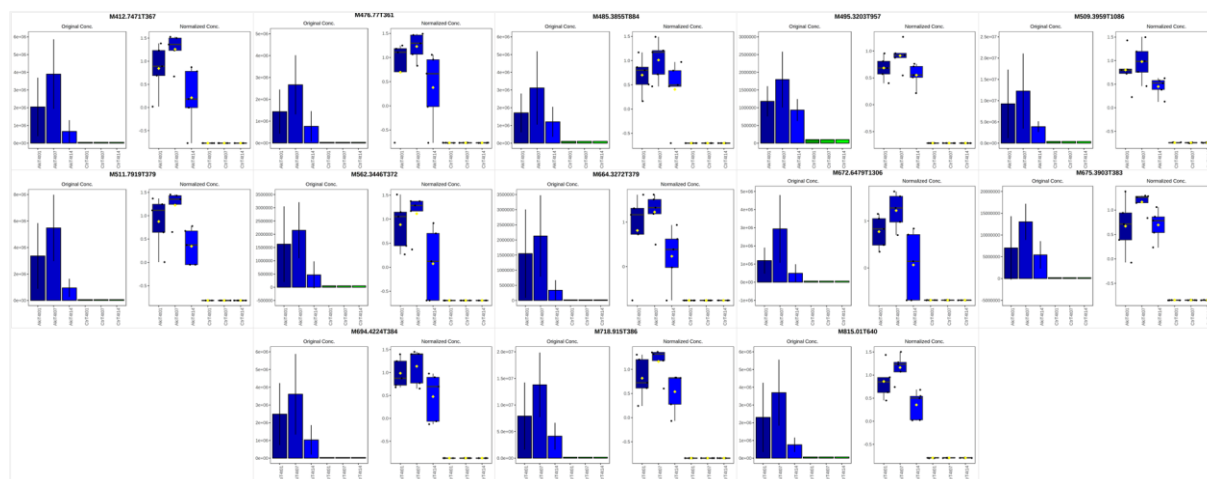
**Figure S5.** Loadings plots of the PCA (**Figure 3**). **A:** Akivi xenometabolites features circled in dark blue are most intense in “Aki” samples 1 day after T4. **B:** Akivi xenometabolites features circled in light blue are most intense in “Aki” samples 7 days after T4.



**Figure S6A.** Boxplots of Akivi xenometabolites features most intense in “Aki” samples 1 day after T4 corresponding to **Figure S5 “A”**.

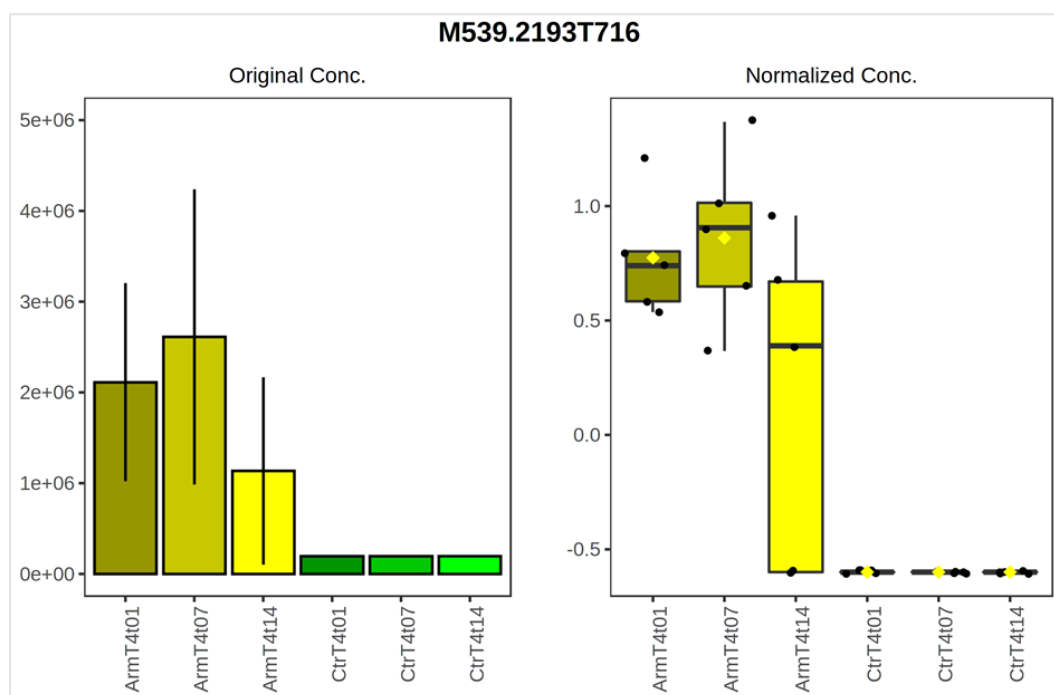
For each feature, boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the “Aki” treated samples collected 1 day after the fourth treatment (T4), 7 days after T4, 14 days after T4 are represented from dark blue to light blue, and boxplots of the “Ctr” samples are represented in green, they are all at an intensity equal to 0 (“No Residue” point).





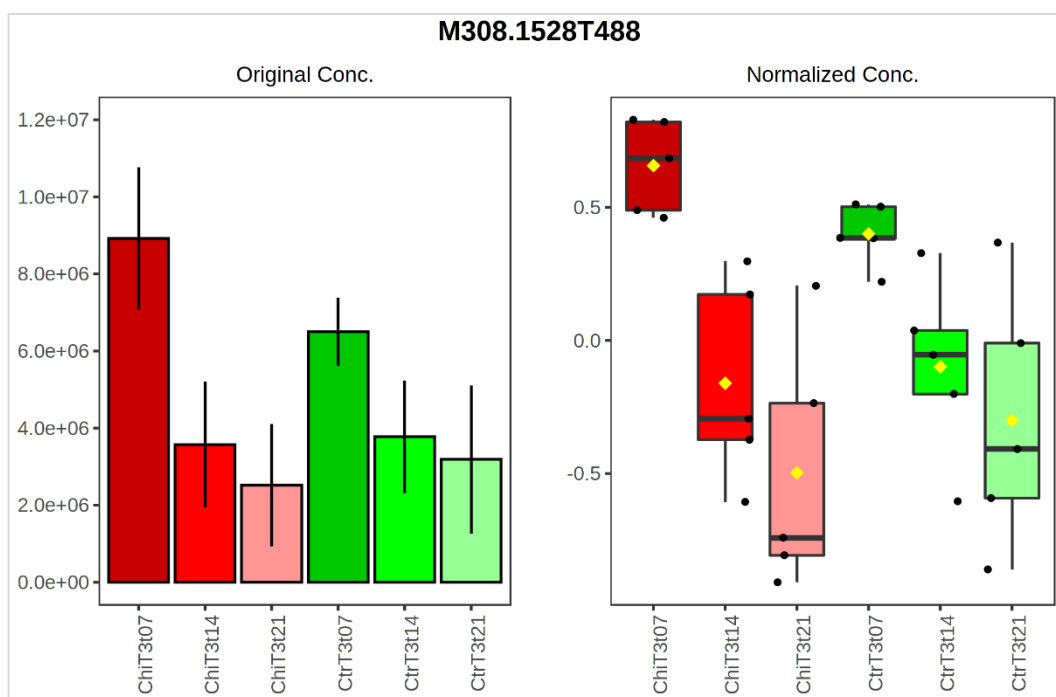
**Figure S6B.** Boxplots of Akivi xenometabolites features most intense in “Aki” samples 7 days after T4 corresponding to **Figure S5 “B”**.

For each feature, boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the “Aki” treated samples collected 1 day after the fourth treatment (T4), 7 days after T4, 14 days after T4 are represented from dark blue to light blue, and boxplots of the “Ctr” samples are represented in green, they are all at an intensity equal to 0 (“No Residue” point).



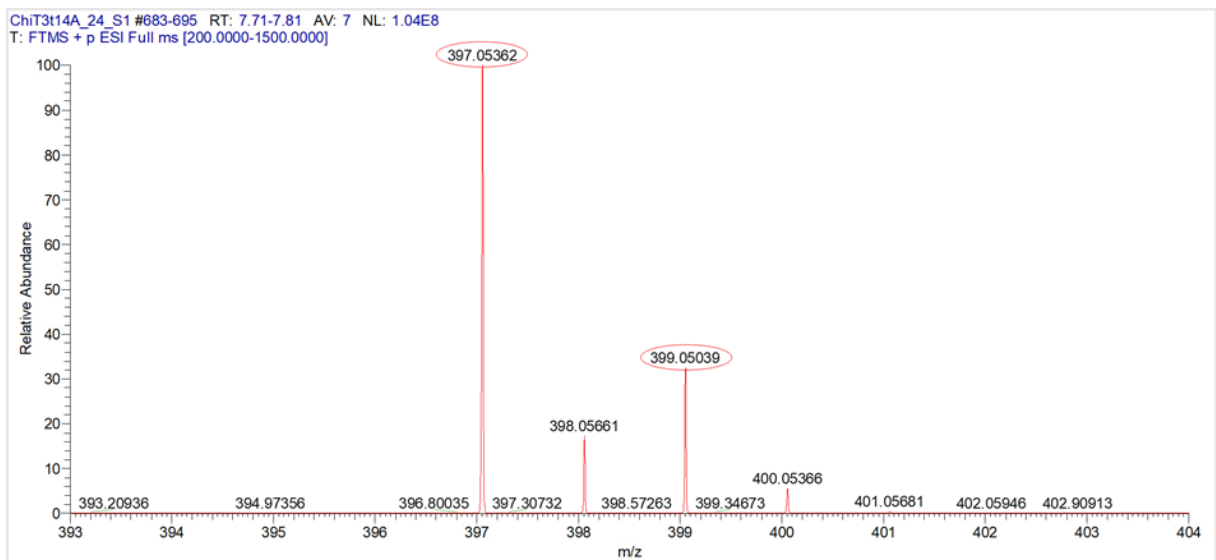
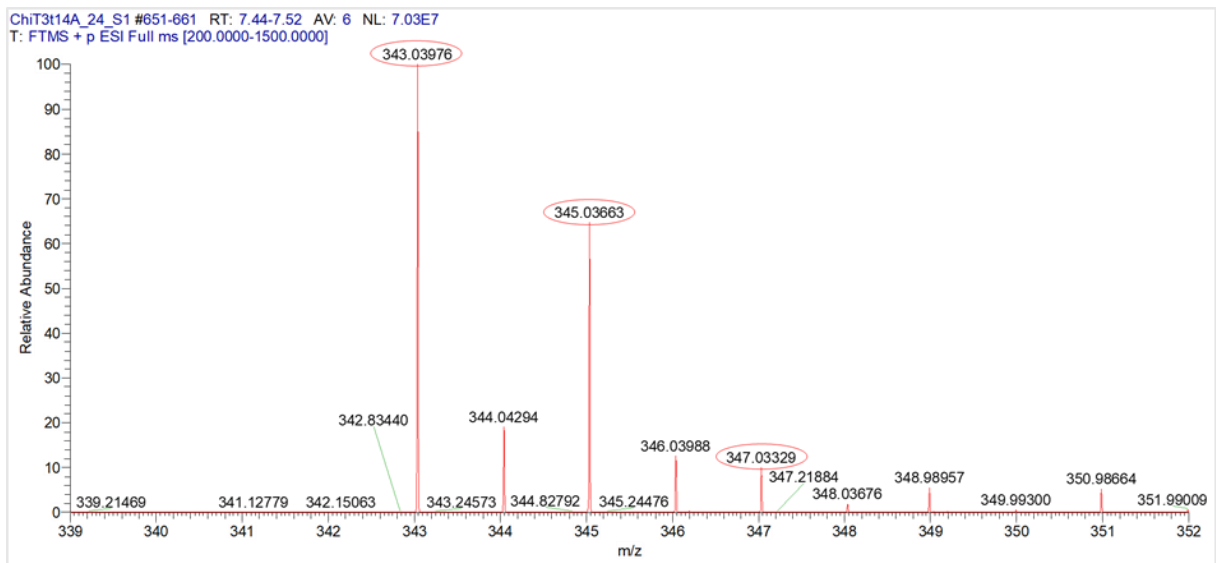
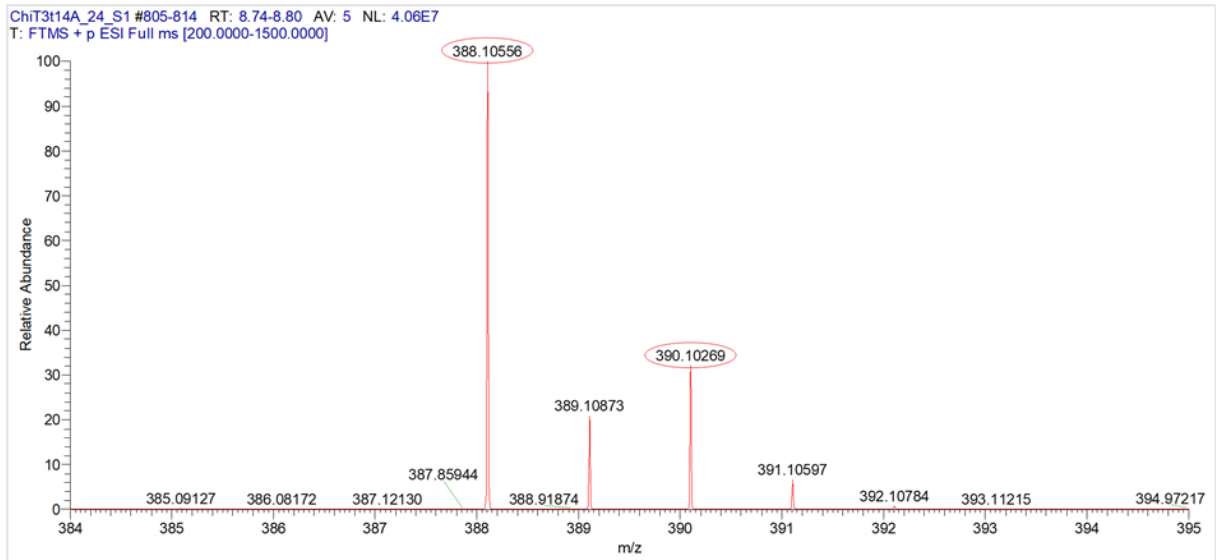
**Figure S7.** Boxplots of Armicarb® xenometabolite feature “Arm” showing a degradation tendency along the sampling time points.

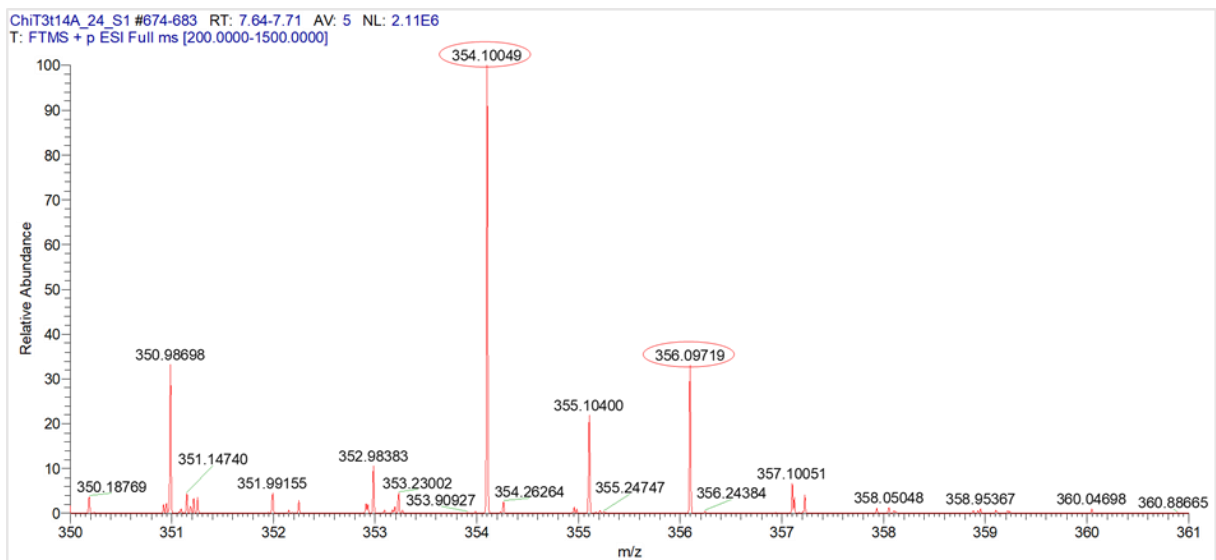
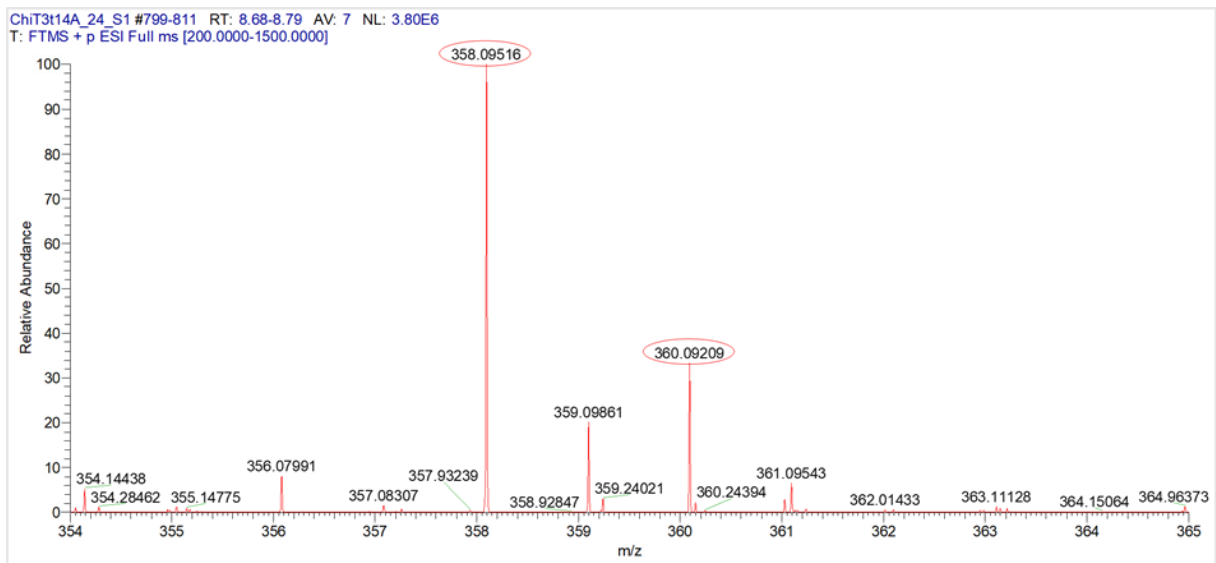
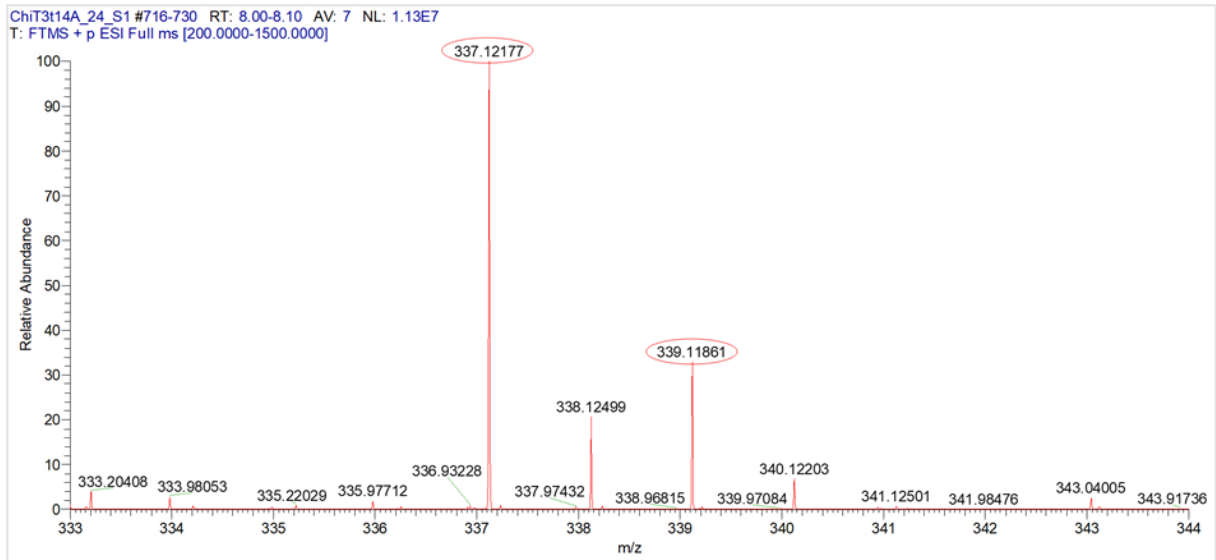
Boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the “Arm” treated samples collected 1 day after the fourth treatment (T4), 7 days after T4, 14 days after T4 are represented from dark yellow to light yellow, and boxplots of the “Ctr” samples are represented in green, they are all at an intensity equal to 0 (“No Residue” point).

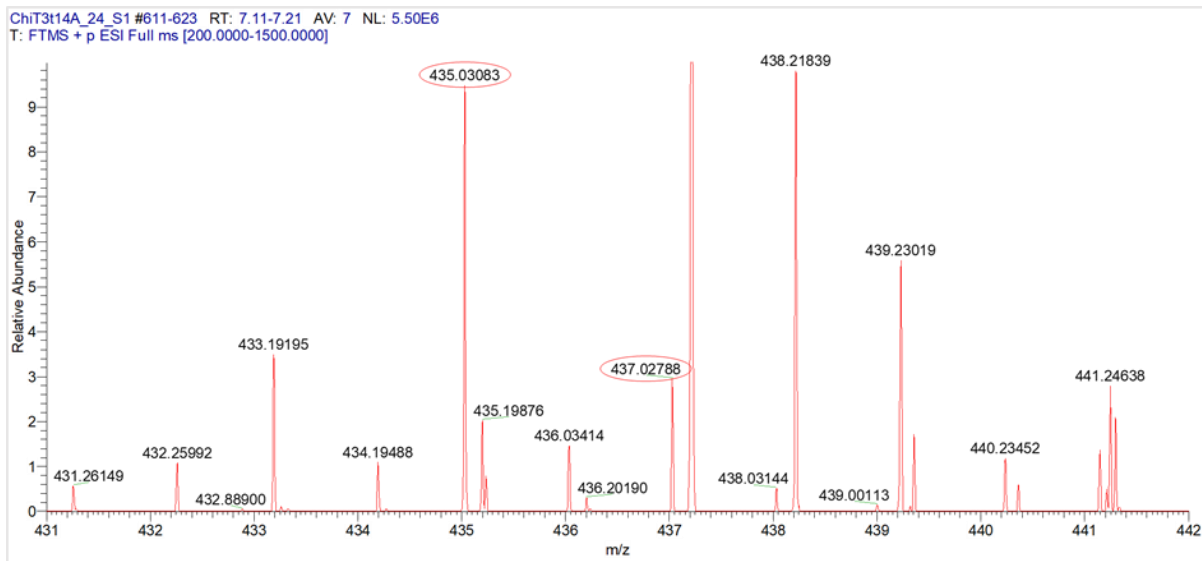
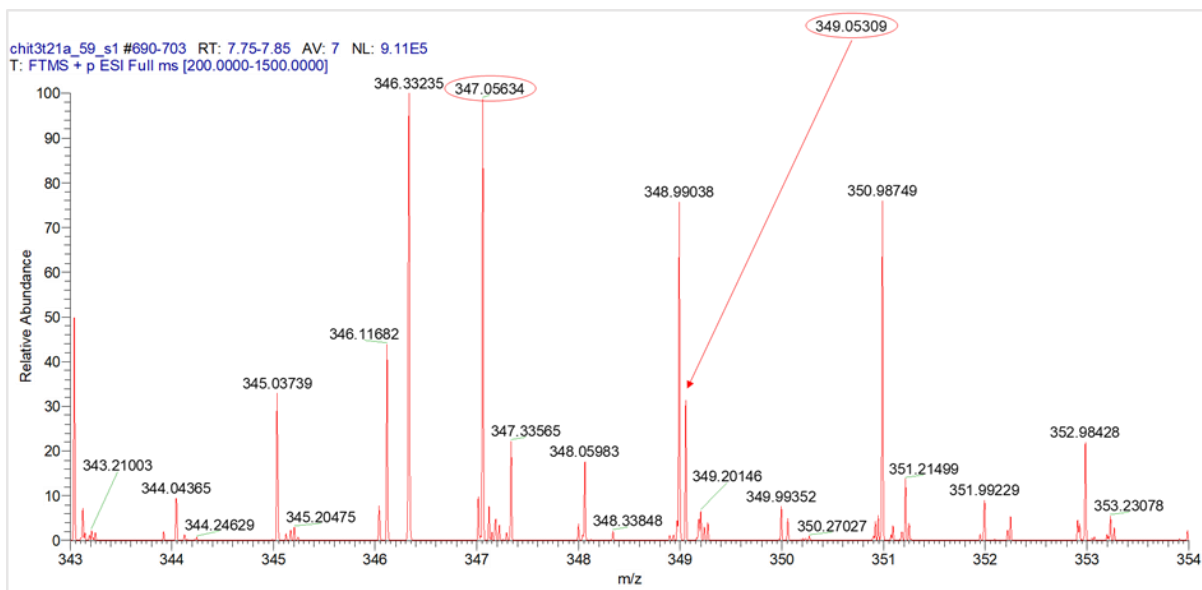
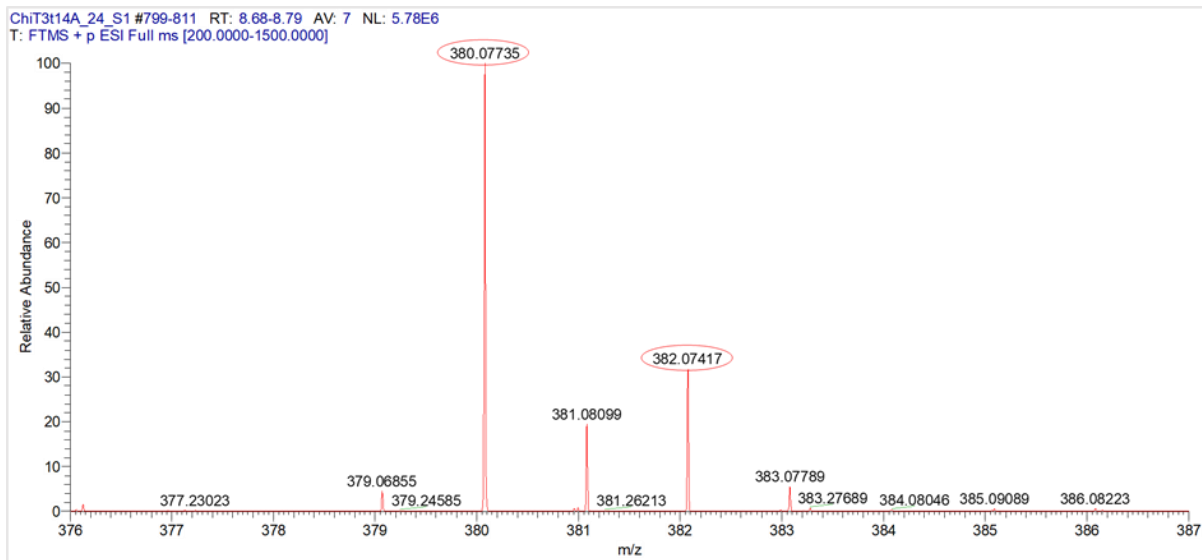


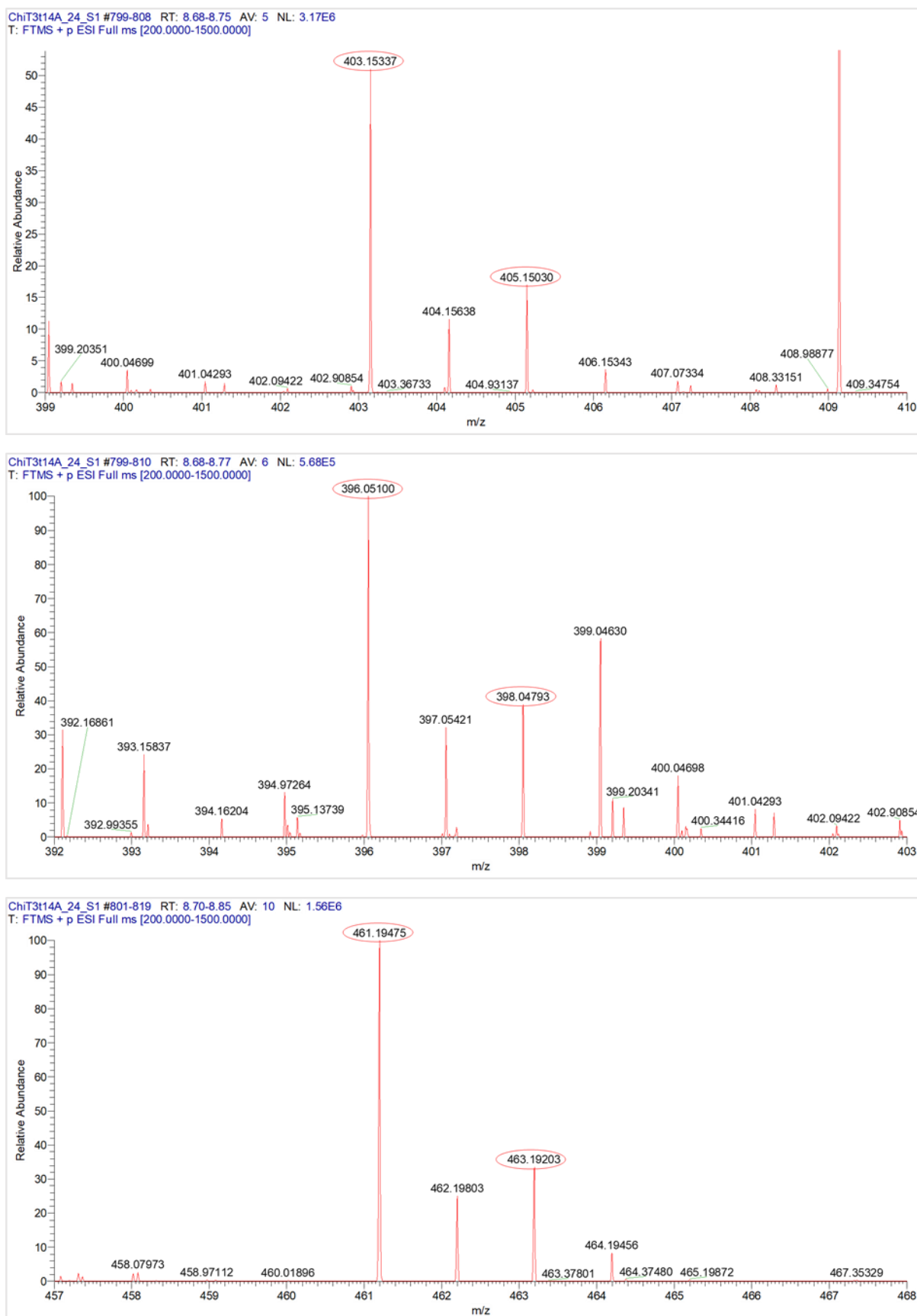
**Figure S8.** Boxplots of putative Tebuconazole highly contaminating the “Ctr” samples and showing a degradation pattern along the sampling time points.

*Boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the samples collected 7 days after the third treatment (T3), 14 days after T3, 21 days after T3 are represented from dark red to light red for the Chemical reference treated samples “Chi”, and from dark green to light green for the Untreated Control samples “Ctr”.*

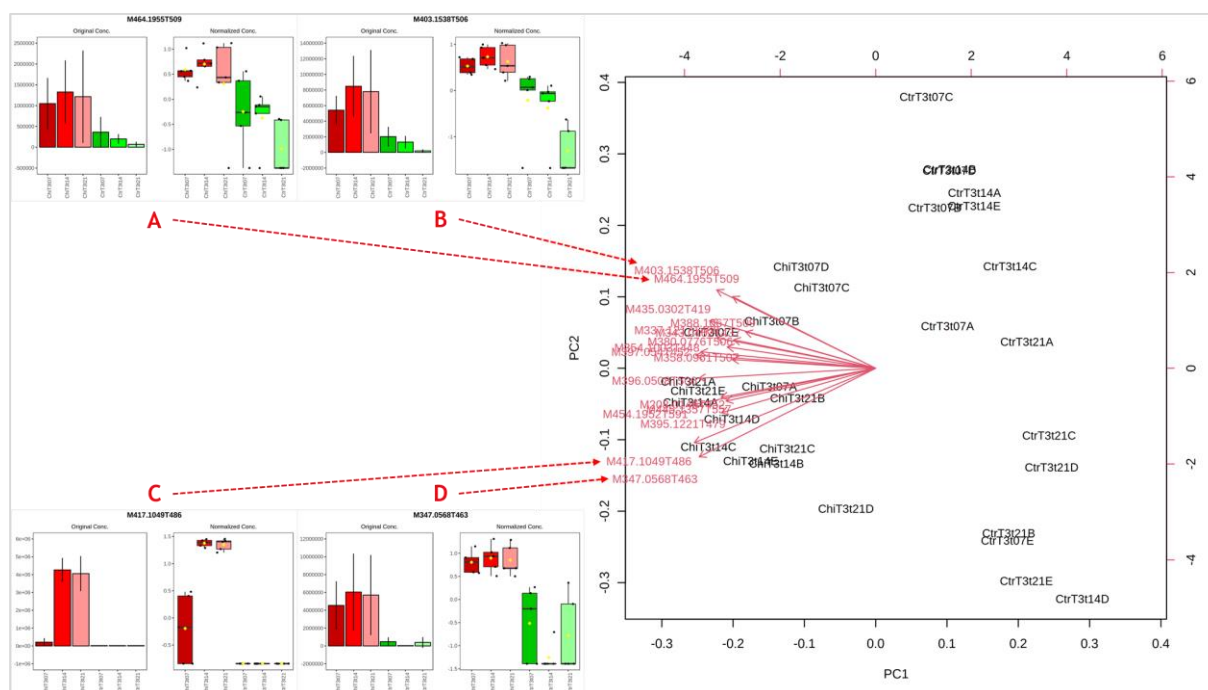






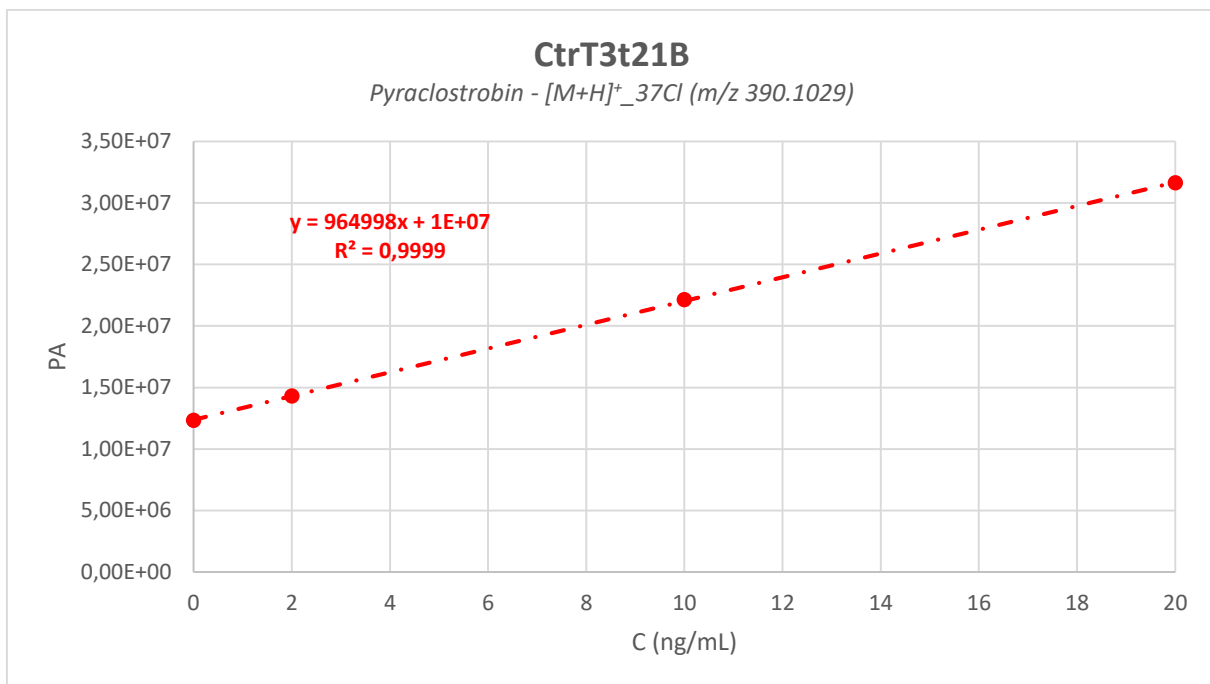
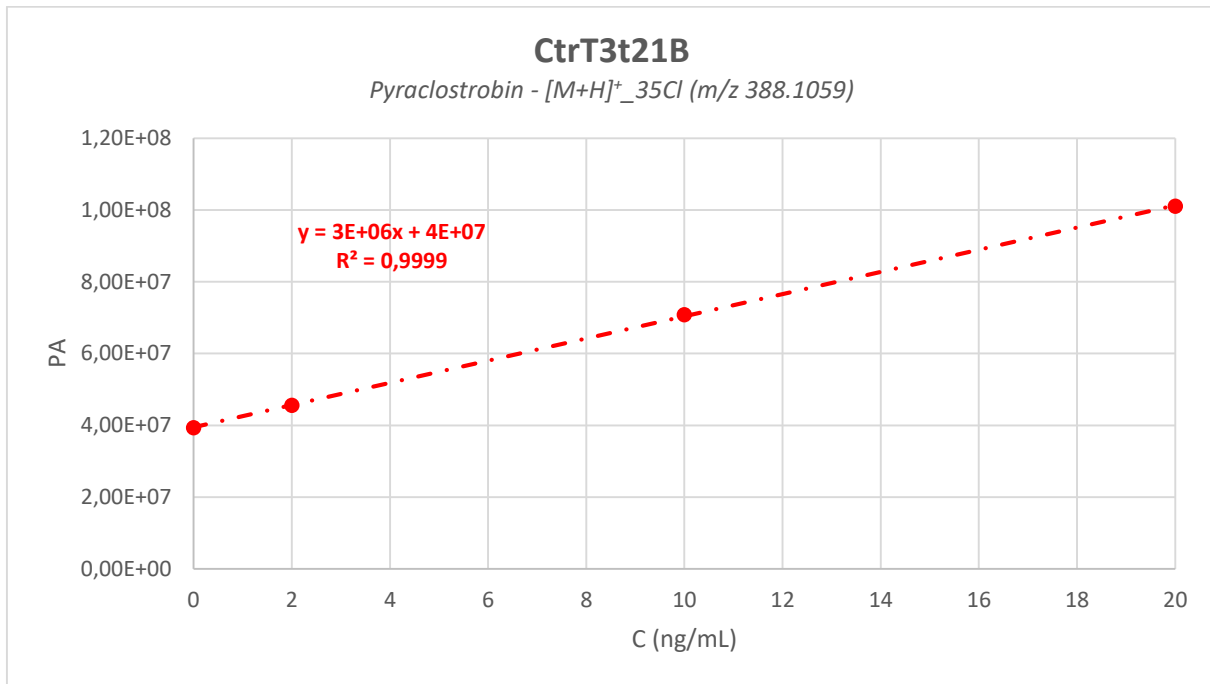


**Figure S9.** MS spectra with Chlorine isotopic patterns belonging to Chemical reference products' xenometabolome "Chi". The  $m/z$  peaks of the principal ions "[M+H]<sup>+</sup><sub>35</sub>Cl" and their "[M+H]<sup>+</sup><sub>37</sub>Cl"/ "[M+H]<sup>+</sup><sub>37</sub>Cl<sub>2</sub>"  $m/z$  peaks are circled in red.

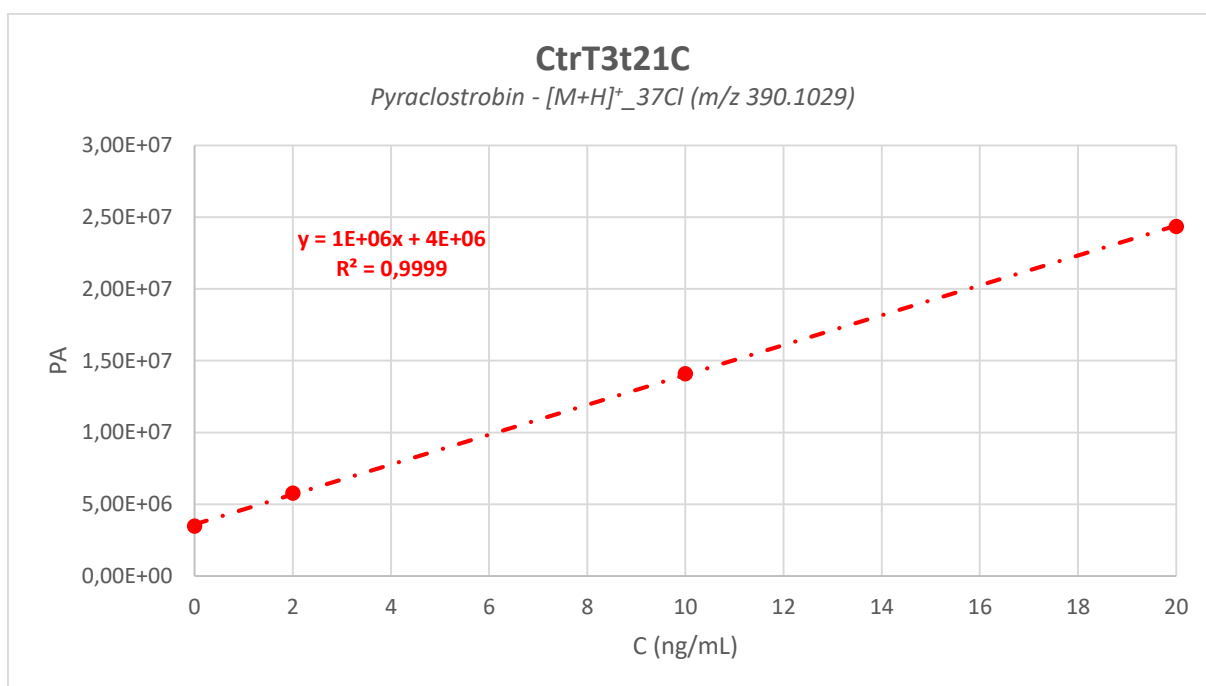
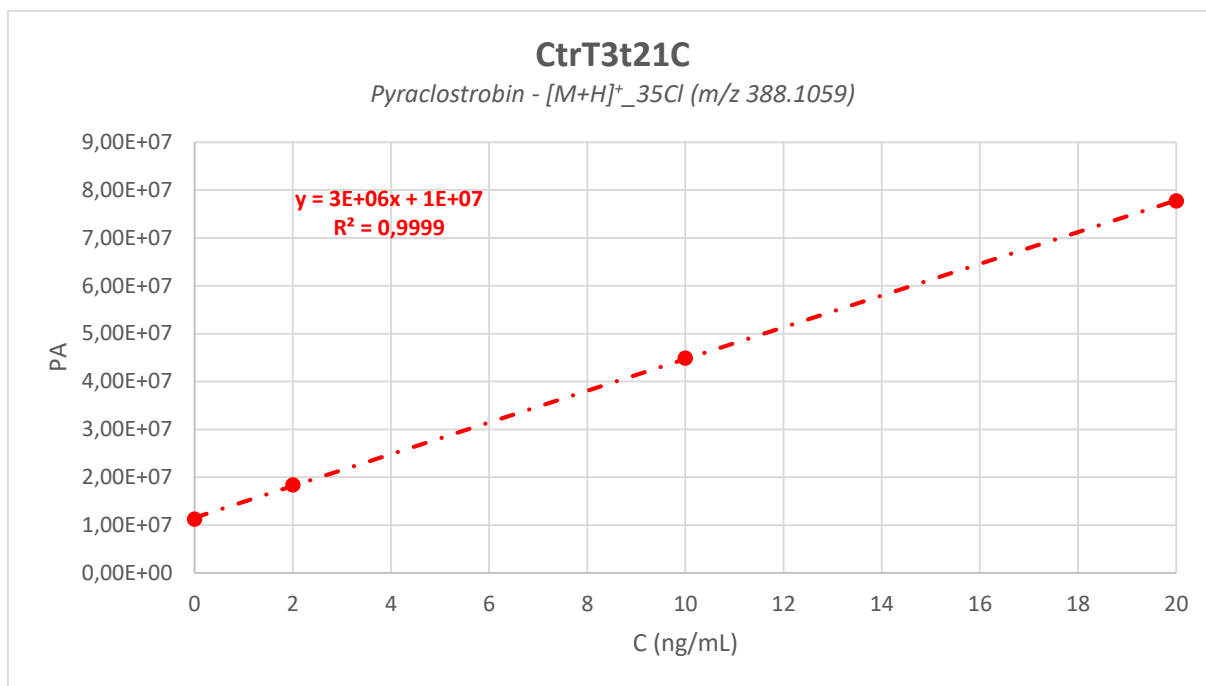


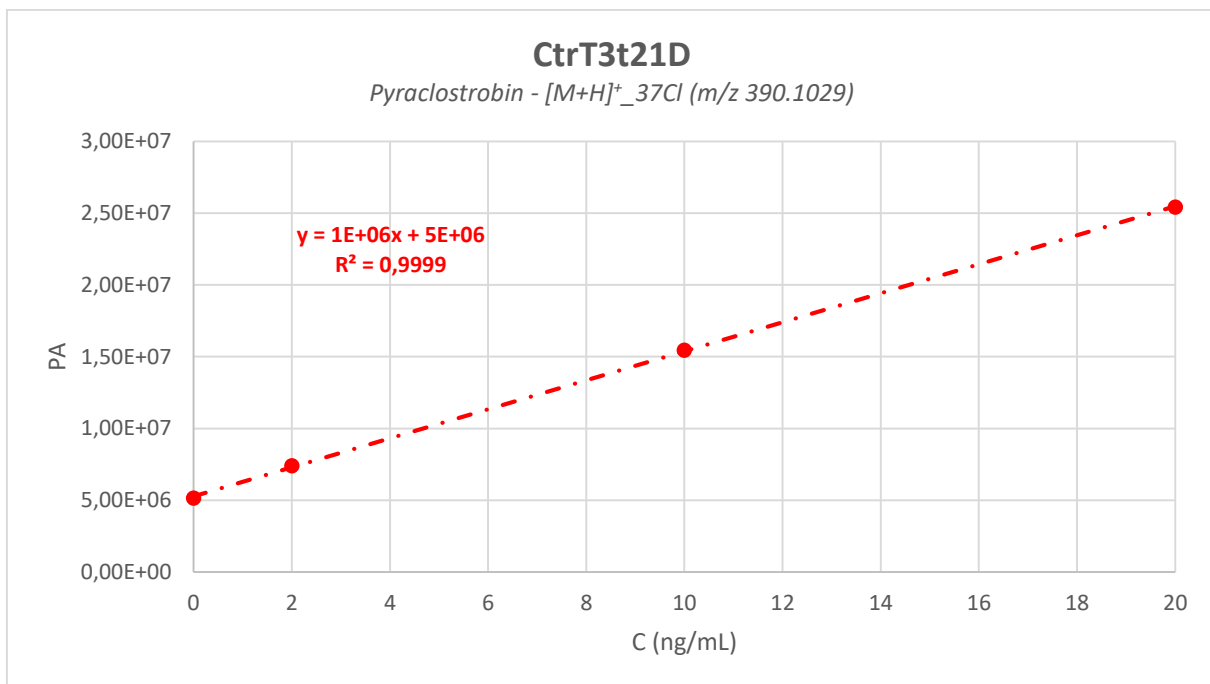
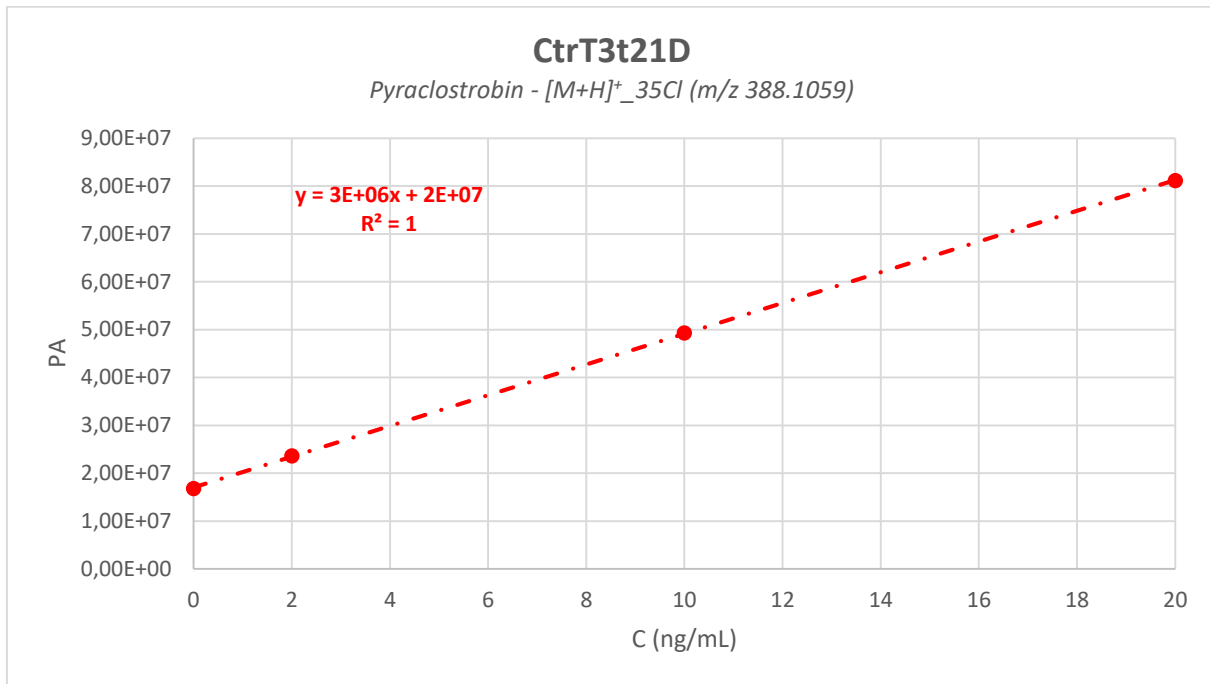
**Figure S10.** Biplot of the PCA of the Chemical reference “Chi” samples *versus* the contaminated Untreated Control samples “Ctr” (**Figure 7**). **A & B:** Boxplots of xenometabolites features highly contaminating the “Ctr”. **C & D:** Boxplots of xenometabolites features showing low or no contamination of the “Ctr”.

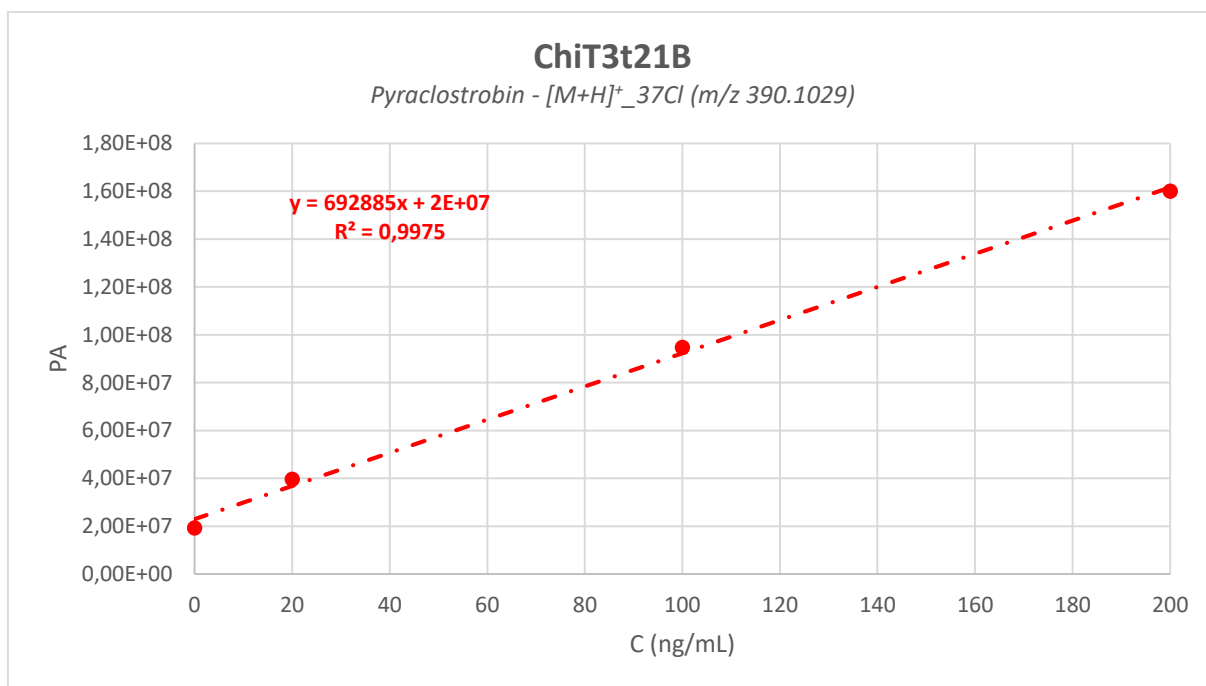
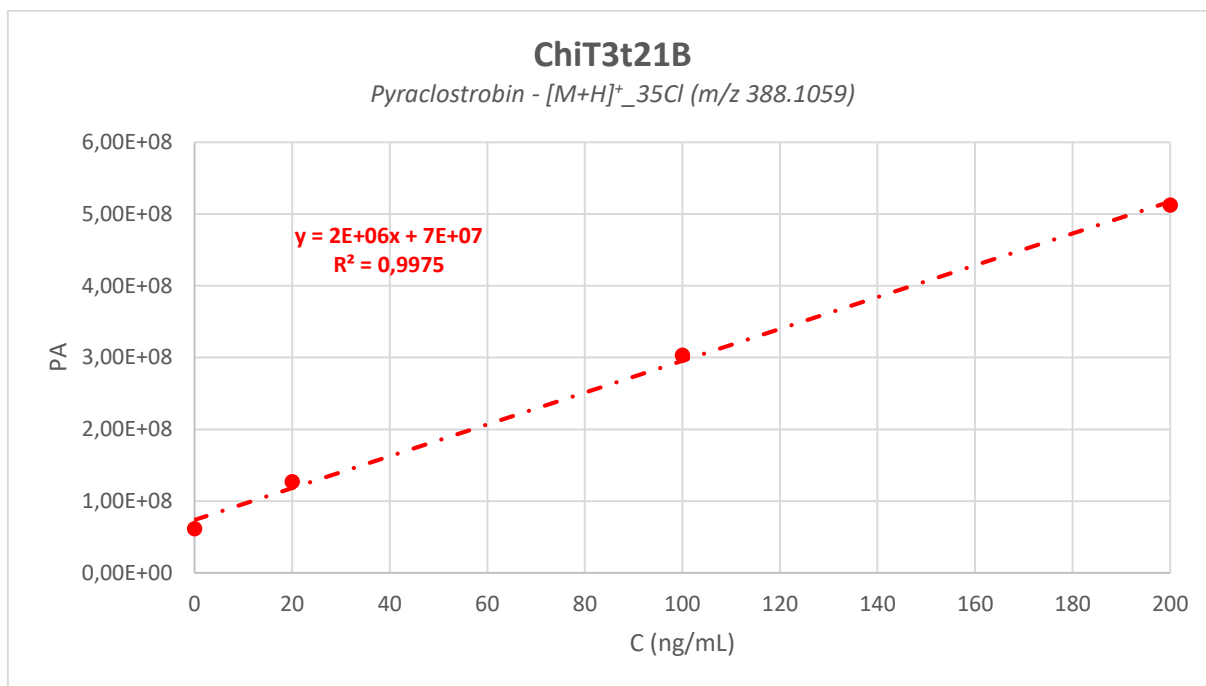
*For each xenometabolite, boxplots of the original relative intensities are represented on the left and boxplots of normalised intensities are represented on the right. Boxplots of the samples collected 7 days after the third treatment (T3), 14 days after T3, 21 days after T3 are represented from dark red to light red for the Chemical reference treated samples “Chi”, and from dark green to light green for the Untreated Control samples “Ctr”.*

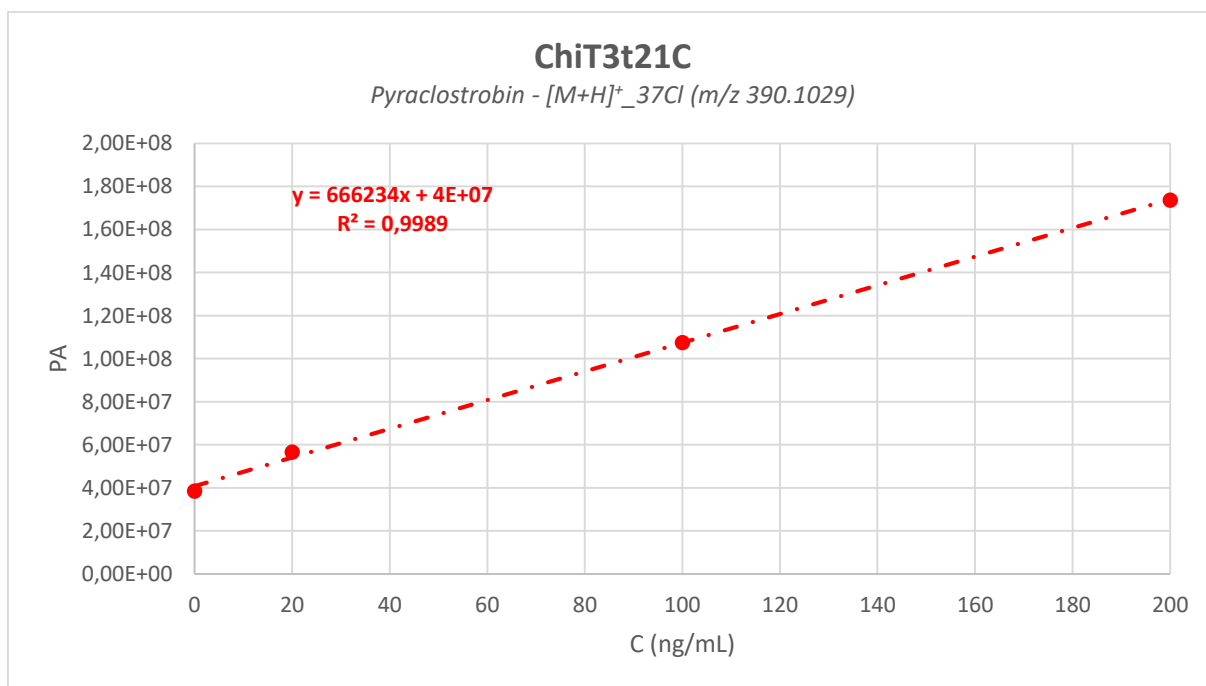
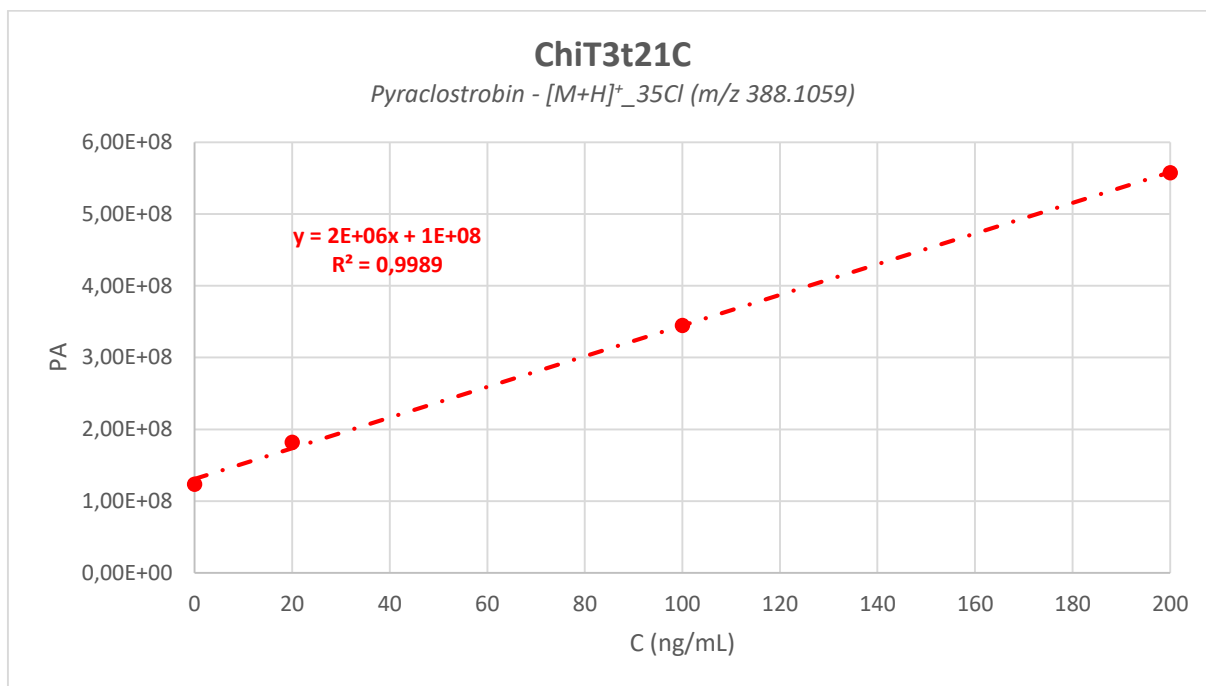


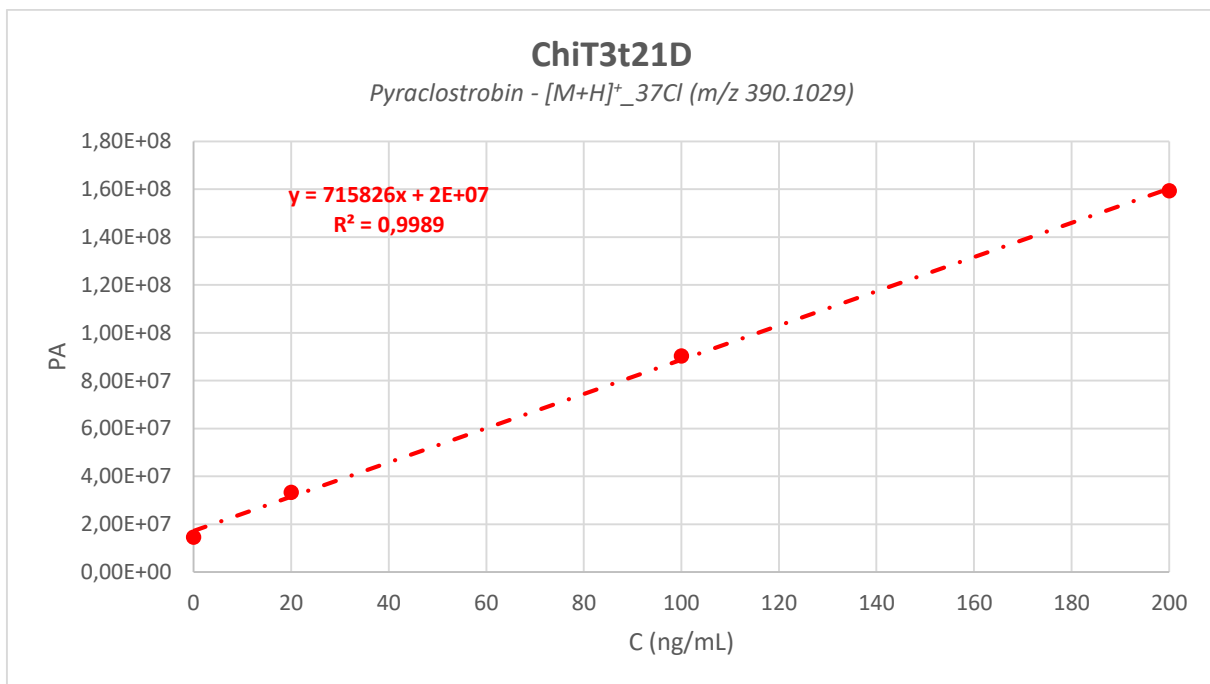
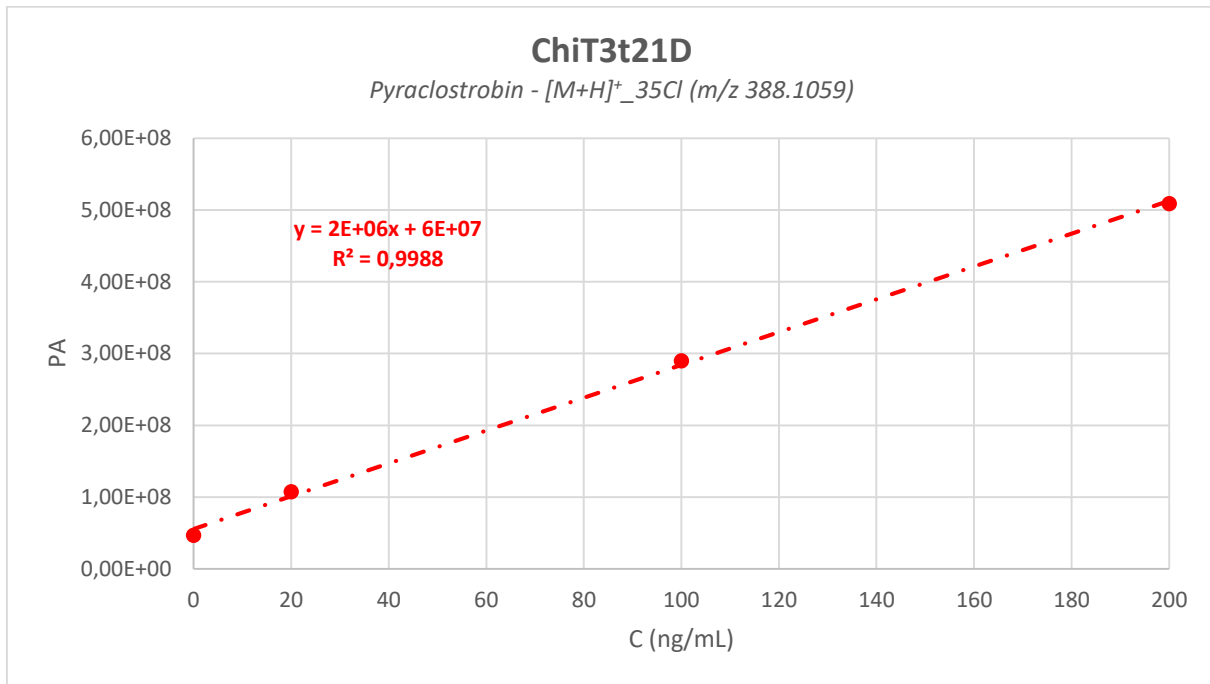


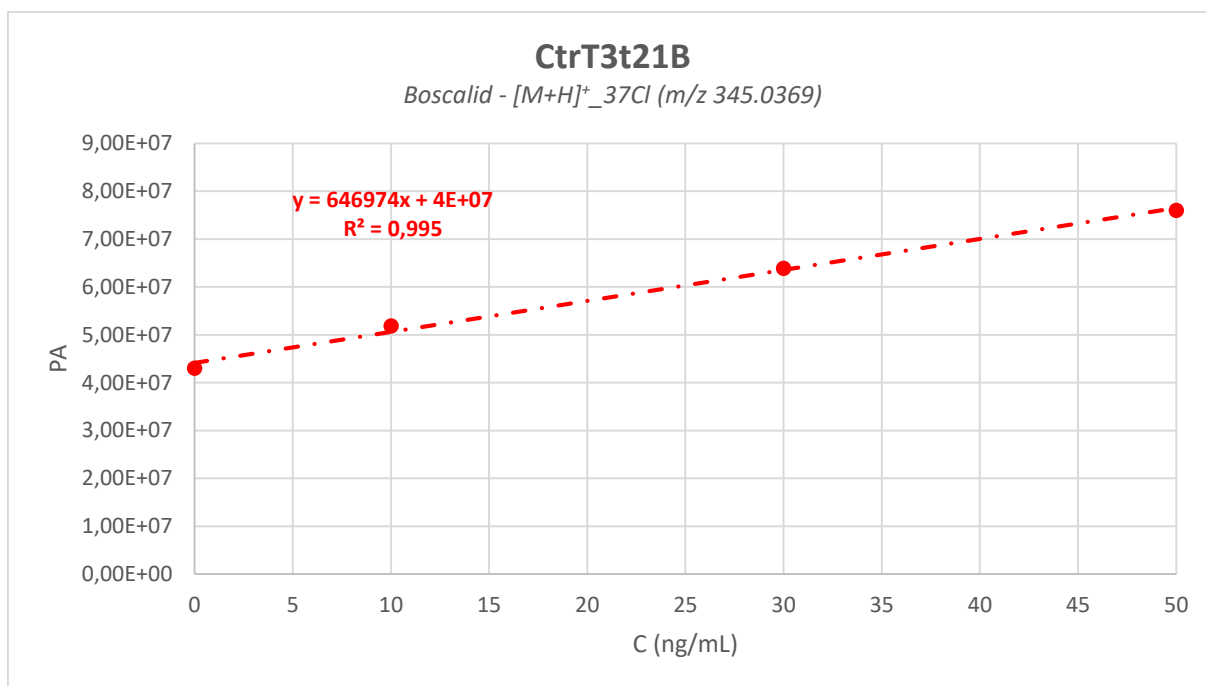
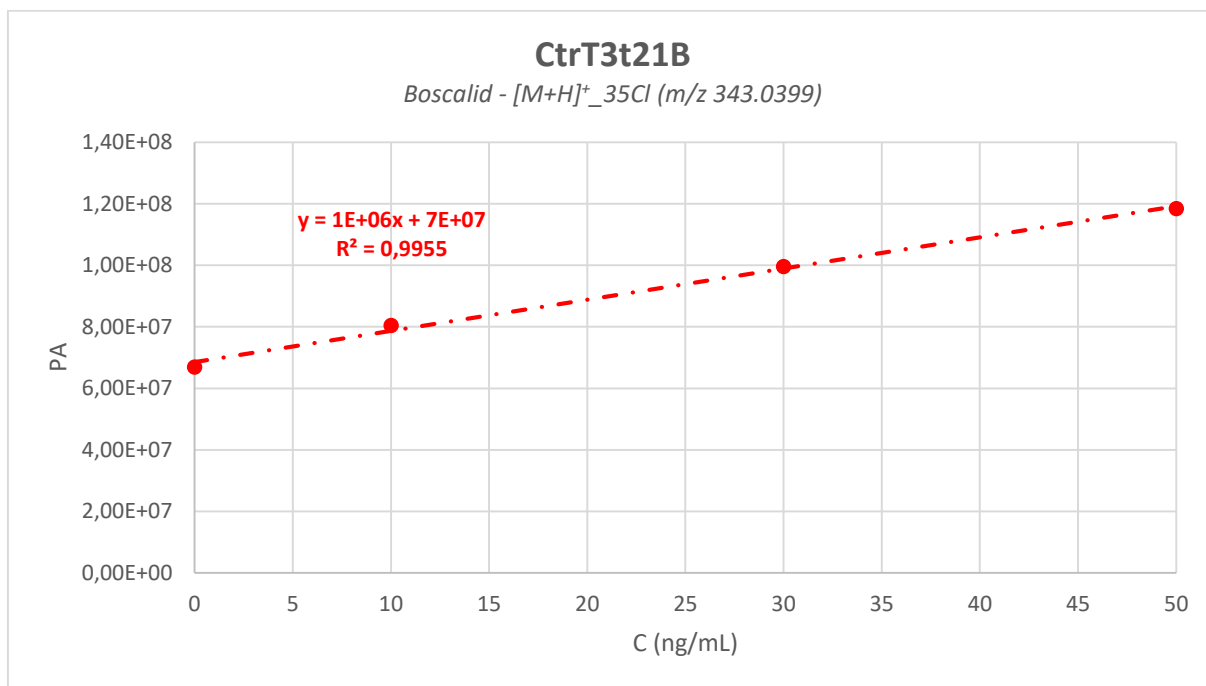


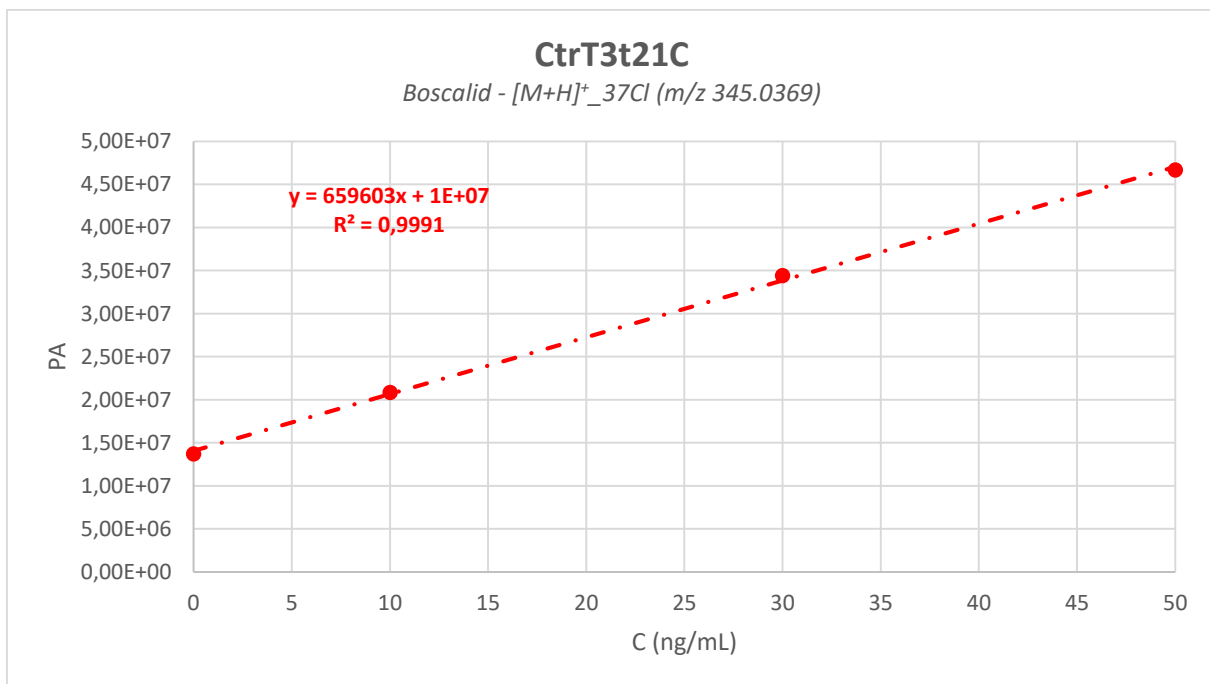
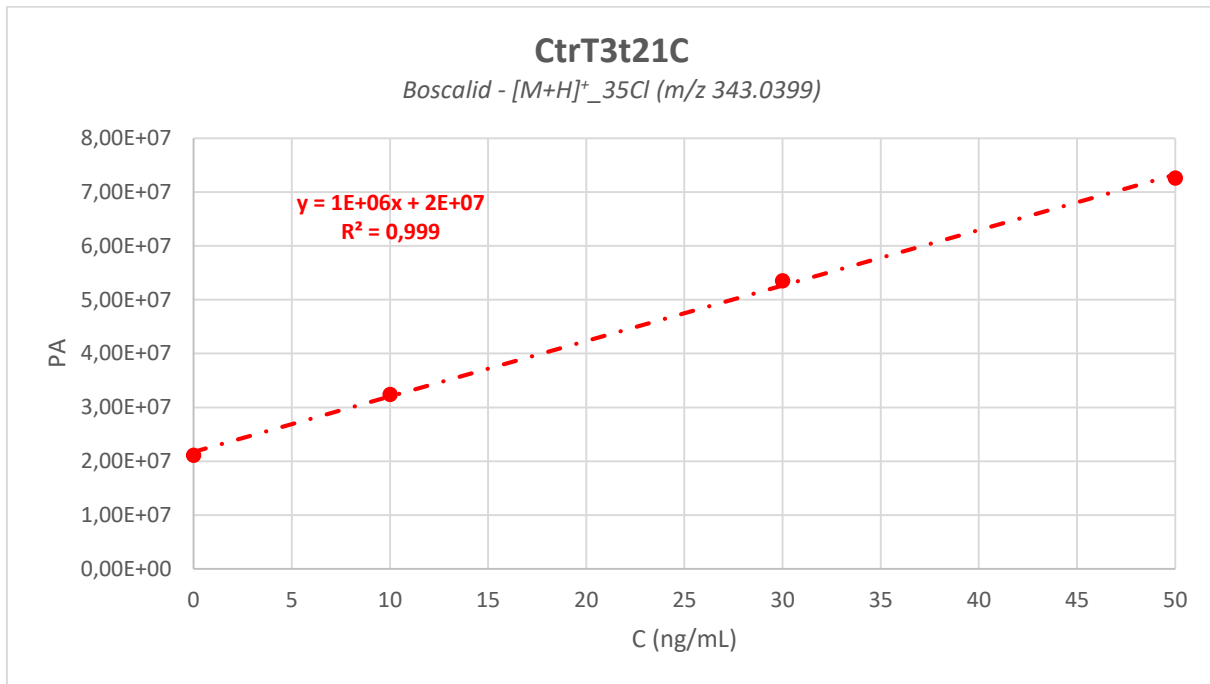


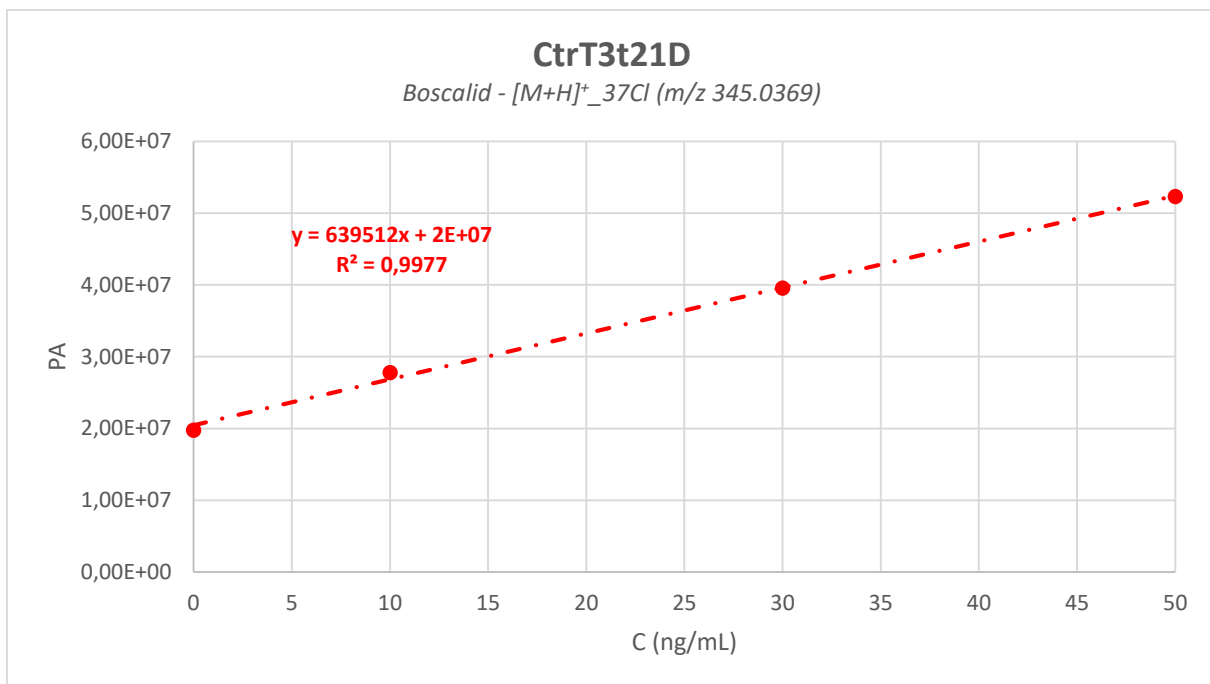
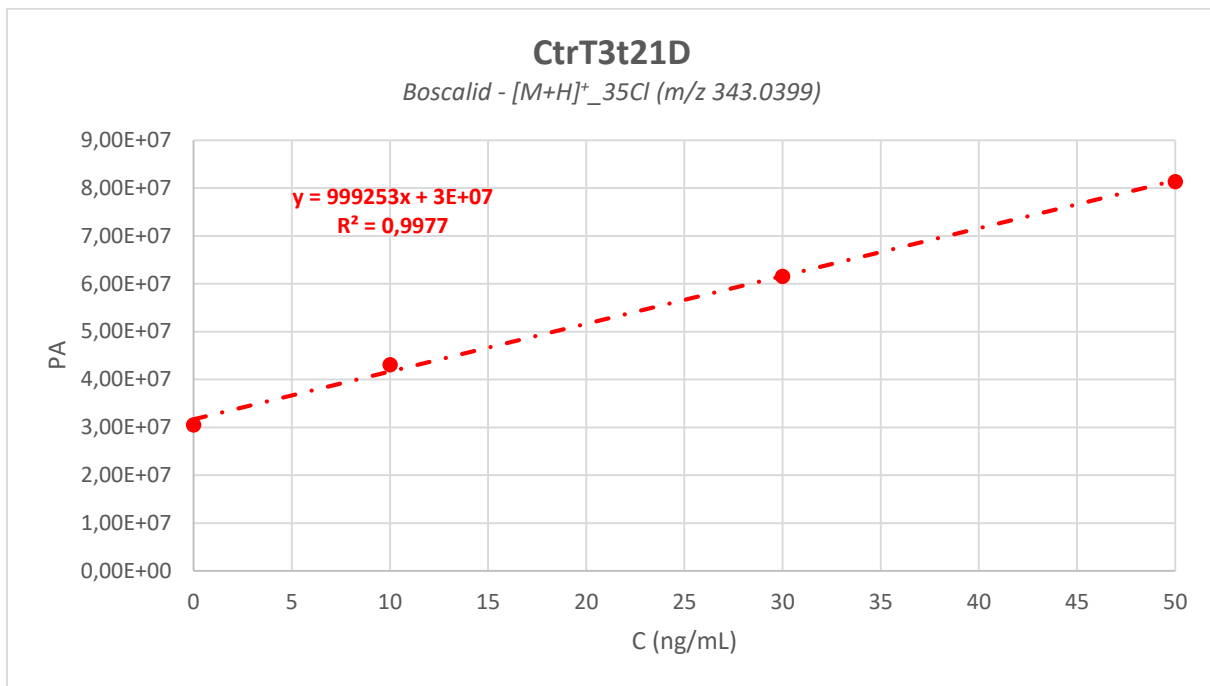




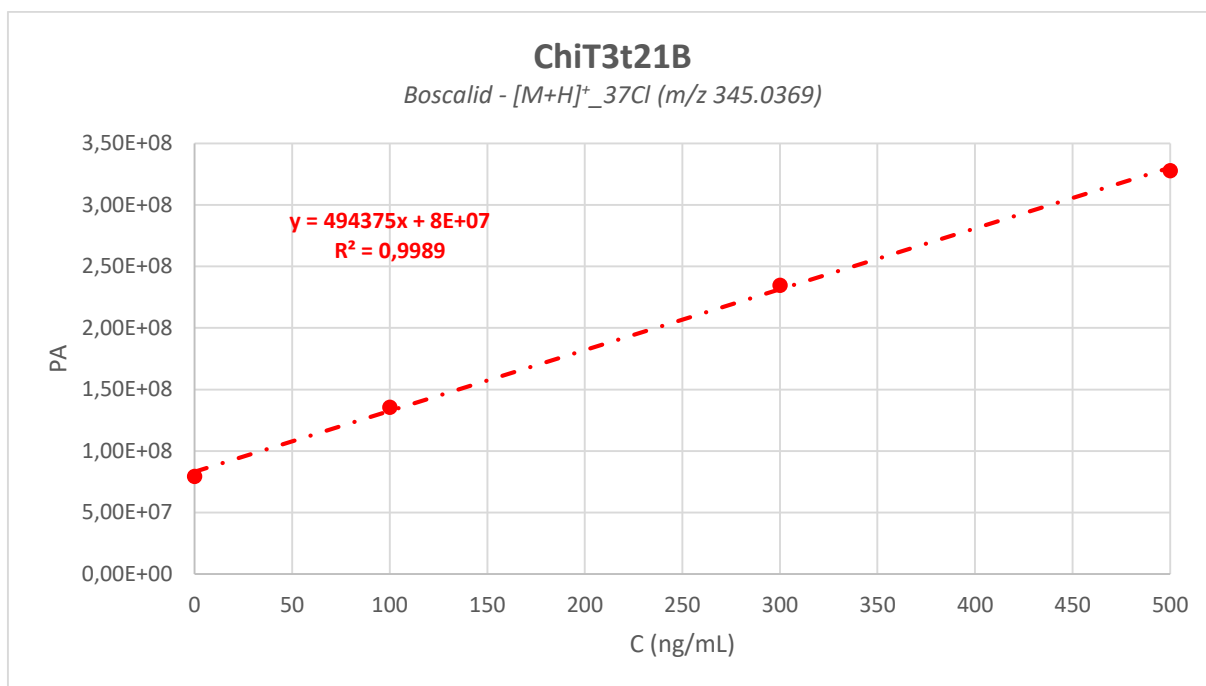
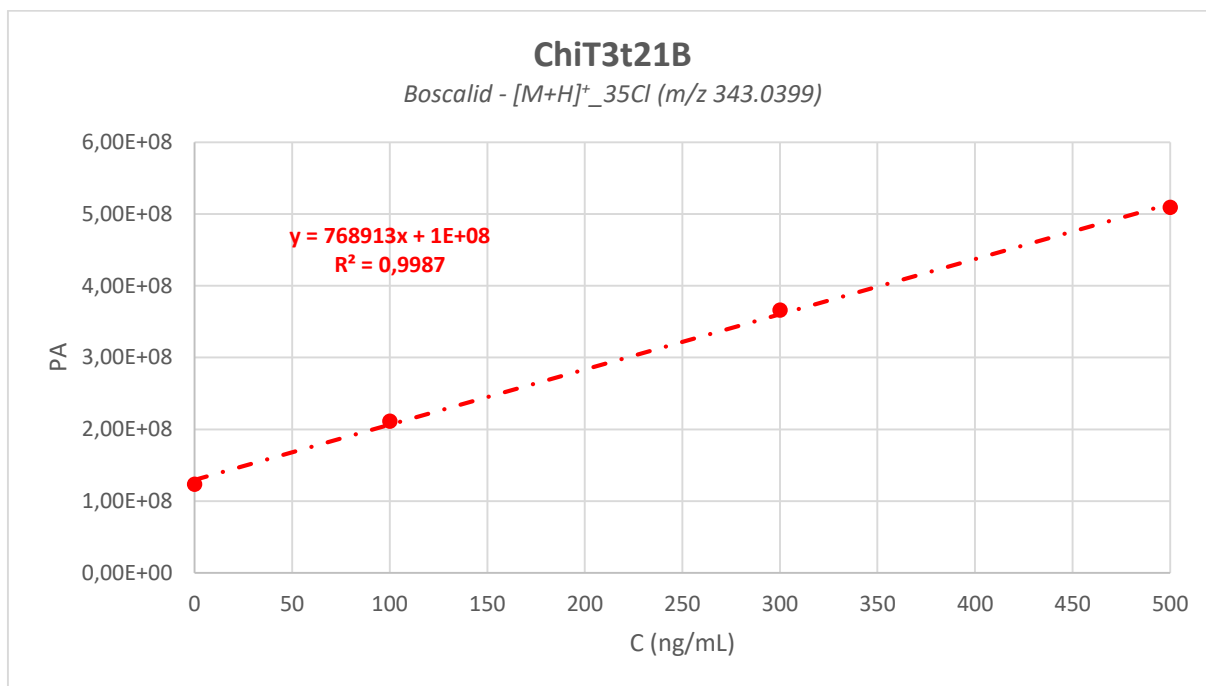


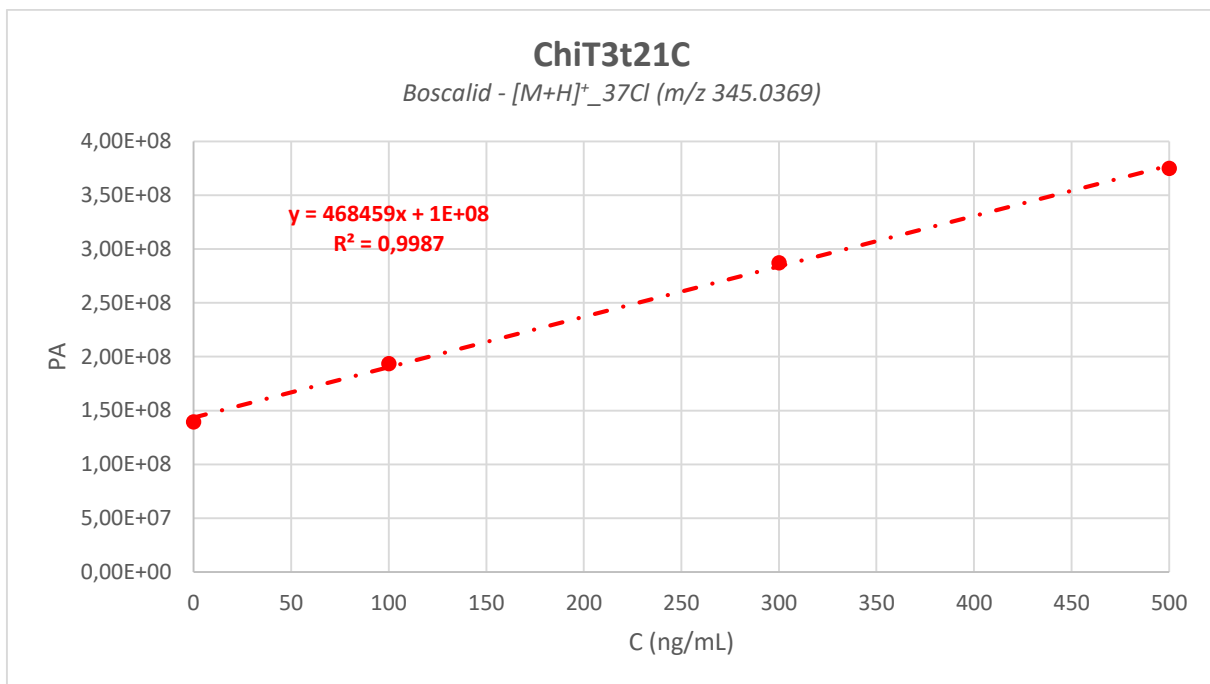
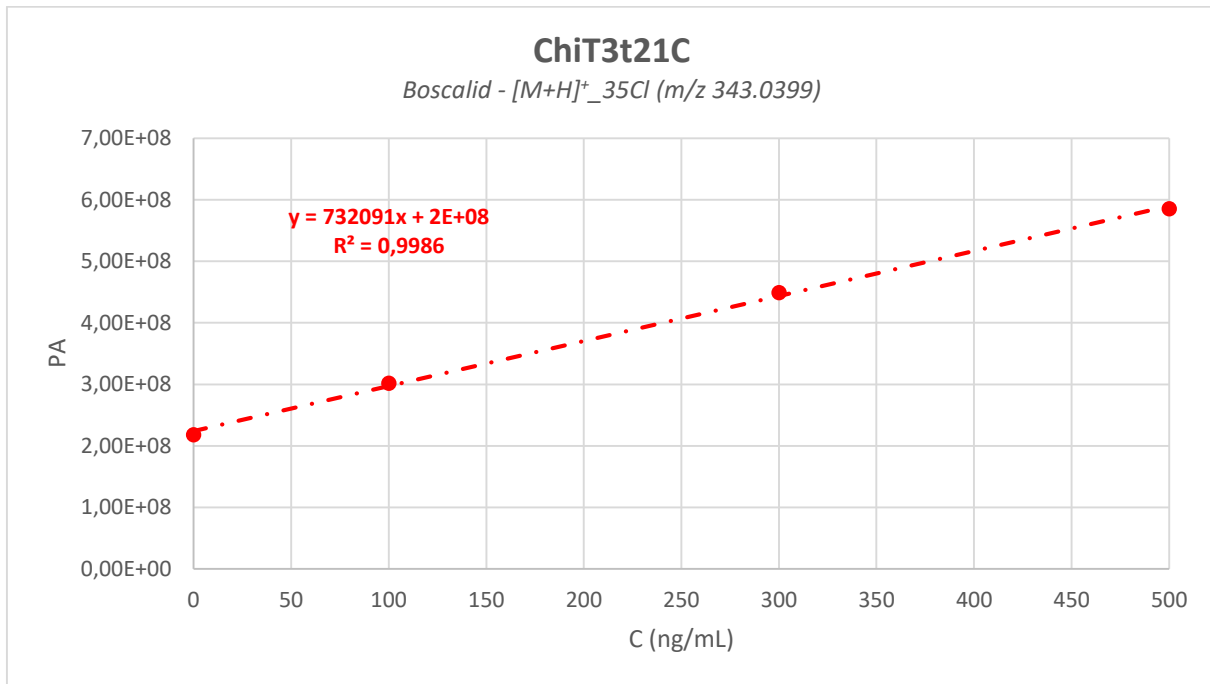


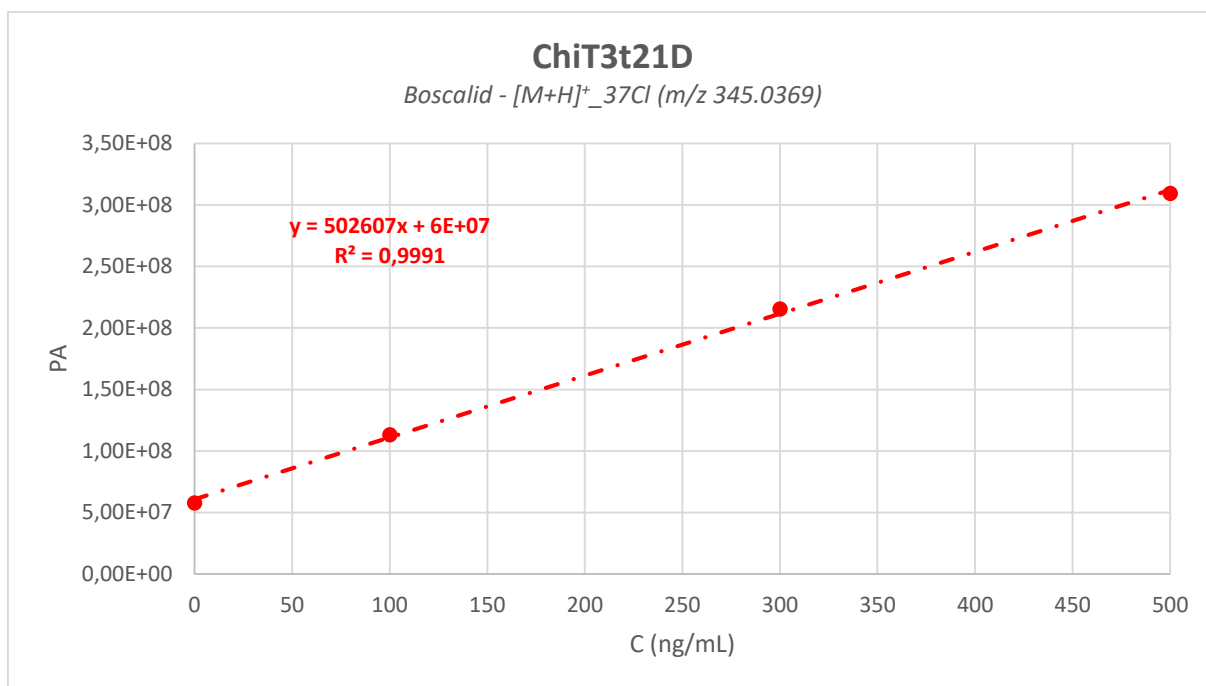
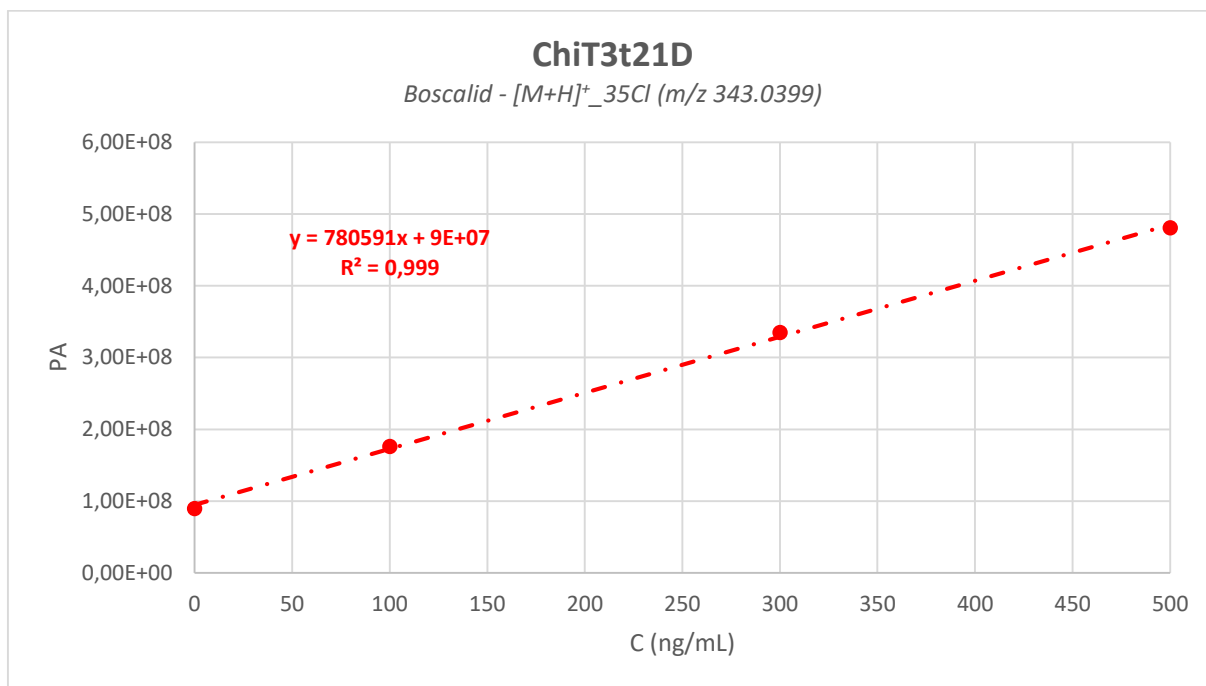


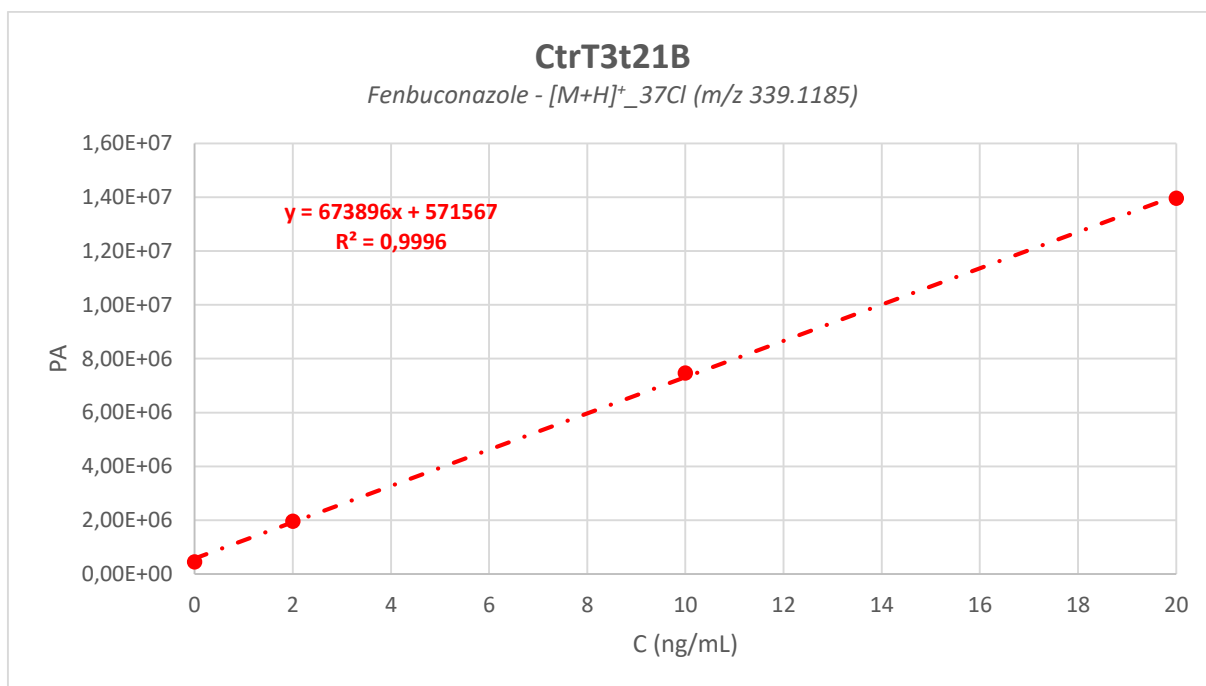
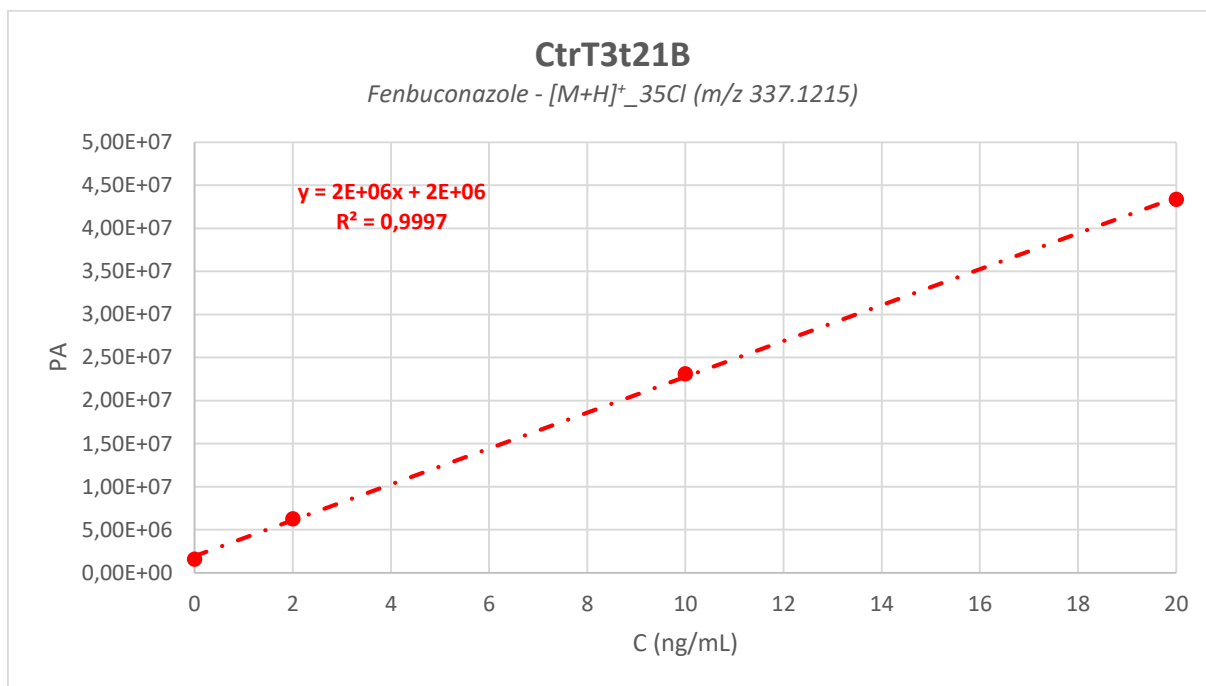


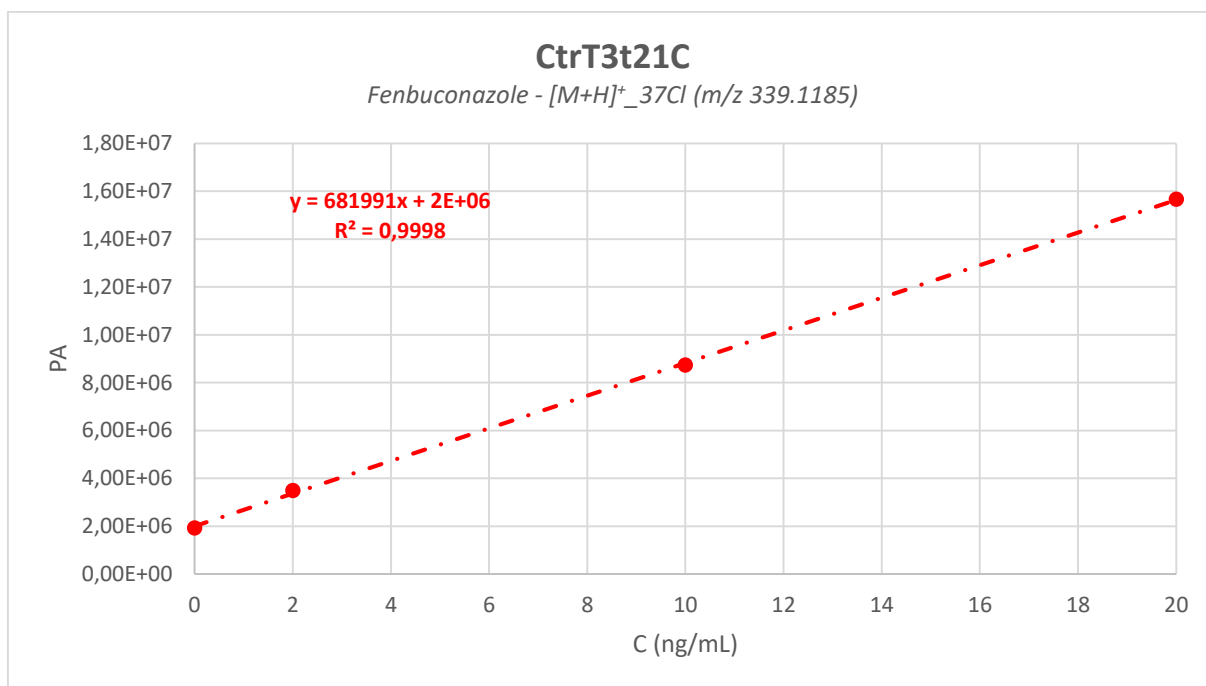
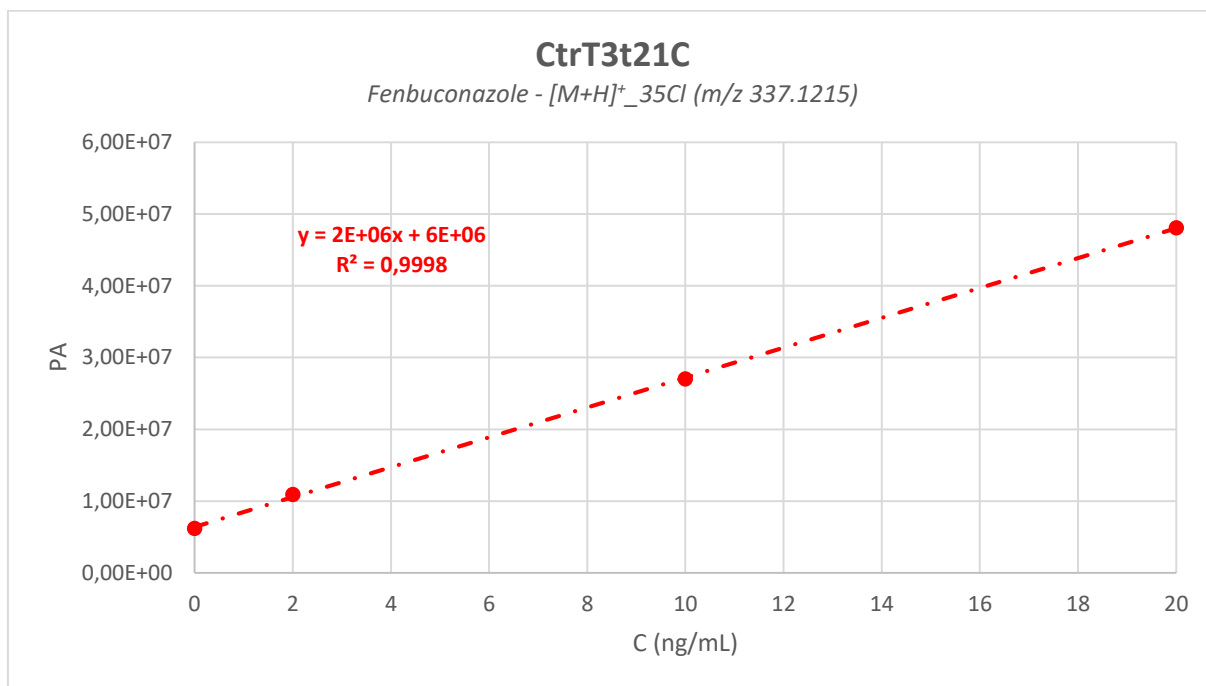


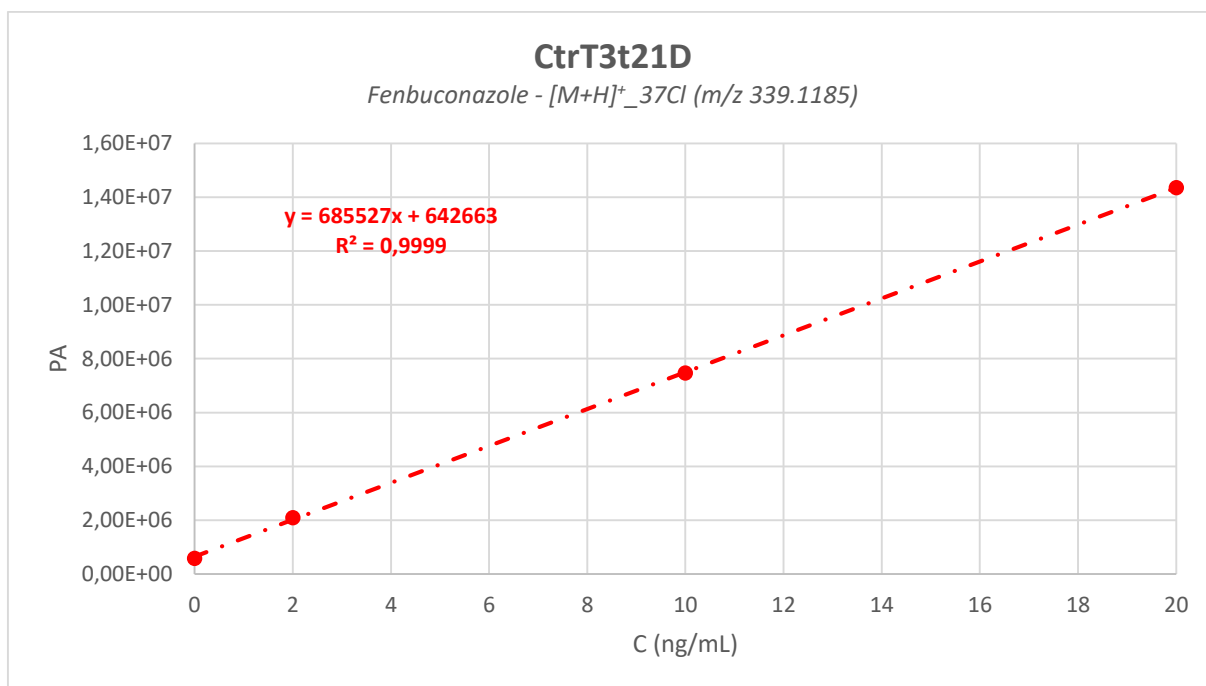
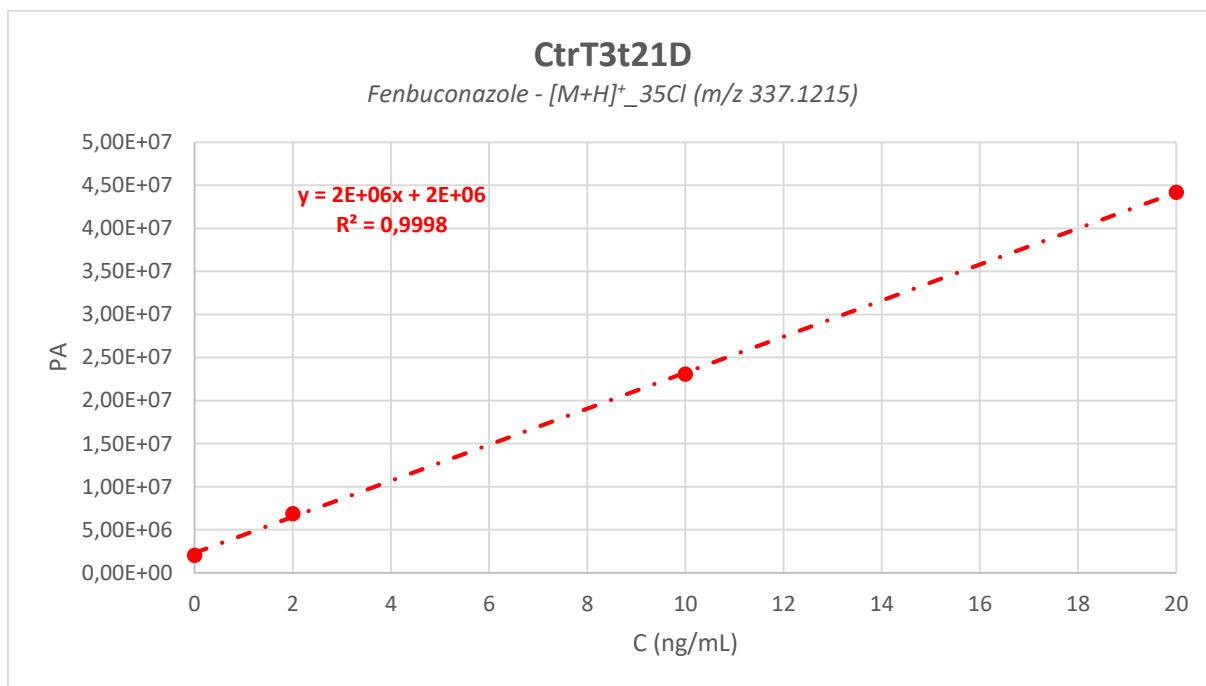


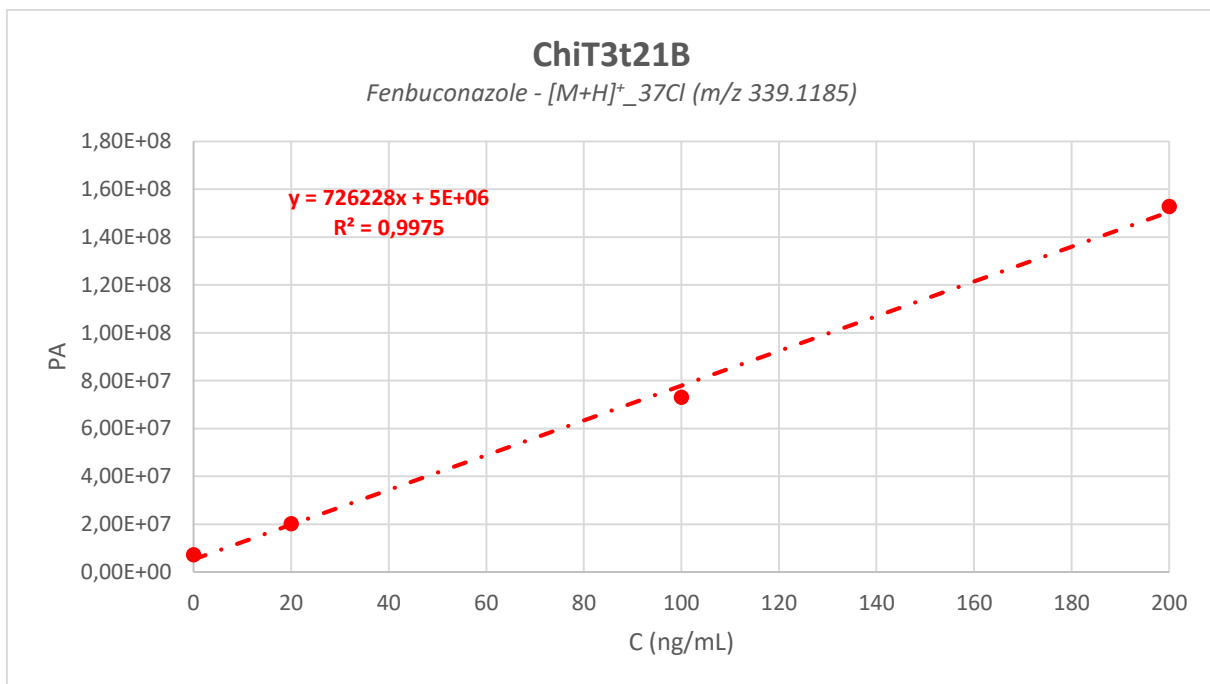
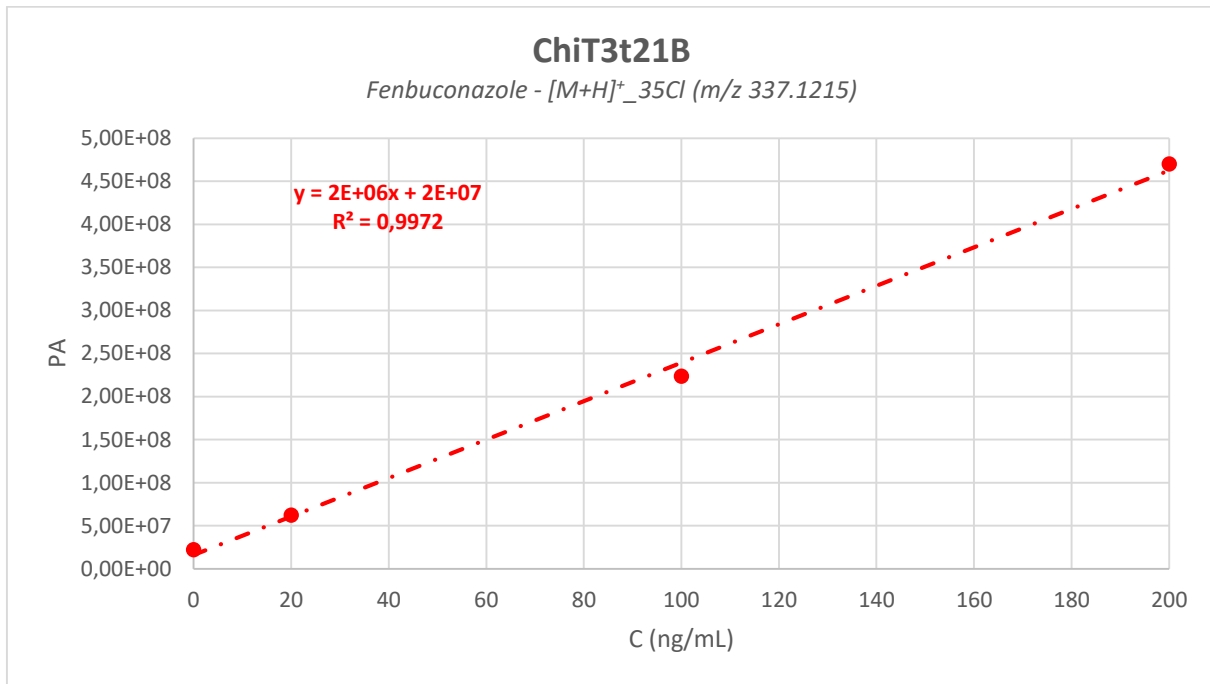


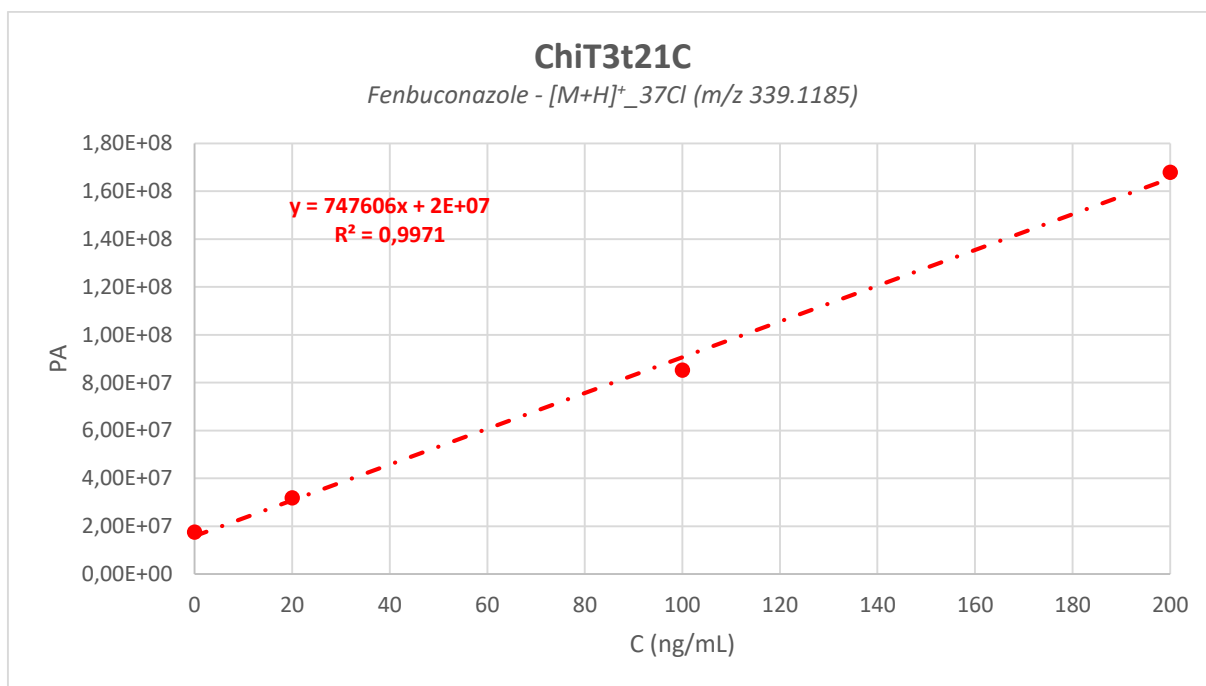
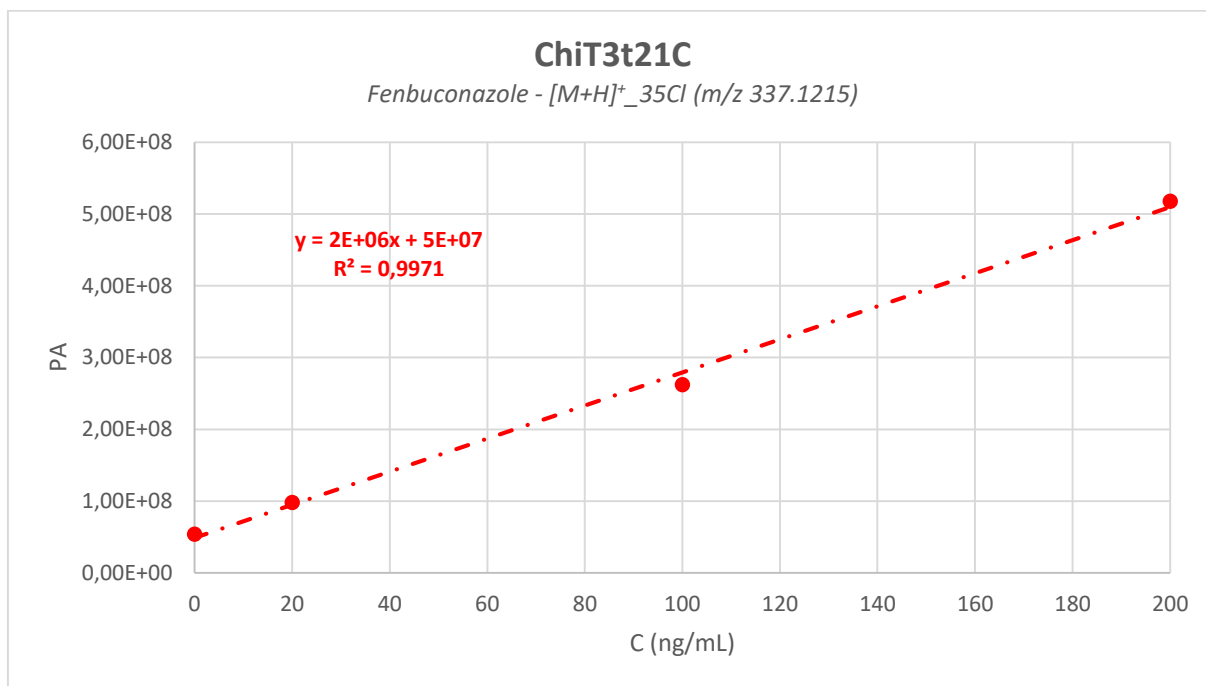




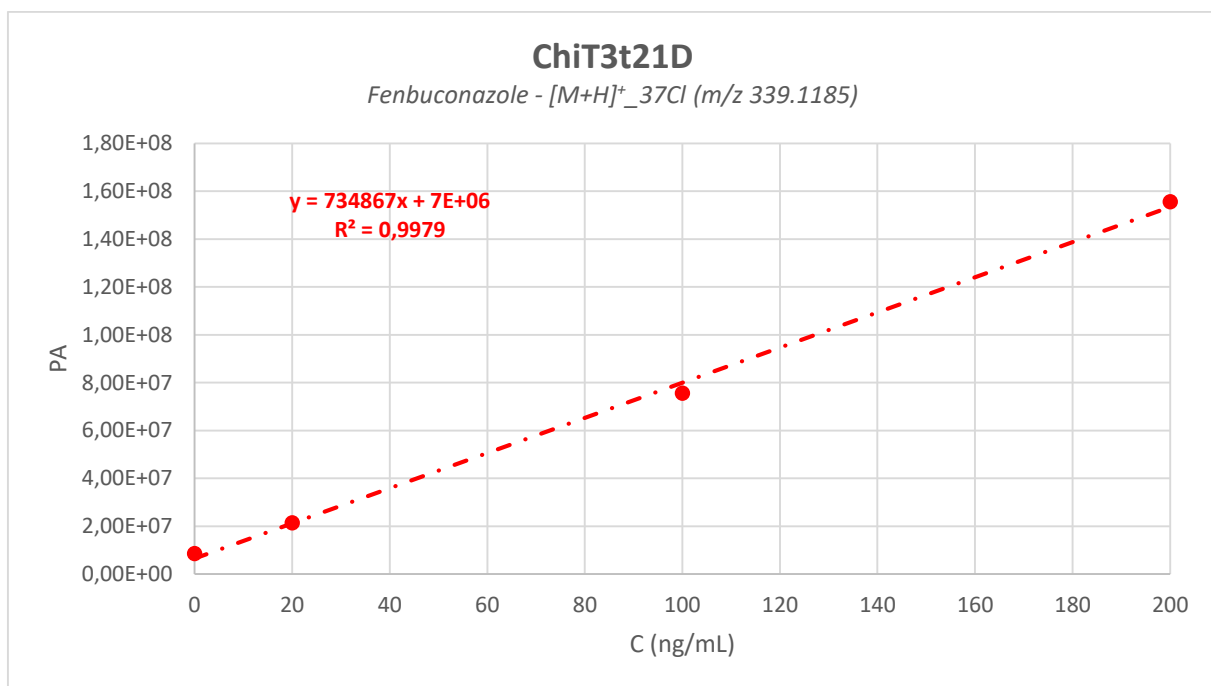
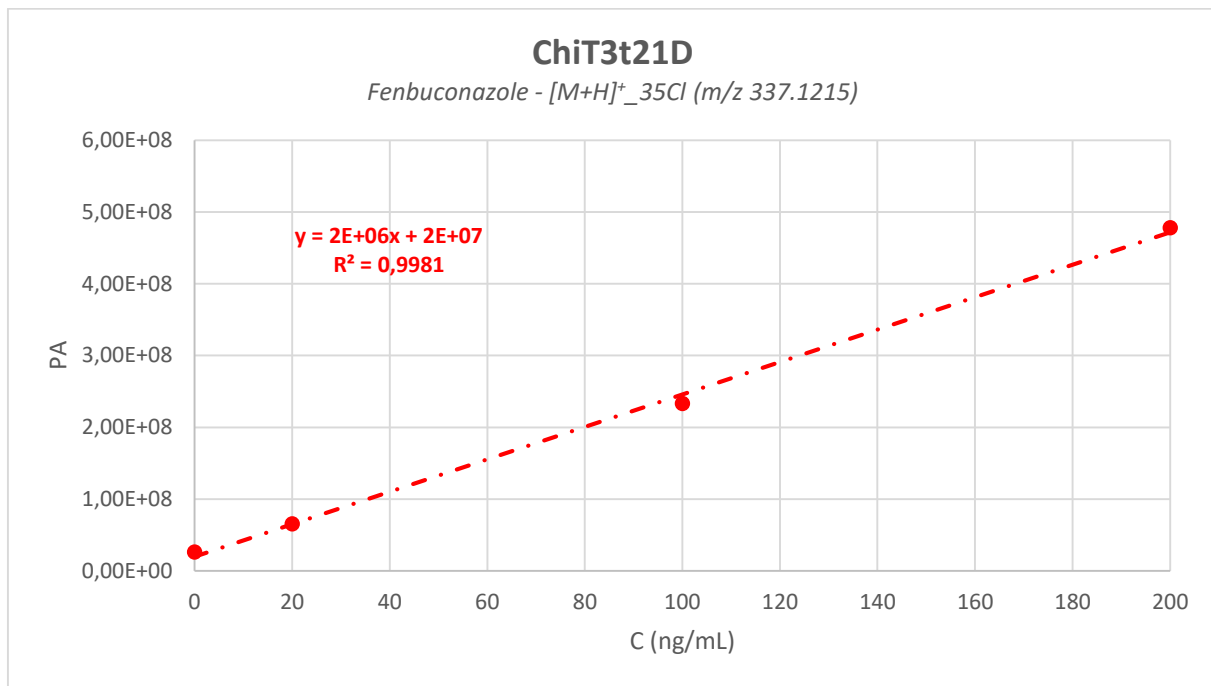


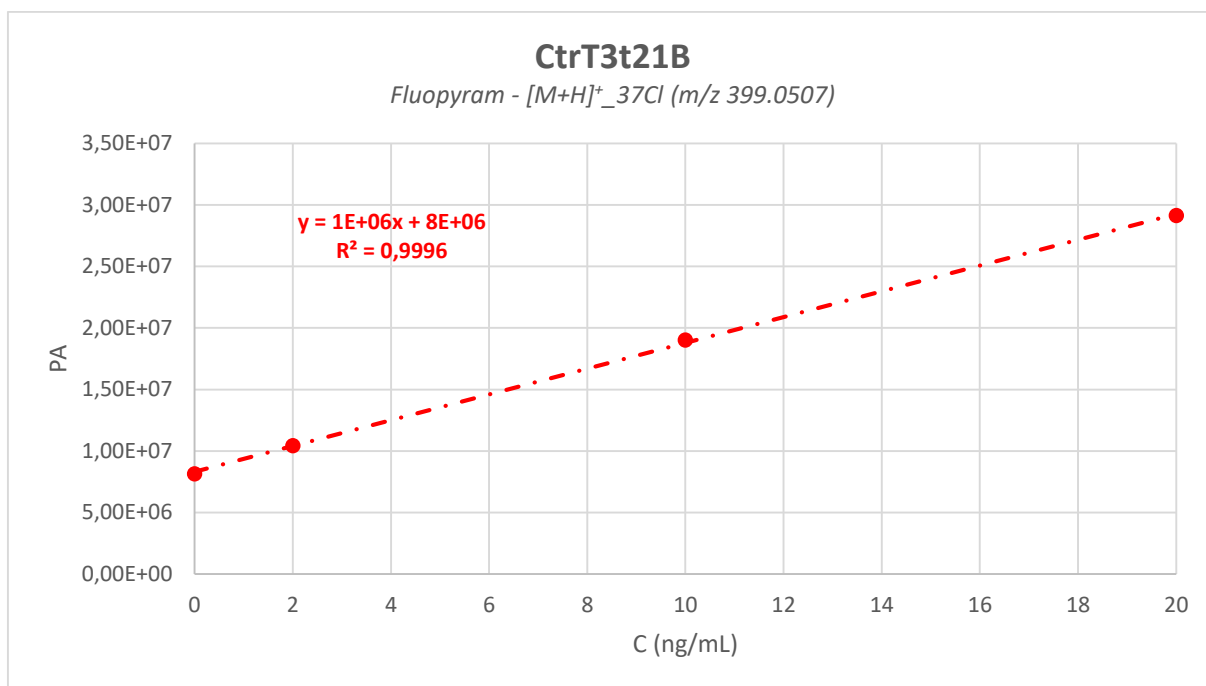
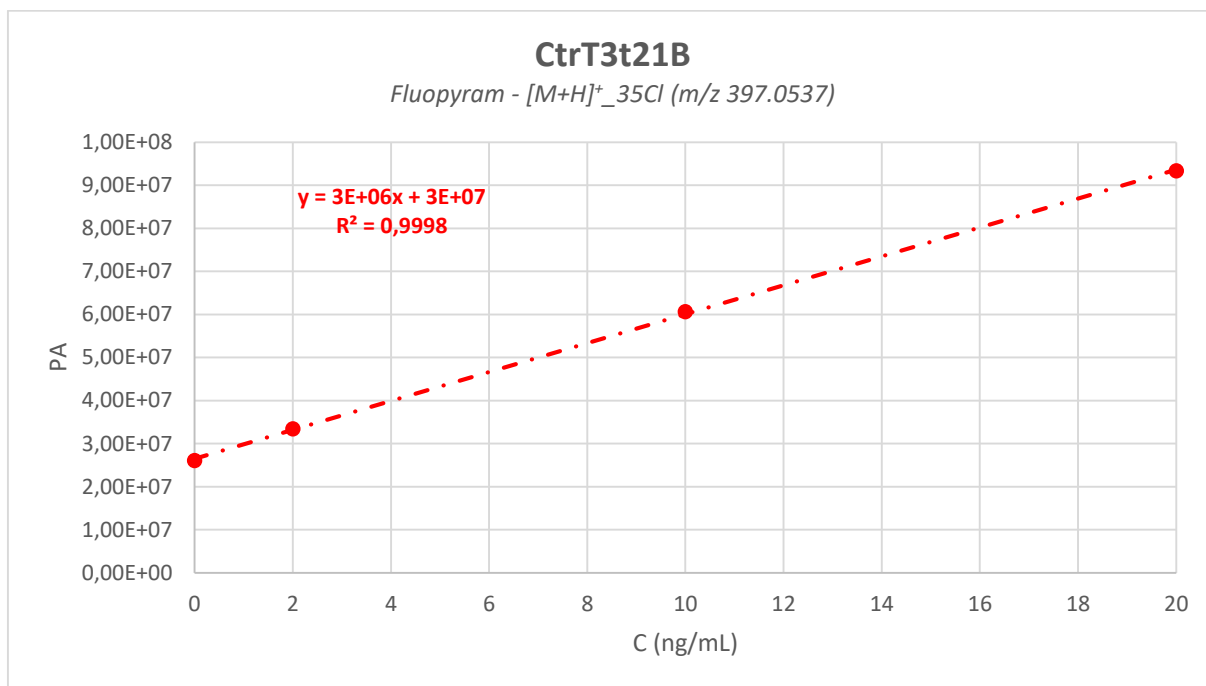


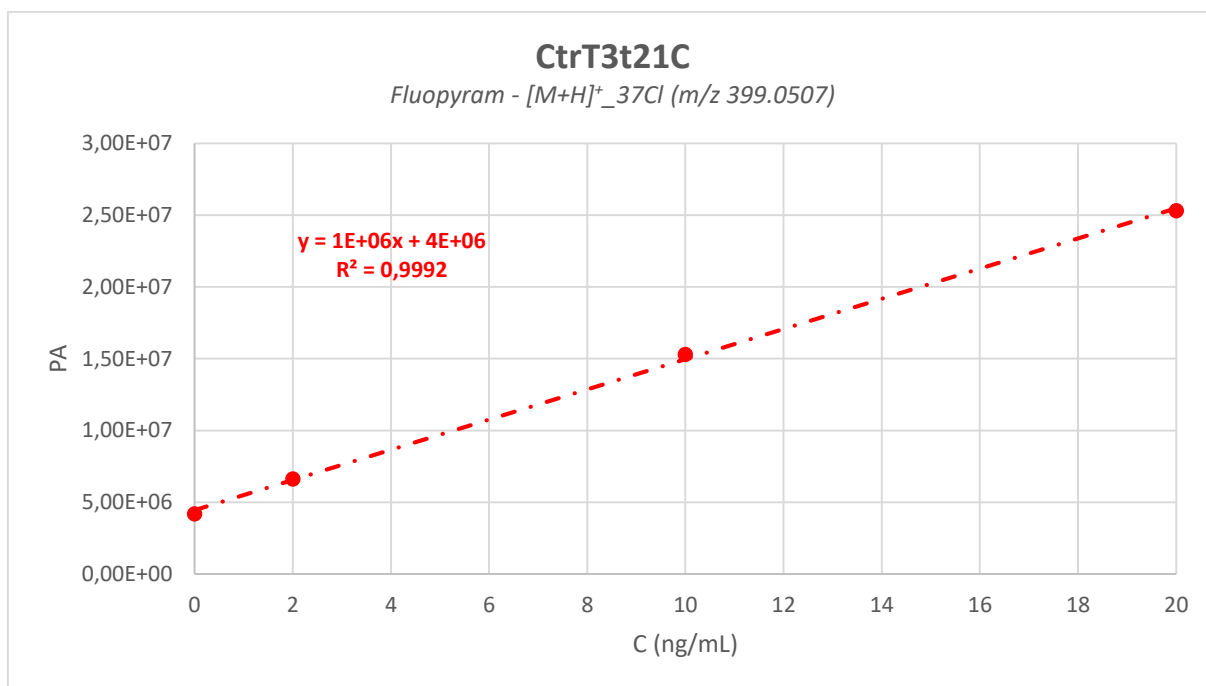
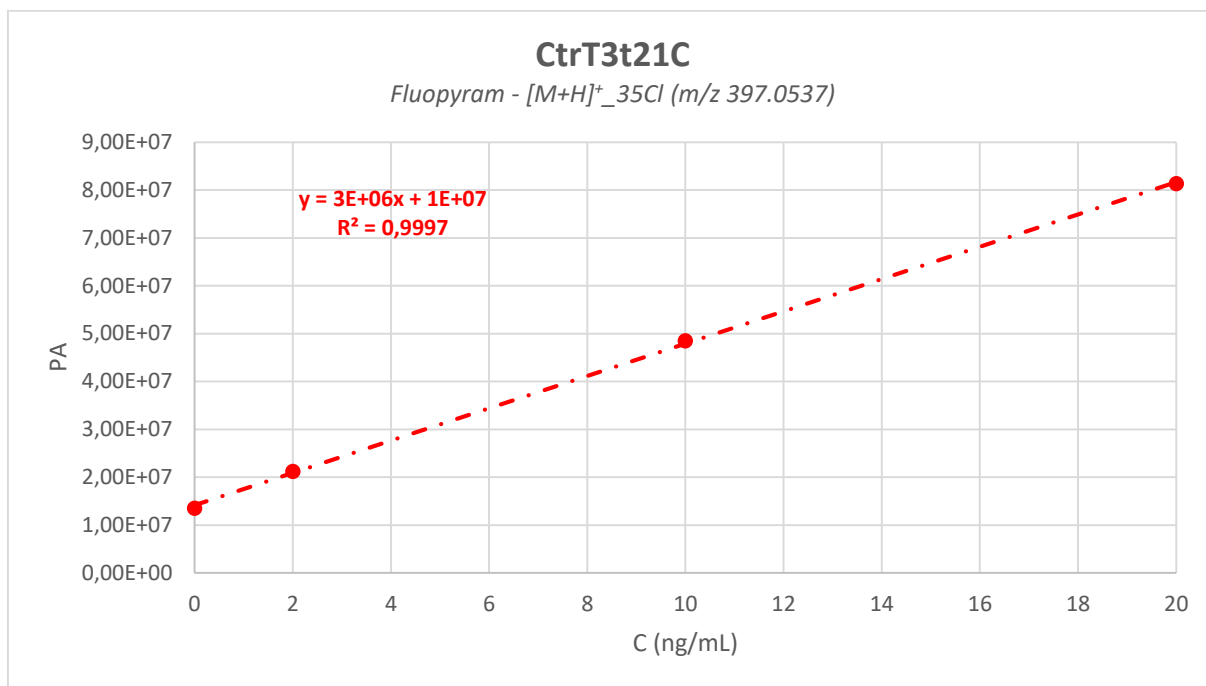


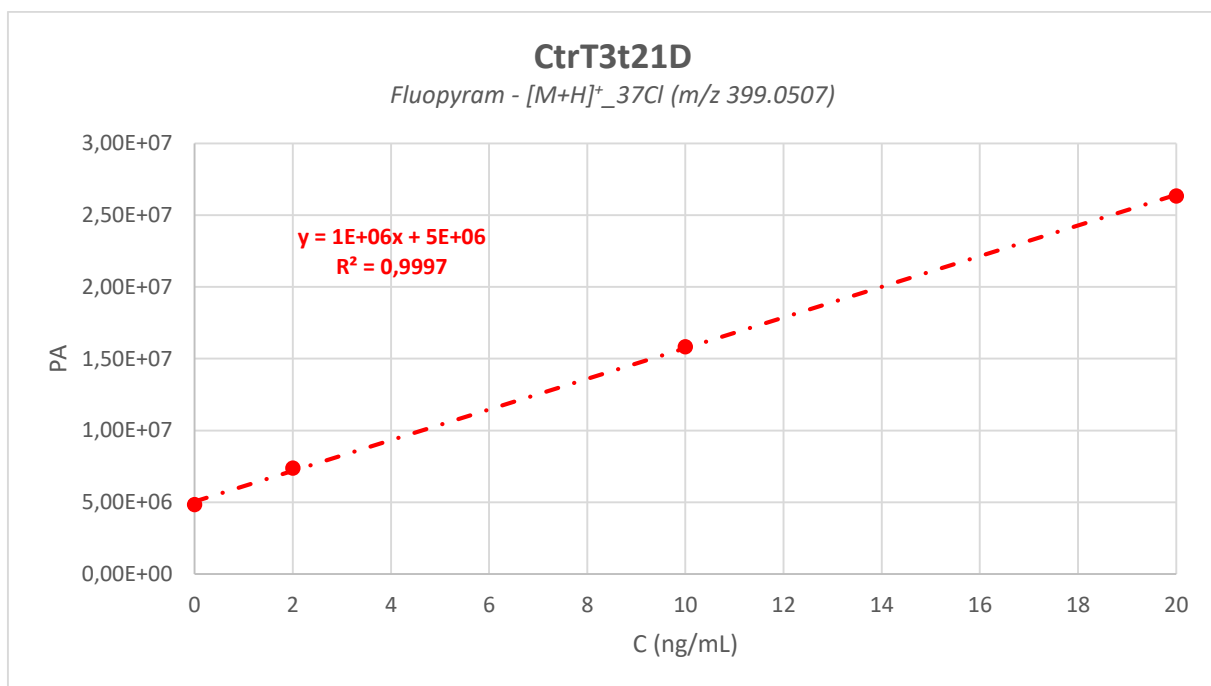
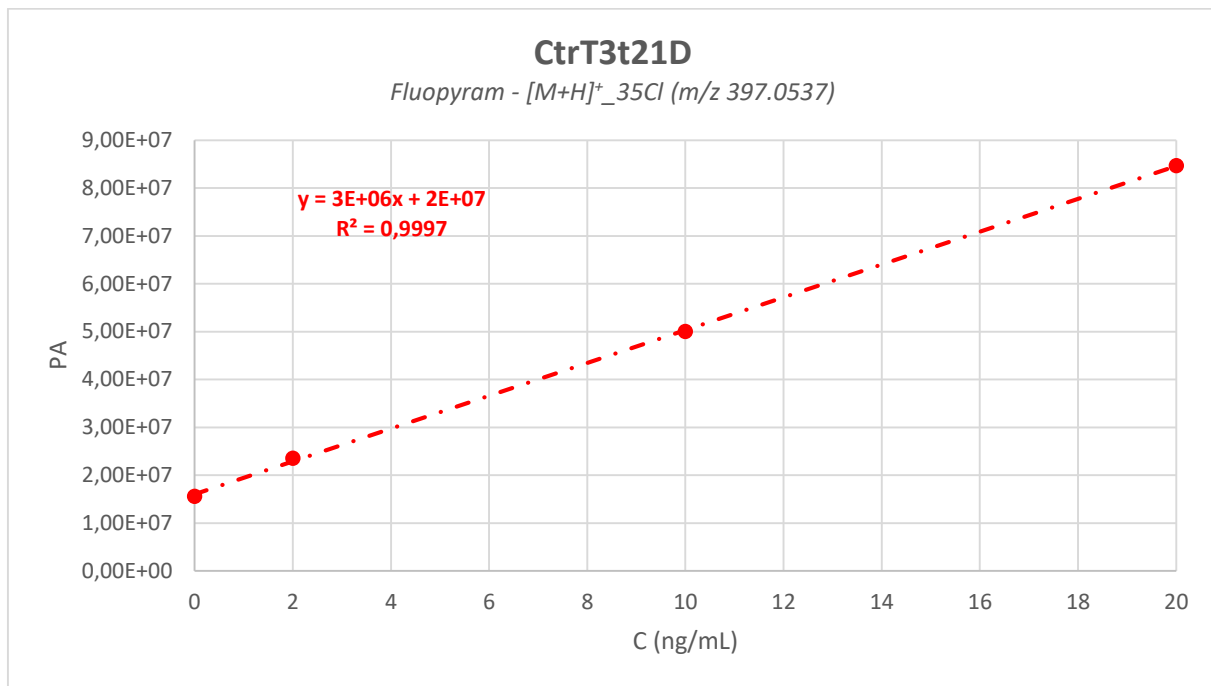


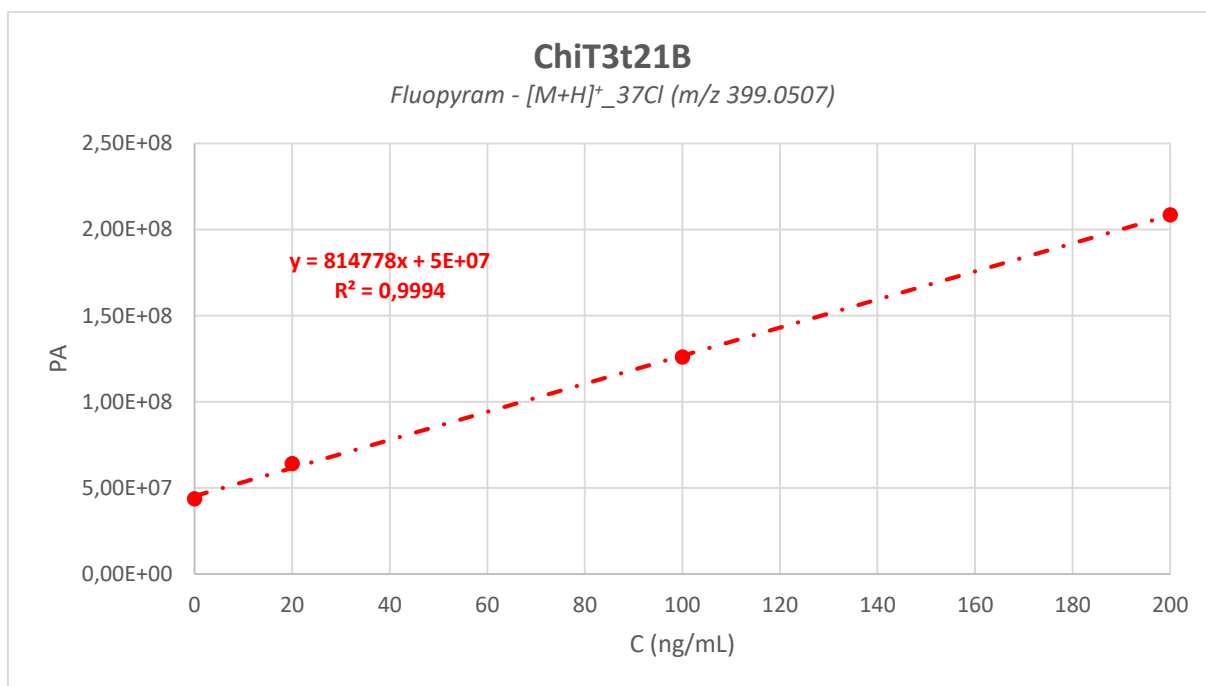
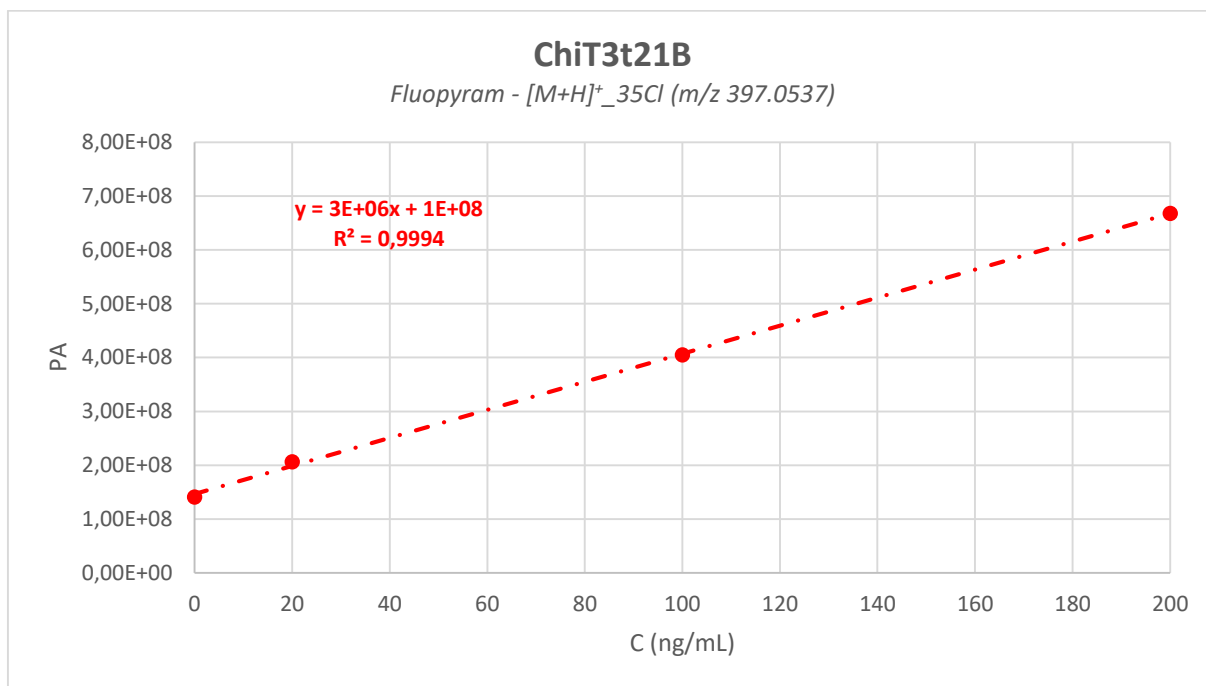


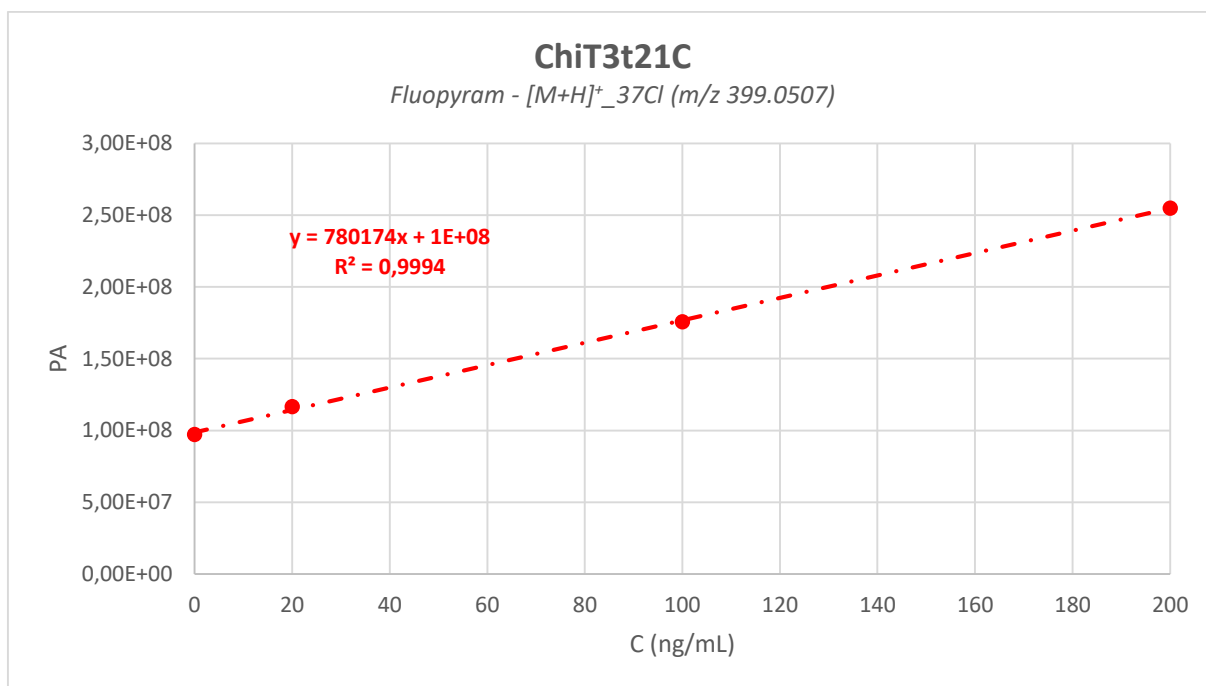
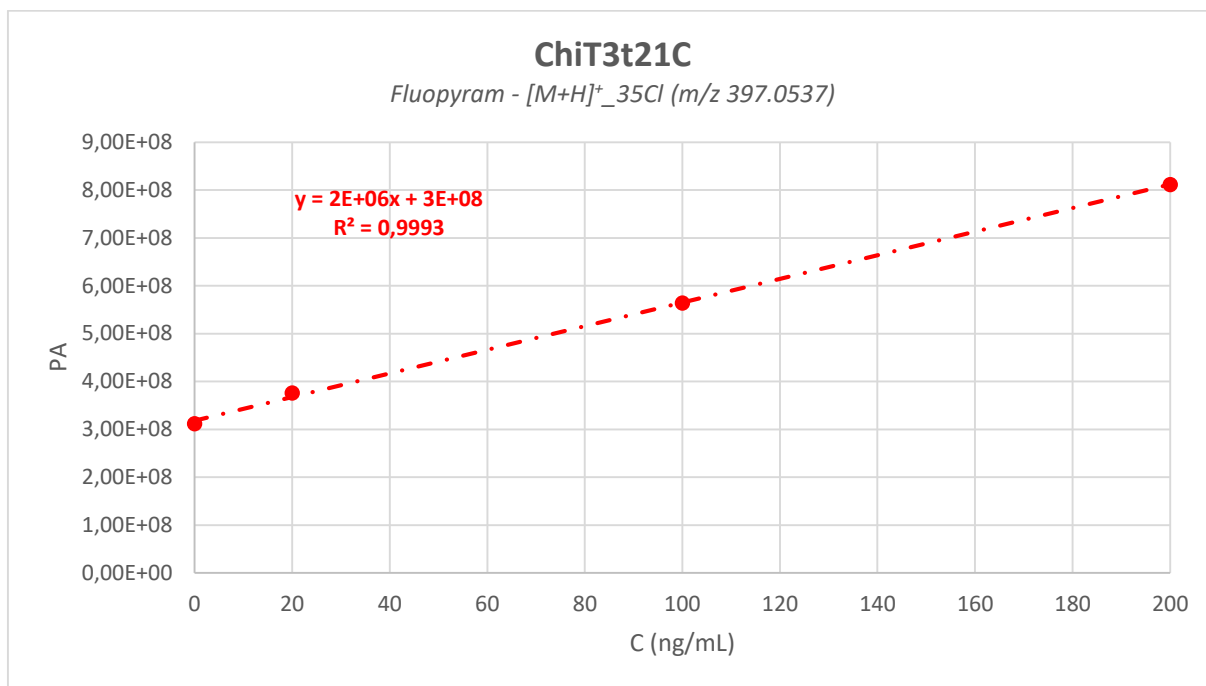


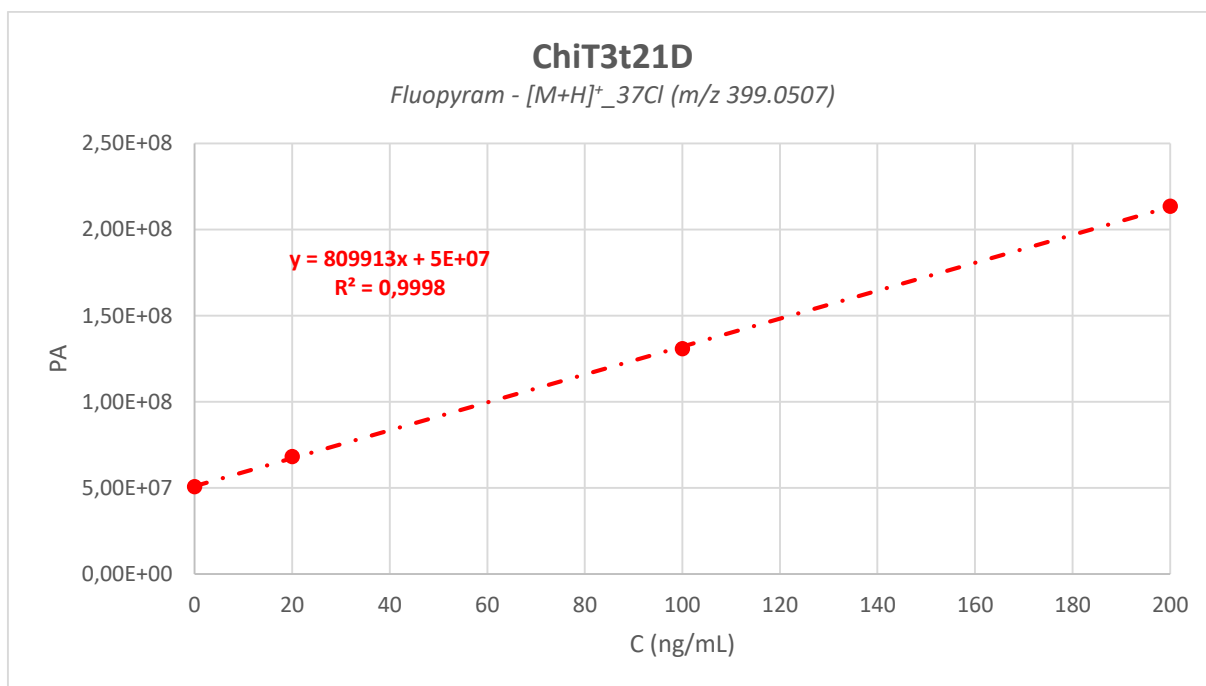
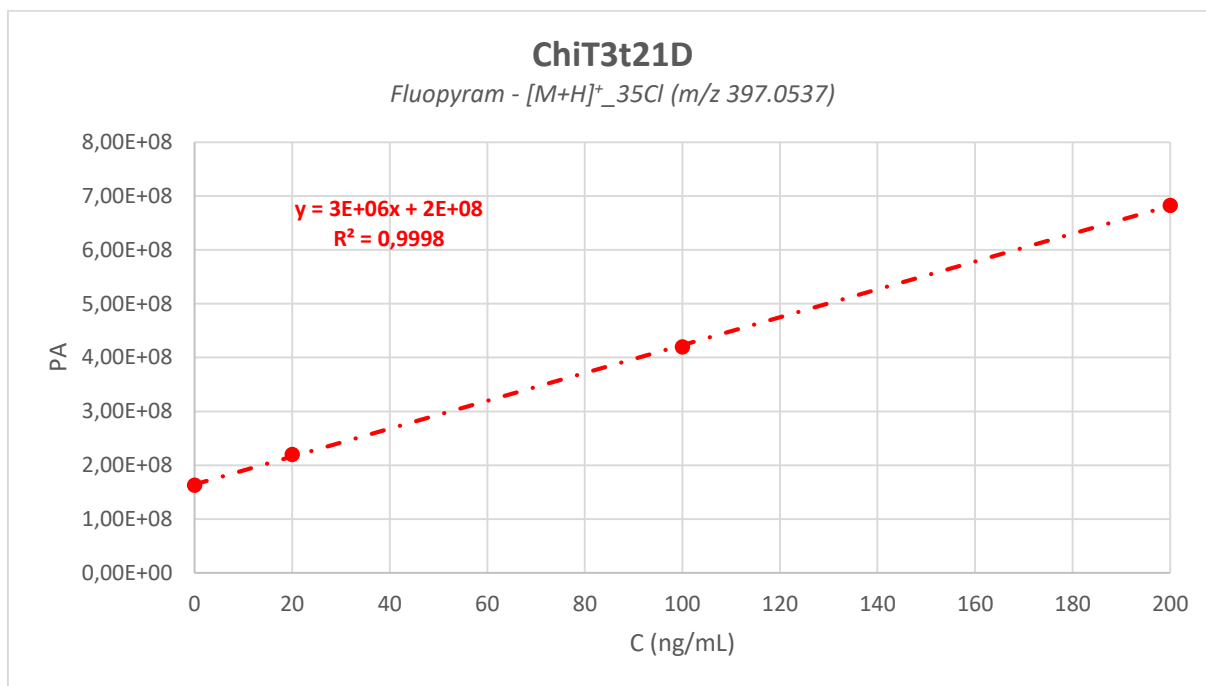


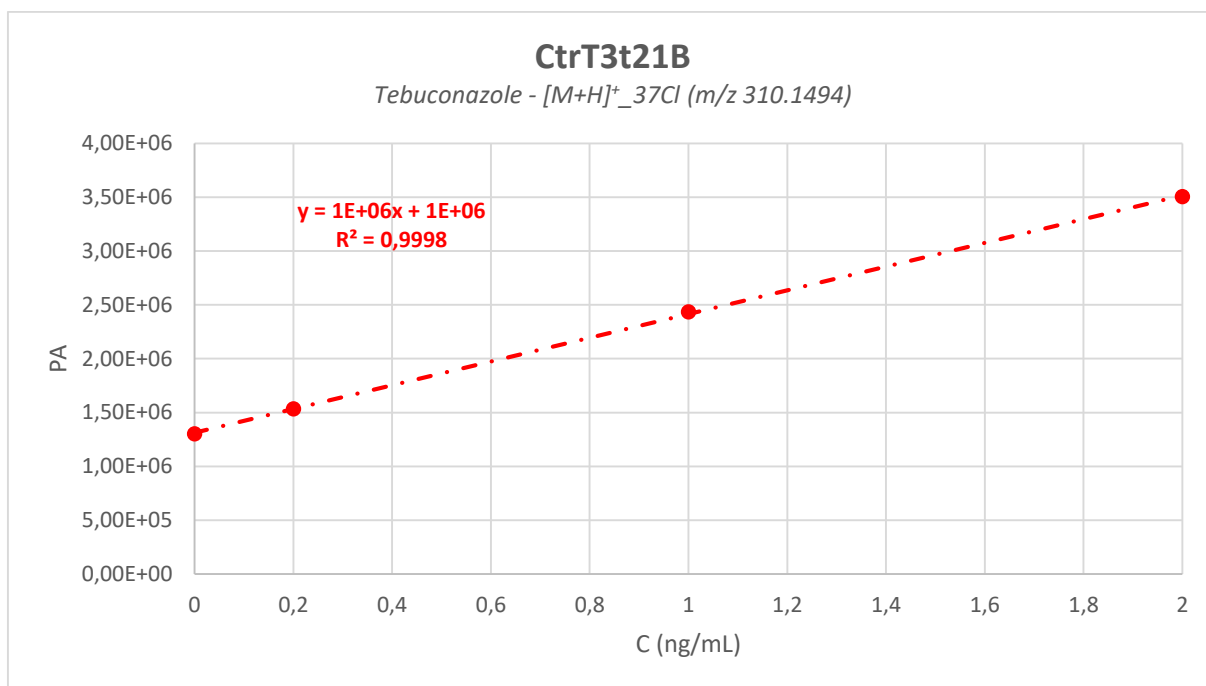
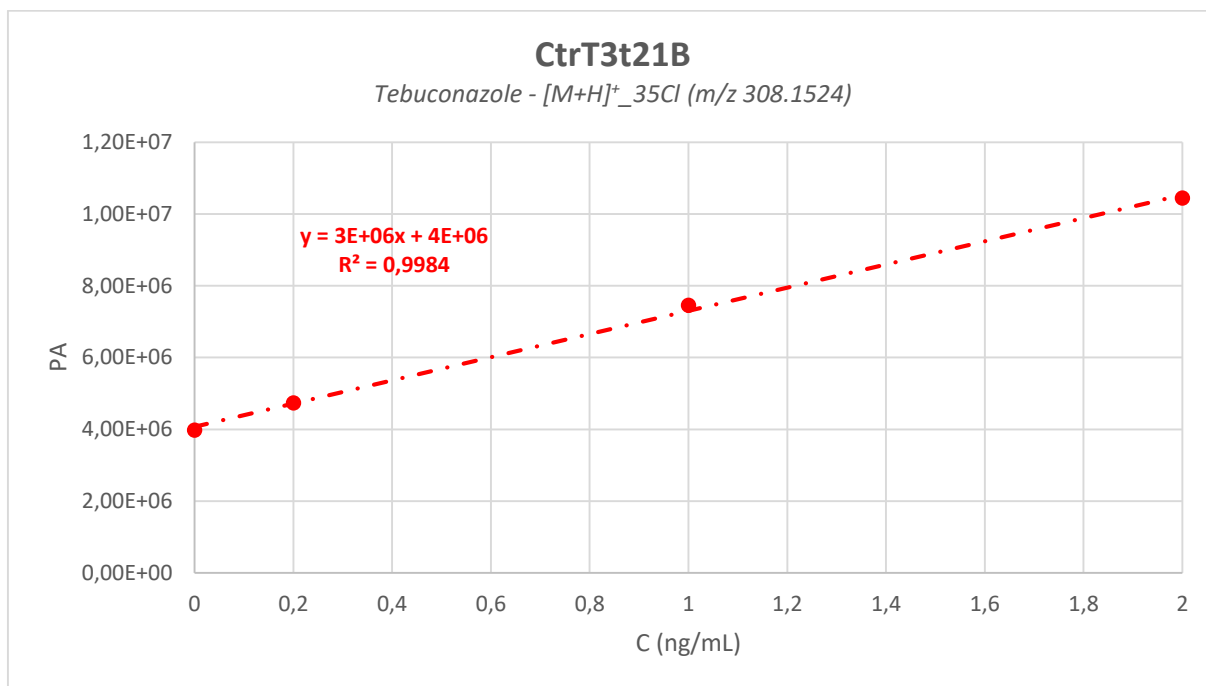




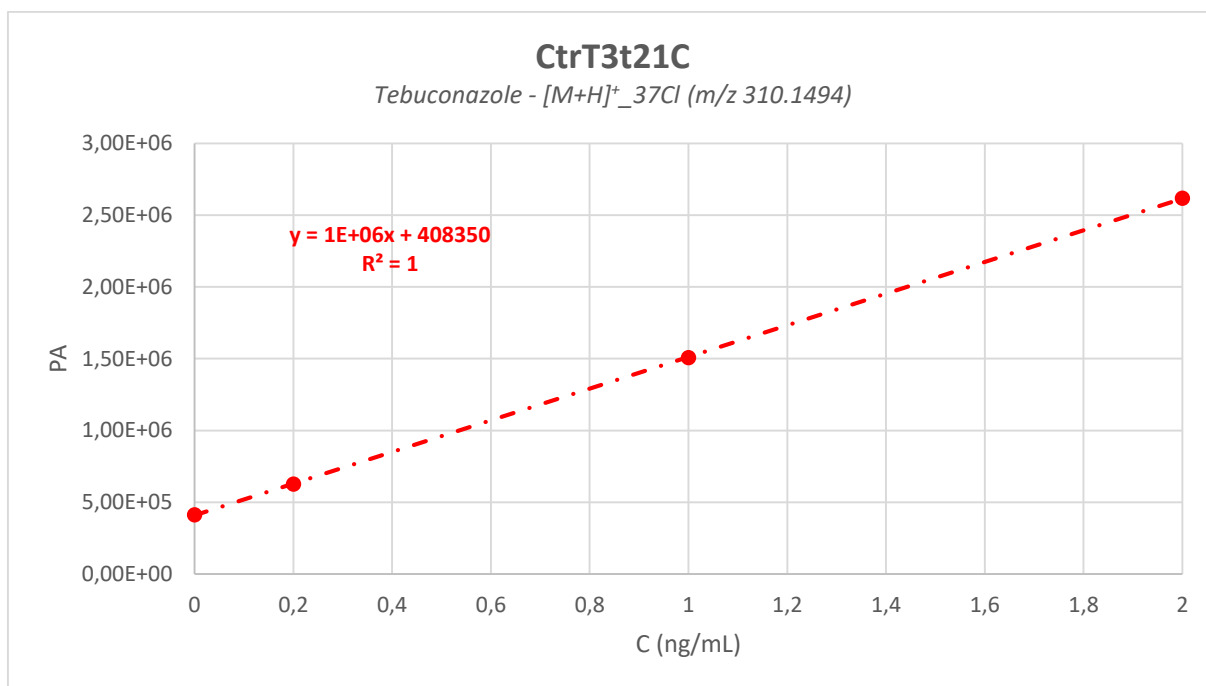
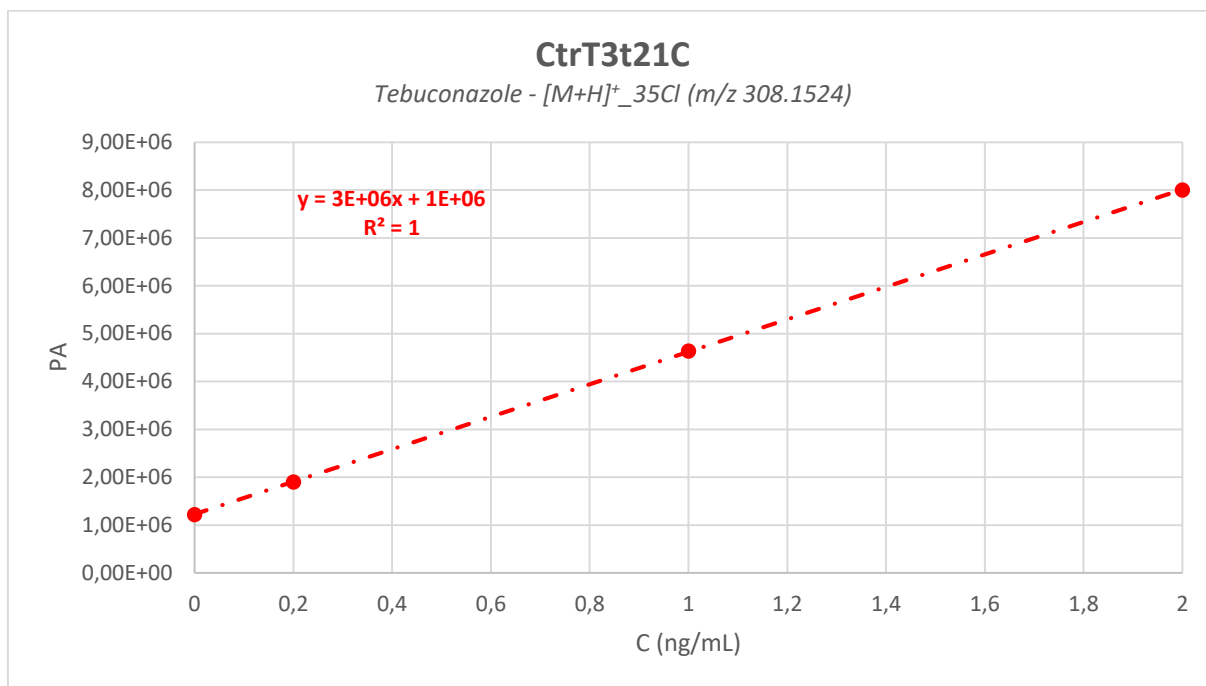


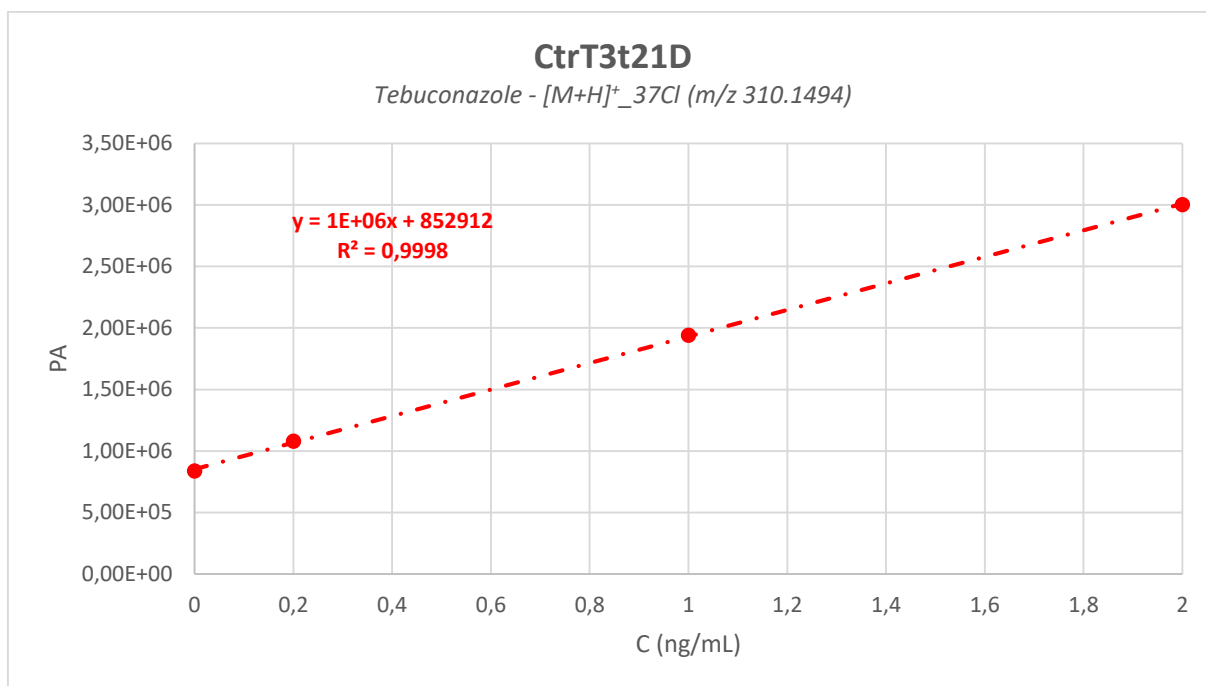
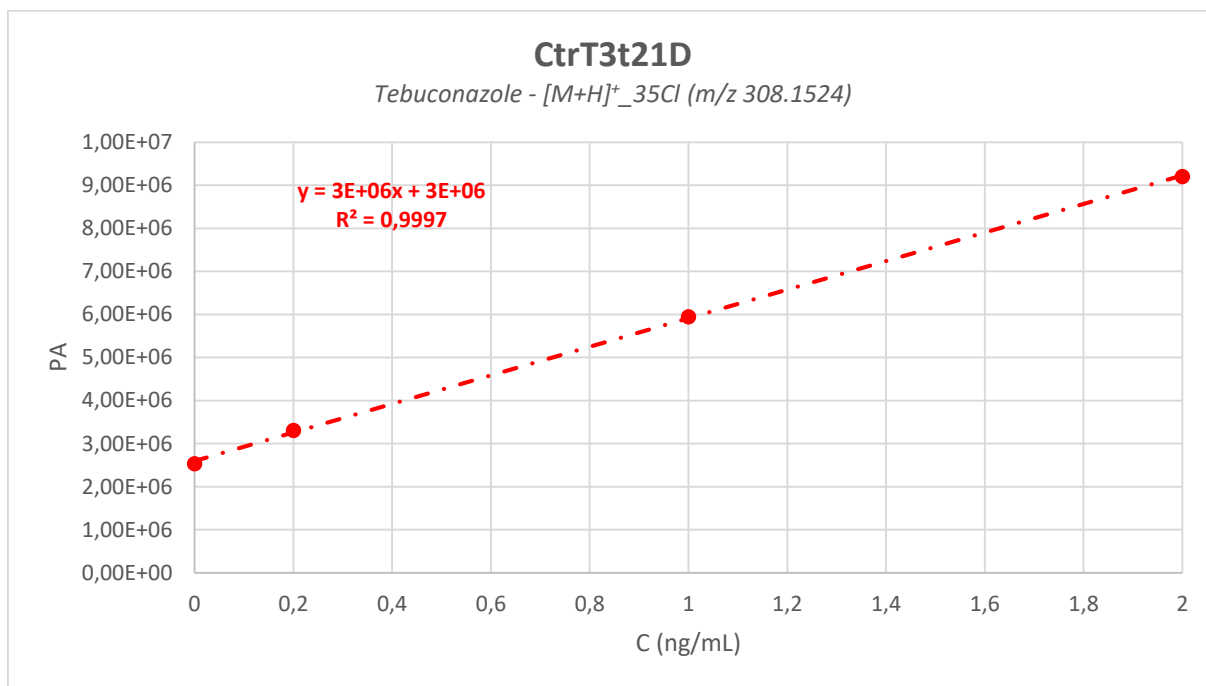


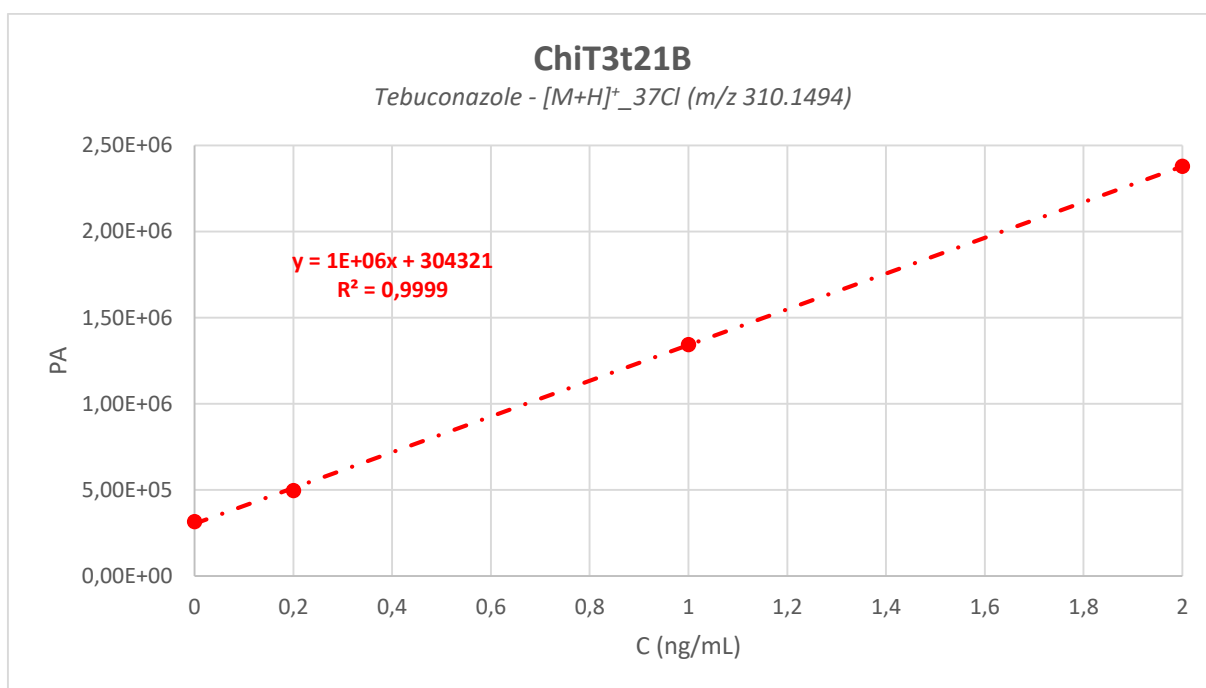
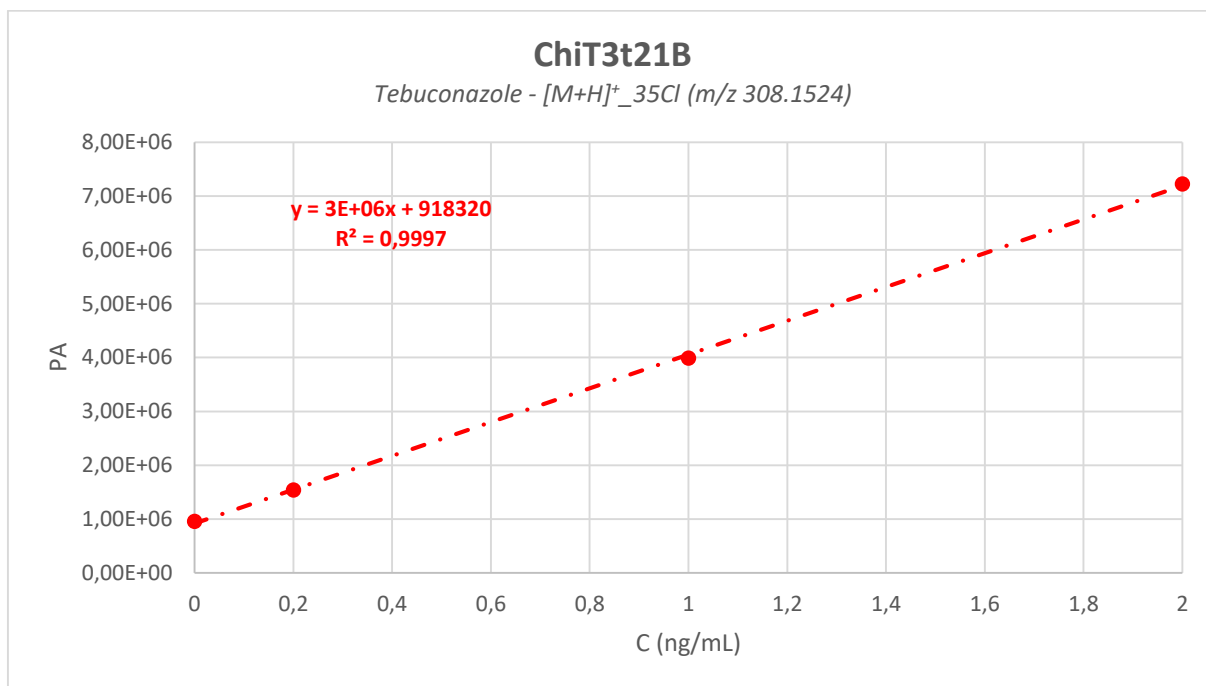


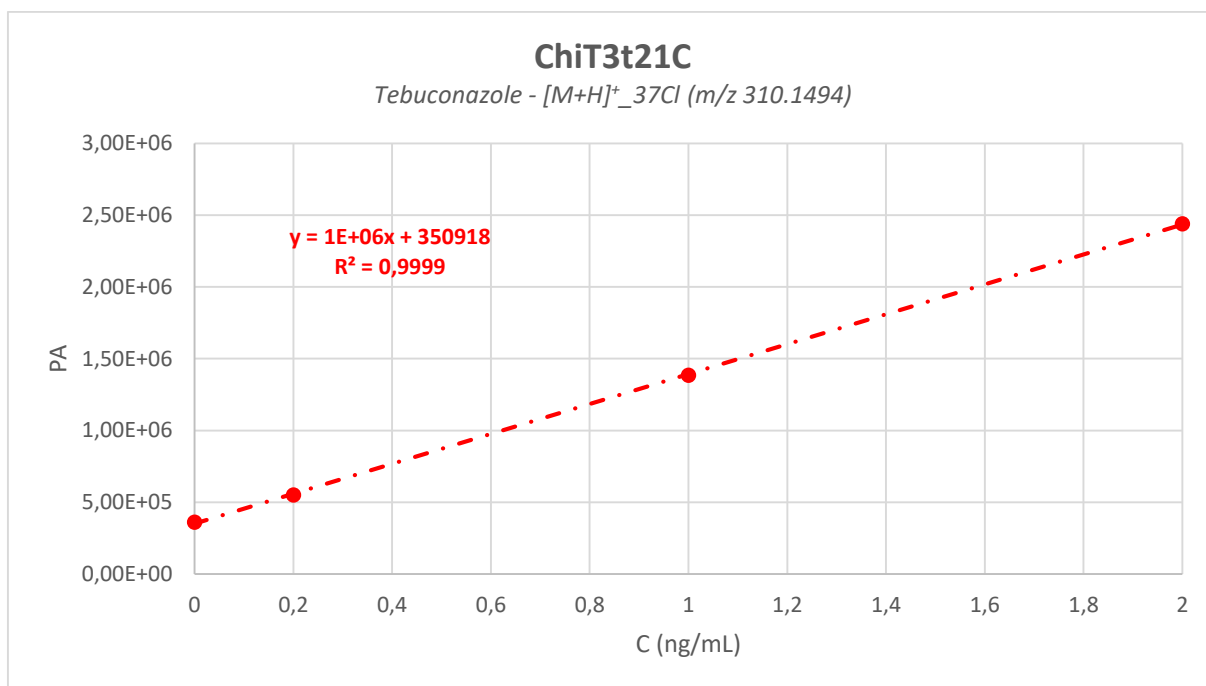
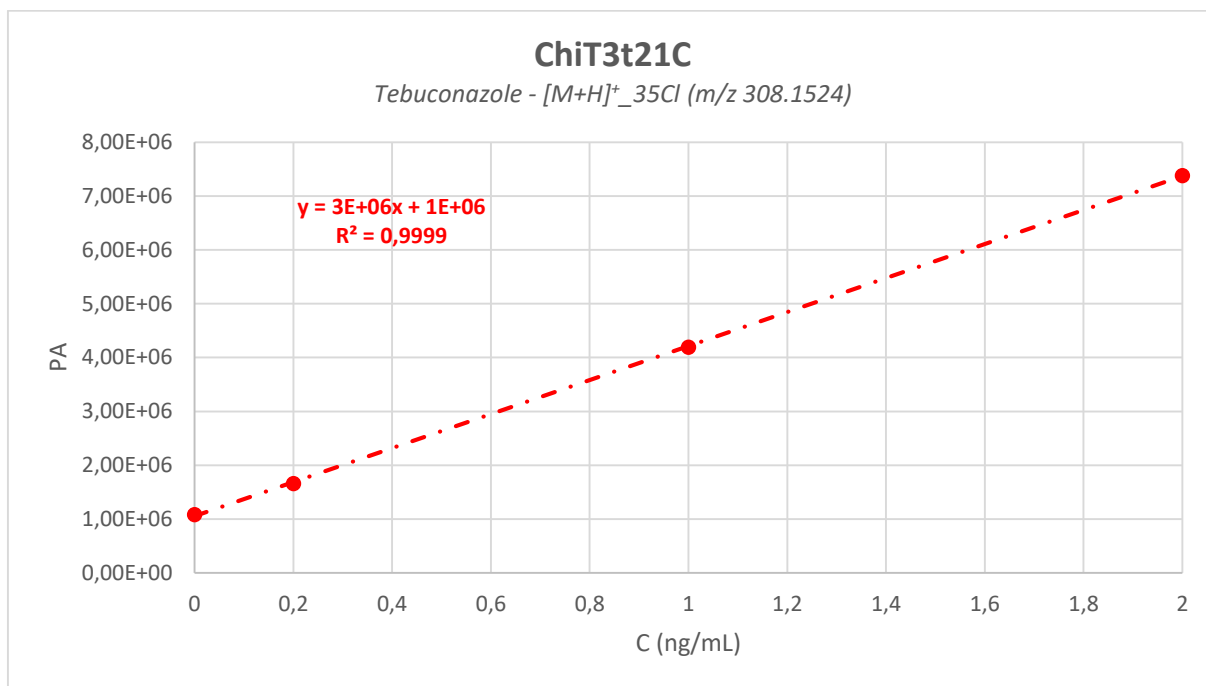


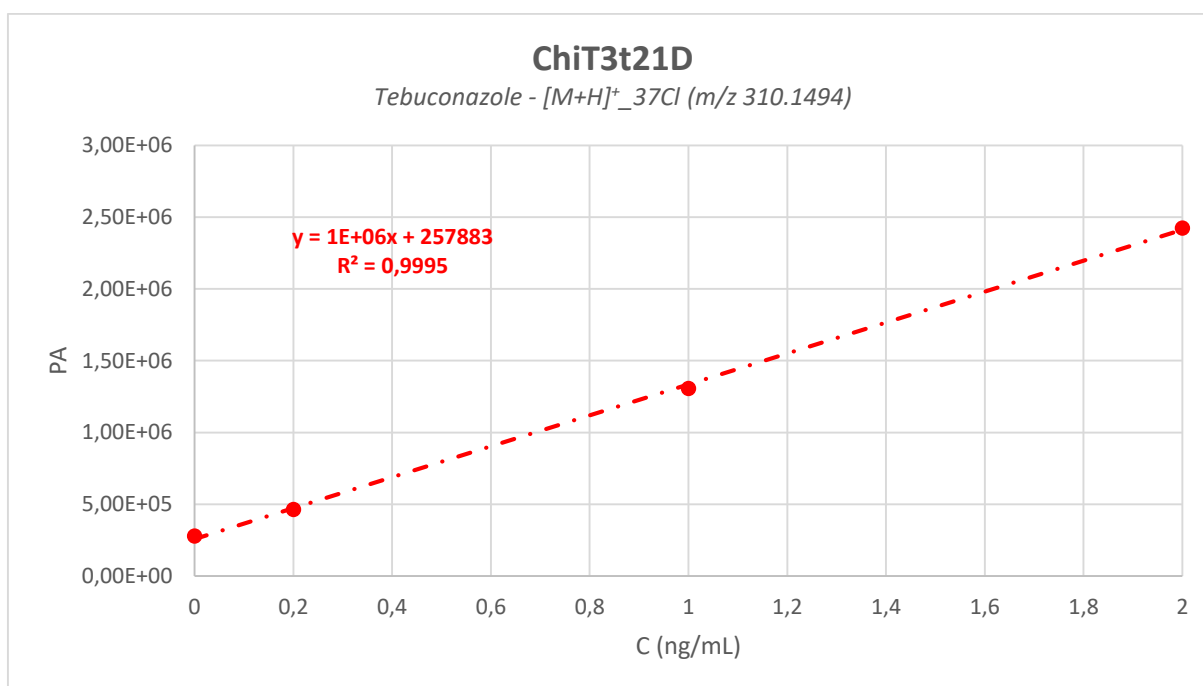
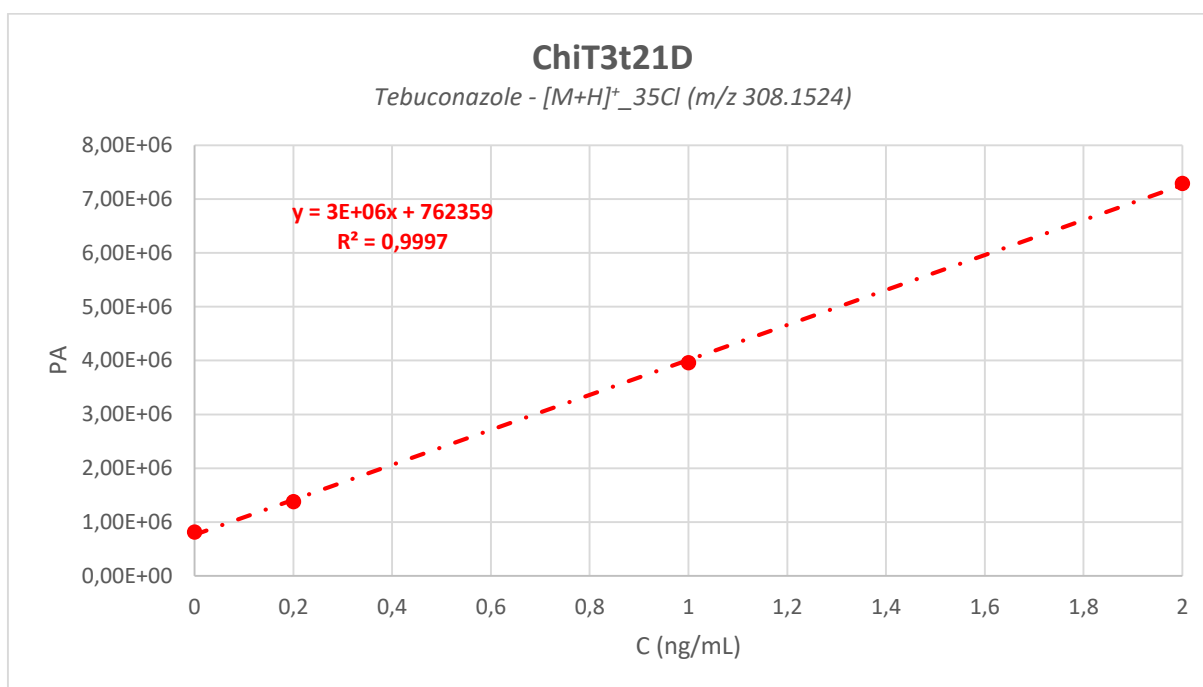












**Figure S11.** Calibration curves of standard addition traced for each standard in each sample integrating the area of the  $[M+H]^+_{35Cl}$  ion and comparing with the  $[M+H]^+_{37Cl}$ . 5 standards were added: Boscalid, Pyraclostrobin, Fenbuconazole, Fluopyram and Tebuconazole. 3 repetitions of “Ctr” contaminated untreated controls samples and 3 repetitions of “Chi” treated samples were used for this experiment.

$$C_f = (C_p \times M_p) / M_f$$

**Formula S1.** Conversion of the concentration per gram of peach peel in concentration per gram of peach (fresh mass).

“*C<sub>f</sub>*”: concentration per gram of peach fresh mass;

“*C<sub>p</sub>*”: concentration per gram of dried peach peel;

“*M<sub>p</sub>*”: mass of dried peel of 3 peaches;

“*M<sub>f</sub>*”: mass of 3 fresh peaches.



## **Chapter 3.**

# **Characterization of the Plant Response to Treatment with Biocontrol Products**





This second part laboratory investigations were realised during the 13 months I spent at the Universitat de Girona (UdG) and the 5 months I spent at the Universidad Autonoma de Barcelona (UAB). As previously mentioned, the main objective of the present PhD work is to characterize new BPs in terms of residue's fate monitoring and plant response to the treatment. More specifically in this Chapter 3 we aim to evaluate the plant response to new BPs treatments to have insights on BPs interaction with the crop and maybe identify the BPs modes of action. As previously described [Chapter 1. - 4.1.], two types of BPs are likely to have special interaction with the treated crop; in fact, natural extracts and beneficial microbials are able to induce resistance to the pathogen by triggering plant defense responses. Thus, a botanical extract formulated prototype (Akivi, AkiNaO), and a microorganism (*Bacillus velezensis*, bacterial strain isolated and produced by the UdG) were investigated in this Chapter 3. These BPs candidates are developed and tested in PALVIP project; they gave promising results during field experiment on grapevine (*V. vinifera*) against powdery mildew (*E. necator*). No previous studies were conducted on grapevine response to these products so transcriptomics, an untargeted holistic approach, was chosen. This investigation was conducted in greenhouse controlled conditions using different local grapevine cultivars also used in PALVIP field experiments: Garnacha Blanca, Garnacha Tinta, and 'Macabeo'. After transcriptomics analysis, targeted metabolomics study was conducted on the same samples to investigate treatment impact on metabolites concentrations.

## GRAPEVINE RESPONSE TO PLANT EXTRACT AND MICROBIAL BIOCONTROL PRODUCTS

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**Keywords:** biocontrol products, transcriptomics, RT-qPCR, gene markers, metabolites, grapevine

### ABSTRACT

The present study aims to evaluate the response of the three Mediterranean local grapevines ‘Garnacha Blanca’, ‘Garnacha Tinta’, and ‘Macabeo’ to treatments with biocontrol products (BPs), a botanical extract formulated prototype (Akivi, *Dittrichia viscosa* extract) and a beneficial microorganism (*Bacillus* UdG, *Bacillus velezensis*). A combination of transcriptomics and metabolomics approaches were chosen in order to study grapevine gene expression and to identify gene marker candidates, as well as, to determine grapevine metabolites differentially concentrated in response to BPs treatments. Grapevine plants were cultivated in greenhouse controlled conditions and submitted to the treatments, and thereafter, leaves were sampled 24h after treatment to conduct gene expression study by RNA-sequencing for ‘Garnacha Blanca’ leaves extract and by RT-qPCR for the three cultivars. Differentially expressed genes (DEGs) were investigated for both treatments and highly influenced DEGs were selected to be tested in the three cultivars as treatment gene markers. In addition, extraction of leaf components was performed to quantify metabolites such as phytohormones, organic acids, and phenols. Considering all the upregulated and downregulated genes and enhanced metabolites concentrations, the treatments had an effect on jasmonic acid, ethylene, and phenylpropanoids defense pathways. In addition, several DEG markers were identified presenting a stable overexpression after the treatments in the three grapevine cultivars. These gene markers could be used to monitor the activity of the products in field treatments in future research. Further research will be necessary to confirm these first results under field conditions.

## 1. INTRODUCTION

The European Union is the main world producer, consumer, and exporter of grapevine for wine-making (*Vitis vinifera*), and the production is mainly concentrated in three countries: Italy (29.7%), Spain (27.1%), and France (24.2%) (European Commission, 2021a). Vineyards are threatened by several diseases, including powdery mildew and gray mold caused by the fungal pathogens *Erysiphe necator* and *Botrytis cinerea*, respectively, and downy mildew caused by the oomycete *Plasmopara viticola* (Boubakri *et al.*, 2012, 2013; Chambre d'Agriculture Rhône-Méditerranée *et al.*, 2014; Krzyzaniak *et al.*, 2018; Rienth *et al.*, 2019; Balestrini *et al.*, 2020). These causal agents are able to infect several grapevine tissues starting from flower and leaves (*E. necator*), from leaves (*P. viticola*), and from berries (*B. cinerea*). If the first infections are not controlled, the diseases spread quickly in the vineyard and mildews can infect berries as well. These diseases can cause severe crop losses depending on the season and the cultivation area, reducing the harvest quality and yield, plant vigor and photosynthesis (Calonnec *et al.*, 2006; Boubakri *et al.*, 2012; Leroy *et al.*, 2013; Krzyzaniak *et al.*, 2018; Balestrini *et al.*, 2020; Beris *et al.*, 2021; Kunova *et al.*, 2021; Mian *et al.*, 2021).

The main grapevine cultivars are susceptible to these diseases, thus, vineyard protection requires intensive treatments with plant protection products (PPPs), such as chemical fungicides from bud burst until ripening (Boubakri *et al.*, 2013). The frequency average of the applied fungicide treatments is around ten treatments per year, which can rise up to 20 treatments under the most critical conditions (Butault *et al.*, 2010; Leroy *et al.*, 2013; Pertot *et al.*, 2017). This intensive use of PPPs can affect the treated crops, the environment, and consumer health as well (Alavanja *et al.*, 2004; Boubakri *et al.*, 2012, 2013; Krzyzaniak *et al.*, 2018; Zambito Marsala *et al.*, 2020). To prevent negative impact of the intensive use of synthetic PPPs, more environmentally friendly compounds, such as biocontrol products (BPs) are promoted by European governments (European Parliament and Council of the European Union, 2009a, 2009b). Among the different types of BPs, there are natural extracts derived from plant, animal, or mineral extracts and beneficial microorganisms able to protect the plant from pests and diseases (Herth, 2010).

Natural extracts as well as beneficial microorganisms used as BPs present modes of action mainly relying on (i) direct action against the pathogen (Bonaterra *et al.*, 2012; Persaud *et al.*, 2019) or (ii) indirect action by stimulating plant defense (Perazzolli *et al.*, 2011, 2012; Pieterse *et al.*, 2014; Rienth *et al.*, 2019; Nishad *et al.*, 2020; Burdziej *et al.*, 2021). It has been reported that a plant extract from *Vitis* cane presents direct activity in grapevine against downy mildew (Schnee *et al.*, 2013); and some beneficial microbials are able to compete against pathogens for space and nutrient supplies (Bonaterra *et al.*, 2012) or to show antagonism

activity against pathogens through antimicrobial or lytic enzyme production (Ongena and Jacques, 2008; Wang *et al.*, 2013; Mora *et al.*, 2015; Vilà *et al.*, 2016). Moreover, laminarin (algae extract) and chito-oligosaccharides associated with oligogalacturonides (COS-OGA) (Van Aubel *et al.*, 2014; Bodin *et al.*, 2020) are already used in vineyards as plant defense stimulators, and protect grapevine against downy mildew and powdery mildew. Some beneficial microbials BPs are already authorized and used in vineyards (Otoguro and Suzuki, 2018), such as a *Bacillus subtilis*-based product that shows antagonism activity against gray mold (Maachia *et al.*, 2015) and a *Saccharomyces cerevisiae* cell wall derivatives based product that induce resistance against downy mildew, gray mold and powdery mildew (De Miccolis Angelini *et al.*, 2019). BPs with a combination between the two types of mechanisms are described as well (Krzyzaniak *et al.*, 2018; Esmael *et al.*, 2020; Zhou *et al.*, 2020).

Plant defense response to biotic stresses relies on different levels of recognition. After pathogen infection, molecular patterns or effectors of the pathogen are recognized leading to (pathogen-associated molecular patterns) PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI). Both PTI and ETI stimulate plant systemic acquired resistance (SAR) (Abdul Malik *et al.*, 2020). Beneficial microbials recognition can also trigger plant defense response called induced systemic resistance (ISR). SAR and ISR responses involve phytohormones, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), being SA more specific to SAR and JA/ET pathway to ISR (Pieterse *et al.*, 2014). It is reported that SA is involved in the defense against biotrophic pathogens, including *P. viticola* and *E. necator*, whereas JA/ET pathway against necrotrophic pathogens, such as *B. cinerea*. However, the two pathways can be activated simultaneously (Burdziej *et al.*, 2021). Direct application of phytohormones or analogues are able to trigger defense response in grapevine against downy mildew (Bodin *et al.*, 2020; Burdziej *et al.*, 2021). However, BPs modes of action are not always well-understood in several plant species and cultivars, although it is important to assess that they have no impact on the treated plants or on the environment.

This study aims to evaluate the response of three Mediterranean local grapevine cultivars: Garnacha Blanca, Garnacha Tinta, and Macabeo to BPs treatments. Two BPs from different sources were investigated: a botanical extract formulated prototype (Akivi, *Dittrichia viscosa* extract) and a beneficial bacterial strain (*Bacillus* UdG, *Bacillus velezensis* living bacteria). These products are still in development, thus, a combination of transcriptomics approach with no *a priori* and a targeted metabolomics approach were chosen to explore grapevine response to them. The objectives of this work are to: (i) study grapevine gene expression response after BP treatment using transcriptomics; (ii) identify robust gene marker candidates presenting stable differential expression after treatment within the three

grapevine cultivars; and (iii) determine grapevine metabolites variations after BP treatment using targeted metabolomics.

## 2. MATERIALS AND METHODS

### 2.1. *Bacillus* UdG Production and Plant Extract

*Bacillus velezensis* UdG strain was isolated from a wild plant collected during a sample screening as reported in Mora *et al.* (2011). *B. velezensis* UdG was routinely cultivated on a Luria-Bertani agar and incubated at 28°C for 24h. For the assays, two different products consisting of lyophilized and fresh cells were prepared.

For lyophilized *Bacillus* (BL), a fermentation process was done in a pilot-scale bioreactor (Biostat® C, Sartorius, Germany) with a working volume of 30 L of production medium for 48 h at 28°C, pH7 and agitation ramp from 50 to 500 rpm. The production medium consisted of a modification of the original recipe of Walker and Abraham (1969). Specifically, the following modifications were considered: 7 g L<sup>-1</sup> instead of 1 g L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub>, 1 g L<sup>-1</sup> instead of 4 g L<sup>-1</sup> of L-monosodium glutamate, 5 g L<sup>-1</sup> of molasses and 1 g L<sup>-1</sup> of soy flour instead of 342 g L<sup>-1</sup> of saccharose, 1 mL L<sup>-1</sup> instead of 5 mL L<sup>-1</sup> of ferric citrate solution, and 1 mL L<sup>-1</sup> of oligoelements solution at 0.1 mg mL<sup>-1</sup> instead of at 0.1 mg L<sup>-1</sup>. After fermentation, the cells were harvested by centrifugation (SA-1-02-175, GEA Westfalia, Granollers, Spain) at 10,000 rpm and the concentrated cell suspension was mixed with skimmed milk (15% final concentration). The bacterial suspension was frozen at -70°C and lyophilized in a laboratory scale freeze-dryer (Unitop HL, VirTis, Gardiner, NY). Dried samples were stored in vacuum sealed plastic-coated aluminium bags.

For fresh *Bacillus* (BF), a fermentation process was carried out in a 2-L Erlenmeyer flask for 48 h at 28°C and shaking at 150 rpm with 500 mL of the original recipe of production medium (modification: oligodynamic solution was used at 0.1 mg mL<sup>-1</sup> instead of at 0.1 mg L<sup>-1</sup>). After fermentation, the cells were harvested by centrifugation at 13,200 g for 10 min (Centrifuge 5810R, Eppendorf) and concentrated 10X with the corresponding volume of supernatant.

The plant extract Akivi (AkiNaO, France) was obtained from *Dittrichia viscosa* composed of a high content of polyphenols and terpenes (Tamm *et al.*, 2017).

### 2.2. Plant Material, Treatments, and Experimental Design

Three grapevine cultivars (*Vitis vinifera* L.), namely Garnacha Blanca, Garnacha Tinta and Macabeo, grafted on rootstock 110R, were obtained from commercial nurseries (Agromillora Iberica and Viveros Villanueva Vides, Spain). One-year-old bench-grafted

grapevine rootlings were planted in a 2 L pot with 80% of the growing media (Prodeasa BV35, Burés Profesional, Spain), 20% of perlite (A-13, Agroteibe, Spain), and 4 g of the fertilizer (Osmocote® Exact Mini 3-4M, ICL Specialty Fertilizers, France). Bench-grafted grapevines were grown in a greenhouse at  $25 \pm 2^\circ\text{C}$ ,  $60 \pm 10\%$  relative humidity and a 16:8 h light:dark photoperiod. Young stocks with at least about 4 to 6 expanded leaves were used for the experiments.

The treatments consisted of Akivi at  $0.521 \text{ g L}^{-1}$  (Aki), and *Bacillus* UdG at  $10^8 \text{ CFU mL}^{-1}$  lyophilized (BL) and fresh (BF). The BF treatment was only used in the experiment with cv. Garnacha Blanca. A non-treated control (NTC) was included in all the experiments. The products were sprayed on adaxial and abaxial leaf surfaces using an airbrush until near run-off.

The experimental design for cv. Garnacha Blanca stocks included 4 randomized blocks corresponding to the different treatment modalities (Aki, BL, BF, and NTC), while for cvs. Garnacha Tinta and Macabeo included 3 blocks (Aki, BL, and NTC). Each block was composed of 4 biological replicates of 5 plants.

### 2.3. Sampling Plant Material and RNA Isolation

Sampling was carried out 24 h after spraying plants with the products. Four biological replicates were sampled for each treatment for RNA-sequencing analysis (RNA-seq), and three biological replicates for reverse transcription quantitative PCR (RT-qPCR) analysis. Two leaves from each plant (5 plants per repetition) were harvested, grounded, and soaked in liquid nitrogen. Each ground leaf sample was added to 2 mL tubes containing two borosilicate glass beads in order to obtain a fine powder using Tissuelyzer II system (Qiagen, USA) for 1 min at 30 Hz.

For total RNA isolation from grapevine leaves, the commercial kit Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) was used (**Supplementary Table 1**) following manufacturer's instructions. Residual DNA was removed using Invitrogen™ TURBO DNA-free™ Kit (Applied Biosystems, USA).

The concentration and purity of RNA was assessed by spectrophotometric measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA quality was evaluated using electrophoresis on 1.0% agarose gels.

Prior to RNA-seq analysis, a R.I.N. measurement was carried out using an Agilent 2100 Bioanalyzer (Agilent technologies, USA) to check RNA integrity from cv. Garnacha Blanca samples and RNA extracted in each sample was quantified by using the Qubit 2.0 Fluorometer (Invitrogen, USA).

## 2.4. RNA-sequencing (RNA-seq) and Reads Mapping

The plant response to treatments using transcriptomic was studied on cv. Garnacha Blanca grapevine leaves after spray application with Aki, BL, BF or water (NTC). A total of 16 samples were used for the library construction.

The RNA-seq transcriptome library was prepared using the TruSeq Stranded mRNA Sample Prep kit (Illumina, USA) following the manufacturer's instructions using 1-2 µg of good quality RNA (R.I.N. > 7) as input. The RNA was fragmented 3 minutes at 94°C and every purification step was performed by using 0.81X Agencourt AMPure XP beads. Final libraries were quantified by using the Qubit 2.0 Fluorometer (Invitrogen, USA) and quality tested by Agilent 2100 Bioanalyzer RNA Nano assay (Agilent technologies, USA). Libraries were then processed with Illumina cBot for cluster generation on the flowcell, following the manufacturer's instructions and sequenced on paired-end (2x150 bp, 30M reads per sample) at the multiplexing level requested on NovaSeq6000 (Illumina). The CASAVA 1.8.2 version of the Illumina pipeline was used to processed raw data for both format conversion and de-multiplexing.

Raw sequence files were first subjected to quality control analysis by using FastQC v0.10.1 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) before trimming and removal of adapters with BBDuk (<https://jgi.doe.gov/data-and-tools/bbtools/>) setting a minimum base quality of 25 and a minimum read length of 35 bp. Reads were then mapped against the *V. vinifera* L. genome (*V. vinifera* cv. Pinot noir var. PN40024) (version 12X Ensembl) with STAR v2.6 (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3530905/>). FeatureCounts v1.6.1 (<https://academic.oup.com/bioinformatics/article/30/7/923/232889>) was then used to obtain raw expression counts for each annotated gene using only uniquely mapping reads (MAPQ>=30). The differential gene expression (DGE) analysis was conducted with the R package edgeR (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796818/>) using the Trimmed mean of M-values (TMM) normalization method and considering as significant the genes with a False Discovery Rate (FDR) ≤ 0.05. Fragments Per Kilobase Million (FPKM) were obtained with edgeR. Gene Ontology Enrichment Analysis was performed using in-house scripts based on the AgriGO publication (<https://academic.oup.com/nar/article/45/W1/W122/3796337>). The main biological functions were selected considering the Gene Ontology (GO) terms that showed at least 4 affected DEGs. Then the selected GO terms were analyzed using REVIGO web platform (<http://revigo.irb.hr/>) in order to summarize GO terms by removing redundancies. For each biological function category, different GO terms clusters (representative groups) that presented semantic similarity were obtained. The affected DEGs corresponding to all the GO terms of each cluster



were added. In addition, GO terms that presented a background number over 1000 genes (BG-Item) were discarded since they are general GO terms. Clusters that showed less than 10 DEGs were joined under the term “other” considering the total number of genes. In addition, metabolic pathways influenced by the treatments were defined using Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation (Kanehisa *et al.*, 2016). KEGG pathways with a corrected p-Value < 0.05 were considered significantly influenced by the treatments.

## 2.5. Screening of Differentially Expressed Genes (DEGs)

Screening of DEGs was carried out for each treatment modality (Aki, BL and BF) in comparison with the NTC. The two *Bacillus* modalities were studied together to identify common genes exclusively due to the bacteria activity, eliminating the effect of the freeze-drying.

Gene expression levels were assessed on the basis of unique mapped genes and were calculated using the FPKM method. FPKM values were used to analyze the differences in gene expression between treatments (Aki, BL, and BF) and NTC, by calculating a Fold-Change (FC) value.

Due to the high biological variability, the DEGs screening was conducted on the three biological replicates that presented less variability between each other, in order to avoid hiding a part of the treatment impact on the plant. For each type of treatment, DEGs exclusively altered by the treatment were targeted. The criteria of selection during the screening were based on DEGs presenting high differential expression value, specifically  $\text{Log}_2(\text{FC}) > |1.4|$  and DEGs presenting good repeatability among the three biological replicates.

## 2.6. Validation of DEGs by RT-qPCR

To confirm the transcriptome data obtained by RNA-seq analysis, 27 DEGs were selected ( $\text{Log}_2(\text{FC}) > |1.4|$  and good repeatability among the three biological replicates) and their expression level was validated by RT-qPCR (**Table 1**). The UBQ gene, coding for the Ubiquitin-conjugating enzyme, was used in this study as the endogenous gene for data normalization. This endogenous gene was previously selected according to the method described by Silver *et al.* (2006) (**Supplementary Figure 1**). The main purpose was to evaluate the 27 DEGs as suitable treatment-related markers on different grapevine cultivars.

**Table 1.** Differentially Expressed Genes (DEGs) and endogenous genes primer sequences used in the present study. The optimized primer concentrations for qPCR analysis are also shown.

Code	Gene ID	Gene description	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	[primer] (nM)
<b>Endogenous genes for expression data normalization</b>					
E1	EC922622	Ubiquitin-conjugating enzyme (UBQ)	GAGGGTCGTCAGGATTTGGA	GCCCTGCACTTACCATCTTTAAG	300
E2	XM_002281110.1	Vacuolar ATPase subunit G (VAG)	TTGCCTGTGTCTCTTGTTTC	TCAATGCTGCCAGAAGTG	300
<b>Selected DEGs impacted by <i>Bacillus</i> treatment</b>					
B1	VIT_16s0022g00860	Invertase/pectin methyl esterase inhibitor	GCTGCAAGAAATGTGGAATGC	TCGACTCTGTGACTTTGTTTTCC	100
B2	VIT_06s0004g07210	CCT motif constans-like	CAAGTGCCAGACACCATCCT	ACCAGCACCGCACATACTTT	100
B3	VIT_16s0100g00740	unknown	CCAGACACGTCTGACTCCAC	CAGCTCCACGGTAACTCCTG	300
B4	VIT_14s0068g01160	Cytokinin-repressed protein CR9	AGAAGCCTGCTTGGCAGATT	CCGGAACACCGTTTTTGCAT	300
B5	VIT_00s1490g00010	5'-adenylylsulfate reductase (APR1)	AAGTTCAGGGCTTGGTGAGG	GGGTCTCACTTCTCACACGG	300
B6	VIT_13s0064g01370	Polygalacturonase inhibiting protein 1 PGIP1	AGGCGAGTTTCATGGAGCAG	GGAATTTTCCCACACAACCTGT	300
B7	VIT_09s0002g04280	Dynein light chain LC6, flagellar outer arm	GGGGAAAATAAGGTGCGGGA	ACAGGGCCCTCATCACAATG	300
B8	VIT_03s0091g00310	Indole-3-acetic acid-amido synthetase GH3.8	TCGCCCTTATGACCCCTACA	AGGACTTGTTTGCGCTCGTA	100
B9	VIT_01s0011g01980	Fasciclin arabinogalactan-protein (FLA21)	TTGCATTGTGCAGCAAACGA	GGATGCCACGTGGTCCATAA	100
B10	VIT_01s0026g02740	Unknown	GGTGACTGCACCAGTGATTG	AGTGGCTGCTCTAACAACCT	300
B11	VIT_08s0058g00430	Ferritin	CCTCTCATCTGCATCTTTCTCGT	TCCCCTGACGACCCTAAGAG	300
B12	VIT_10s0116g00530	Thiazole biosynthetic enzyme, chloroplast (ARA6)	TGGCCAGGCCTAATGTGAAG	AACCAAAGCCCAGTTGGTGA	300
B13	VIT_00s0480g00060	Polyphenol oxidase	GCTTTTCTTCCCTTCCACCG	CGGCATTTGCATTCCAGGAG	100
B14	VIT_07s0031g02610	NAC domain containing protein 2	CTCTCCAAGGGACCGCAAAT	AATTCCGACCGTCTTGGGTC	300
B15	VIT_13s0067g02130	Dehydration-induced protein (ERD15)	TATCGGACGGTGGAGGACTT	AGCCAGTAATCGCGAAACCA	300
<b>Selected DEGs impacted by <i>Akivi</i> treatment</b>					
A1	VIT_12s0059g02600	Receptor protein kinase RK20-1	TGTGTCACTGAGGCAACCAA	TCGTACCAAATGATCGCTCC	100
A2	VIT_06s0004g03350	Lateral organ boundaries protein 1	GCGAGCTTCAAGCGCAATTA	AGGTTTGCTTGCTGGCATTG	300

Continue

**Table 1.** (continued)

Code	Gene ID	Gene description	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	[primer] (nM)
A3	VIT_05s0077g00520	Gibberellin 2-oxidase	AATGGGAGGTTTGTGAGCGT	GAAGGCCTCTCAGGTGTGAC	300
A4	VIT_17s0000g00200	Ethylene-responsive transcription factor ERF114	AAGTGGGCAGCTGAGATACG	TAGGCAAGTGCAGCATCCTC	300
A5	VIT_08s0058g00970	Cationic peroxidase	CTCCGCTTGACACCAAAAGC	ACTTGAGAATCCGTGGAGCC	300
A6	VIT_12s0055g01010	Peroxidase	CGCAAAGTGTGCTCTGCAAT	AGTGCATGTGAGAAGTTACGGA	100
A7	VIT_00s0372g00040	1,8-cineole synthase, chloroplast	CAAGGCACAGATGGATGGGT	GCAGCATCTCCTTCTGGTGT	100
A8	VIT_04s0023g02240	S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase	GGGACACCAGTTACGCAGAA	GGTCCAGAAGAACAGCCCAA	300
A9	VIT_12s0034g01140	Plastocyanin domain-containing protein	TAGCCCTTCGGCTCACAATG	AATAGTTGGCCCCCTTCACC	300
A10	VIT_19s0090g00660	Lipase GDSL	AATTGGGCTTACCATCCGCA	TCAAAGATTCCGGCACCTCC	300
A11	VIT_03s0088g00810	Pathogenesis-related protein 1 precursor (PRP 1)	CAATGGAGGGTGGTTTCGTCA	CACCATGCTCTAACAGTACCCA	100
A12	VIT_07s0005g06090	Pore-forming toxin-like protein Hfr-2	TTTACGTTGGCGTGAAGTGC	CAAGGAAGGGGGATTTCGACC	300

Standard curves for DEGs and the endogenous gene were obtained using decimal dilutions of extracted recombinant plasmid DNA (target sequences were cloned into a vector pSpark® in *Escherichia coli* DH5α cells) corresponding to copy numbers ranging between  $10^2$  and  $10^7$ . Ct values in each dilution were measured in triplicate and a negative non-template control was included in each run. Real-time PCR reactions included 10 μL SYBR® Green PCR Master Mix (Applied Biosystems), 6 μL RNase-free water, 1 μL of each forward and reverse primer (**Table 1**) at the corresponding concentration, and 2 μL DNA in a final volume of 20 μL. The optimal primer concentration (100, 300 or 600 nM) was previously defined. The thermal cycling conditions were as follows: 10 min at 95°C for initial denaturation; 40 cycles of 15 s at 95°C, and 1 min at 60°C; and a final melting curve program of 60 to 95°C with a heating rate of 0.5°C s<sup>-1</sup>. Ct values were plotted against the logarithm of their initial template copy numbers and each standard curve was generated by a linear regression of the plotted points. The efficiency of each standard curve was calculated using the formula  $E = (10^{(-1/a)} - 1) \times 100$ , where “a” is the slope of the curve.

For RT-qPCR, total RNA was extracted from leaf samples of treated plants using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) as explained above. First-strand of cDNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The absence of chromosomal DNA contamination was confirmed by minus-reverse transcriptase control in qPCR. Quantitative PCR was carried out in a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) to assess the transcriptional level of 27 DEGs. All the information of selected genes and primers designed by Primer-BLAST tool from the National Centre for Biotechnology Information (NCBI) are shown in **Table 1**. Optimized qPCR reactions and the thermal cycling conditions were described above. Each qPCR assay included duplicates of each cDNA sample, no-template and RNA controls to check for contamination. Ct values from three biological replicates were averaged, and UBQ gene was used for data normalization.

The comparative critical threshold ( $\Delta\Delta Ct$ ) method was used to assess the relative quantification of gene expression. Similar amplification efficiencies of all gene primer pairs were checked (**Supplementary Table 2**) making the  $\Delta\Delta Ct$  method appropriate to calculate the Fold-Change (FC). The  $\Delta Ct$  of the NTC leaf samples was used as the calibrating condition to calculate the FC. Genes were considered to be up- or down-regulated if their FC were at least two-fold ( $FC = 2^1$  or  $2^{-1}$ ) higher or less than the calibrator condition (Livak and Schmittgen, 2001).

## 2.7. Metabolite Analysis

Metabolite extractions were carried out from powdered samples of grapevine leaves of cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo obtained 24 h after spraying them with Aki, BL, or water (NTC) as explained above.

The phytohormones were extracted and purified according to Llugany *et al.* (2013). Briefly, 250 mg of fresh grapevine leaf powder was grounded in an ice-cold mortar with 750  $\mu$ L of extraction solution constituted by methanol:isopropanol:acetic acid (20:79:1 by vol.). Then, the supernatant was collected after centrifugation at 1000 g for 5 min at 4°C. These steps were repeated two more times and pooled supernatants were lyophilized. Finally, samples were dissolved in 250  $\mu$ L pure methanol and filtered with a Spin-X centrifuge tube filter of 0.22  $\mu$ m cellulose acetate (Costar, Corning Incorporated, USA). Phytohormone quantification was done using a standard addition calibration curve spiking control plant samples with the standard solutions of gibberellin A1 (GA1), gibberellin A4 (GA4), methyl jasmonate (MeJA), salicylic acid (SA), ( $\pm$ )-jasmonic acid (JA), (+)-cis,trans-abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) ranging from 5 to 250 ppb and extracting as described above. Deuterated hormones jasmonic acid-d5 (JA-d5) and salicylic acid-d6 (SA-d6) at 30 ppb and 300 ppb, respectively, were used as internal standards in all the samples and standards measurements. Standards were purchased from Merk (Germany).

Plant hormones were analyzed by LC-ESI-MS/MS system in multiple reaction monitoring mode (MRM) according to Segarra *et al.* (2006). Phytohormones were separated using HPLC Acquity (Waters, USA) on a Luna Omega C18 column 1.6  $\mu$ m 100 Å 50 x 2.1 mm (Phenomenex, USA) at 50°C at a constant flow rate of 0.8 mL min<sup>-1</sup> and 10  $\mu$ L injected volume. The elution gradient was carried out with a binary solvent system consisting of 0,1% of formic acid in methanol (solvent A) and 0,1% formic acid in milli-Q H<sub>2</sub>O (solvent B) with the following proportions (v/v) of solvent A (t (min), %A): (0, 2) (0.2, 2), (1.6, 100), (2, 100), (2.1, 2) and (3, 2). MS/MS experiments were performed on an ABI 4000 Qtrap mass spectrometer (Sciex). All the analyses were performed using the Turbo Ionspray source in negative ion mode except for MeJA and ACC.

Quantification was made by injection of extracted and spiked samples in multiple reaction monitoring (MRM) mode. Identification of phytohormones was based on retention time and presence of peak in the MRM trace compared with those of the standards.

Organic acids (OA) were extracted with a classical extraction protocol. Briefly, 250 mg of fresh grapevine leave powder was grounded in an ice-cold mortar with 2 mL of hydrochloric acid (0.025N). Then, the supernatant was collected after centrifugation at 1000 g for 15 min at 4°C. Meanwhile, Sep-Pack C18 cartridges (Waters, USA) were activated with (i) 1.4 mL of

methanol, (ii) 0.7 mL of milli-Q water, and (iii) 1.4 mL of hydrochloric acid (0.025N). Supernatant (1.4 mL) were passed through the cartridge in order to recover 0.7 mL of clean extract. Finally, samples were filtered at 0.22  $\mu\text{m}$ .

OA were analyzed by HPLC-UV system (Shimazu, Japan). OA were separated on a YMC-Pack ODS-A HPLC column 5 $\mu\text{m}$  120 $\text{\AA}$  250 x 4.6 mm (YMC, Germany) at a constant flow rate of 0.8 mL min<sup>-1</sup> and 10  $\mu\text{l}$  injected volume. The injection method is 15 min of isocratic flow of 50 nM Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) adjusted at a pH of 2.8 using Phosphoric acid (H<sub>3</sub>PO<sub>4</sub>).

The following standards were used for OA measurements: acetic acid, cis-aconitic acid, trans-aconitic acid, ascorbic acid, citric acid, isocitric acid, formic acid, fumaric acid, galacturonic acid, gluconic acid, glucuronic acid, glutamic acid, glycine, glycolic acid, glyoxylic acid, lactic acid, maleic acid, malic acid, malonic acid, oxalic acid, oxoglutaric acid, pyruvic acid, quinic acid, succinic acid, tanic acid, and tartaric acid.

Four peaks corresponding to OA were detected on samples HPLC-UV chromatograms. Then, these peaks' retention times were compared with the retention times of 26 standards injected in the same conditions, and the identification was confirmed by standard enrichment injection within the grapevine samples. The four OA were identified (oxalic acid, tartaric acid, malic acid, and oxoglutaric acid) and quantified thanks to calibration curves. Calibration curves: malic acid ( $y=1.2967x+7.0154$ ,  $R^2=0.9967$ ), oxalic acid ( $y=0.2891x+4.7116$ ,  $R^2=0.9993$ ), oxoglutaric acid ( $y=1.4261x+17.324$ ,  $R^2=0.9972$ ), tartaric acid ( $y=2.6801x+2.4512$ ,  $R^2=0.9998$ )

Putatively identification was carried out by comparing the retention time of the standards with the peaks obtained in the grapevine leaves samples. The standard addition to the samples was done to check that the standard matches the targeted peak in leave matrix conditions. Calibration curves were done at an appropriate range for each putatively identified organic acid,  $R^2$  must be above  $>0.99$  to allow quantification. Quantification was made within the samples using the calibration curves.

Phenolic compounds were extracted according to Solecka *et al.* (1999), with modifications (Kidd *et al.* 2001). Briefly, shoots were extracted with 70% methanol and after centrifugation (10 min, 5000 x g) the supernatant was re-extracted three times with ethyl ether to eliminate ether soluble lipids. The remaining water phase was treated with 2 M HCl for acid hydrolysis of soluble conjugated phenolic compounds. After extraction with ethyl acetate and drying, the residue was re-dissolved in 50% methanol. Total phenolic compounds levels were determined by spectrophotometry (Shimadzu UV-2450, Duisburg, Germany) following the method of Folin-Ciocalteau (Slinkard and Singleton 1977), using gallic acid

(Sigma, Steinheim, Germany) as the standard with detection at 765 nm. The results are expressed in Gallic Acid Equivalents (GAE).

## 2.8. Statistical Analysis

Principal Component Analysis (PCA) was applied to the RNA-seq data comparing the biological replicates of each treatment modality with the non-treated control (NTC). The statistical analysis of the RT-qPCR data was done using REST2009 Software (Pfaffl *et al.*, 2002). DEGs standard curves for genes expression quantification were made by linear regression on Excel. Validation of the DEGs was performed by a correlation study between the gene expression measured by RNA-seq and RT-qPCR techniques. Pearson correlation analysis was applied to the data for each treatment modality and the test was made using R software (R version 3.5.2).

For metabolite measurements, to identify significant differences between treated (Aki and BL) and NTC leaves, several statistical tests were performed. All tests were performed on R software (R version 3.5.2) with a significant level of p-Value < 0.05. First, each of the metabolite datasets were tested (Shapiro-Wilk and Bartlett tests) to determine the suitability of parametric or non-parametric tests. For parametric tests, one-way analysis of variance was applied (ANOVA) followed by a Tukey's multiple comparisons test to compare simultaneously the means of every sample. For non-parametric tests, Kruskal-Wallis test followed by Dunn test were applied to compare simultaneously the means of every sample.

## 3. RESULTS

### 3.1. Quality Assessment of RNA-seq Data and Gene Expression Estimation

The results of the 16 sequencing samples produced on average around 39 million of total sequencing raw reads for NTC and Aki treatments, while around 36 million for BL and BF treatments (**Supplementary Table 3**). Following the filtering and trimming process, we obtained an average of 33 (NTC and Aki) and 31 (BL and BF) millions of cleaned reads (85 and 86%, respectively, of the total sequencing reads).

When the reads were paired and aligned to the reference *V. vinifera* L. PN40024 genome, around 14.7 (BL and BF) and 15.8 (NTC and Aki) million reads in average from each treatment could be mapped, (94.9 and 94.6%, respectively, of the input paired reads) (**Supplementary Table 4**). Moreover, between 85.6 (NTC and Aki) and 86.7% (BL and BF) of the input paired reads were assigned to genes.

The overall quality of the experiment was evaluated considering the consistency between biological replicates using the normalized gene expression values (normalization of the FPKM) from each treatment. The PCA analysis revealed that one out of four biological replicates of each treatment (Aki\_R1, BL\_R1, BF\_R1, NTC\_R3) did not cluster as expected from the experimental design (**Supplementary Figure 2**). This variability among replicates could hide some of the treatment effect on gene expression, thus, this replicate was not included in further analysis.

PCA on normalized gene expression using the three biological replicates retained showed that the two first principal components explained 83.57% (Aki), 84.21% (BL) and 85.43% (BF) of the variance. In addition, the PC1 explained 63.33% (Aki), 60.5% (BL) and 49.38% (BF) of the variability in gene expression between each treatment and the NTC.

The RNA-seq raw transcriptomic data were submitted to the National Center for Biotechnology Information (NCBI) (in process).



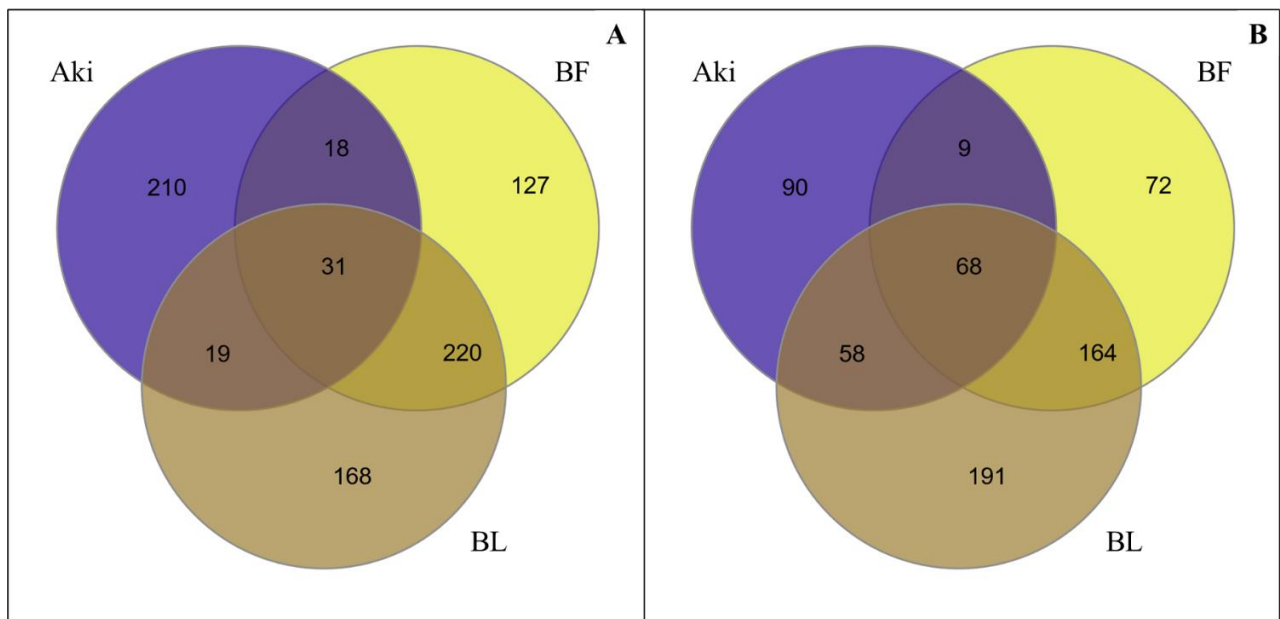
### 3.2. Analysis of the Differential Expression of Genes (DEG) After the Treatments

Gene transcription in cv. Garnacha Blanca grapevine leaves was triggered by Aki, BL and BF treatments to varying degrees. The volcano plots show the degree of variation of the Differential Expression of Genes (DEGs) based on red and green dots (**Supplementary Figure 3**). The relationship between the fold-change ( $\text{Log}_2(\text{FC})$ ) and the statistical significance of the differential expression test ( $-\text{Log}_{10}(\text{FDR})$ ) is displayed.

Plot similarities within *Bacillus* treatments (BL and BF) were observed since the most of genes were distributed between  $\text{Log}_2(\text{FC})$  values of -4 and 4 and with significance values ( $-\text{Log}_{10}(\text{FDR})$ ) up to 75 (downregulated genes) and 50 (upregulated genes). However, Akivi plot differed from *Bacillus* ones since the main of genes were distributed between  $\text{Log}_2(\text{FC})$  values of -3 and 3 and with lower significance values of 20 (downregulated genes) and 60 (upregulated genes).

Additionally, heatmaps of these DEGs for each treatment effect are shown in **Supplementary Figure 4**. The expression patterns of DEGs were consistent within biological replicates but differed between treatments in comparison with the NTC. It is worth to mention that after Aki and BL treatments, the number of genes that resulted over-expressed (red) and down-expressed (green) in comparison with the NTC were equivalent. However, after BF treatment a higher number of genes resulted over-expressed (red) in comparison with the NTC.

As is shown in Venn diagrams (**Figure 1**), 793 genes were upregulated and 652 genes were downregulated ( $\log_2\text{FC} > |1.4|$ ) within the different treatments (Aki, BL and BF) in grapevine leaves after the treatments. *Bacillus* treatments (BL and BF) altered the expression level of a higher number of genes than the botanical extract Akivi treatment (Aki). BL and BF treatments showed 438 and 396 upregulated DEGs, respectively, and 481 and 313 downregulated DEGs, respectively, whereas Aki treatment showed a total of 278 upregulated and 225 downregulated DEGs. In addition, the plant response towards *Bacillus* (both BL and BF) and Akivi (Aki) treatments was fair particular since only 31 upregulated and 68 downregulated genes were common to all three treatments.



**Figure 1.** Venn diagram showing the relationship between up-regulated (A) and down-regulated (B) differentially expressed genes (DEGs) identified in leaves of cv. Garnacha Blanca grapevine. Data correspond to 24h after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC).

However, the *Bacillus* treatments, both lyophilized and fresh, shared a high number of up- (43.1%) and downregulated (41.3%) genes. These genes were altered by the *Bacillus* treatments, independently of being the product lyophilized or not. Therefore, these shared genes were used for the following validation of RNA-seq results by RT-qPCR. From the 583 upregulated genes after either BL or BF treatments, 251 genes were shared. From the rest of genes, 187 and 145 were only upregulated after BL and BF treatments, respectively. Whereas from the 562 downregulated genes after BL or BF treatments, 232 genes were shared. From the remaining genes, 249 and 81 were only upregulated after BL and BF, respectively.

### 3.3. Functional Analysis of DEGs in Grapevine After Treatments

To understand the biological significance of DEGs, Gene Ontology (GO) and KEGG enrichment analysis were performed.

#### 3.3.1. GO Analysis of DEGs

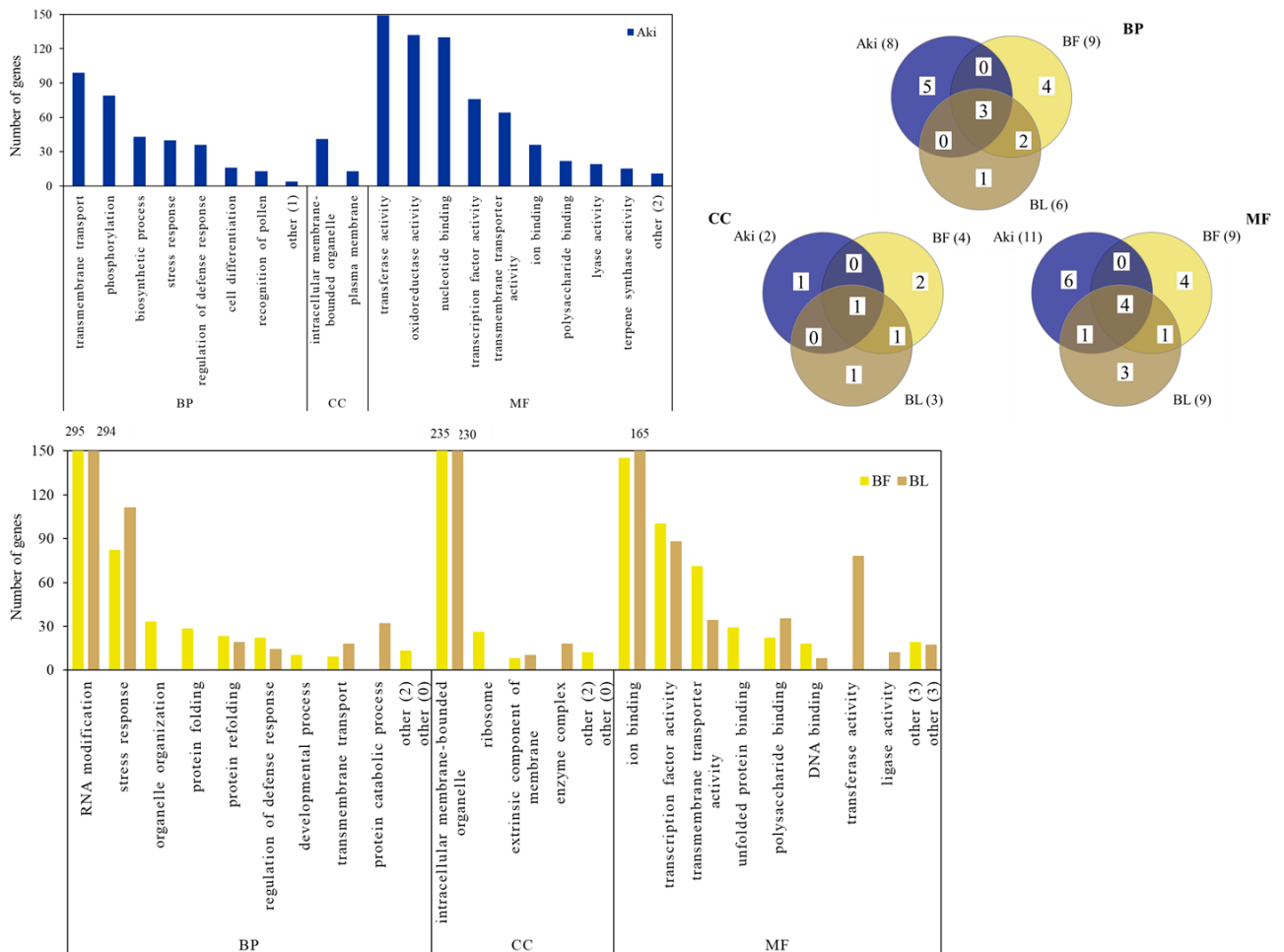
GO enrichment analysis was carried out using a threshold value ( $p$ -Value < 0.05) to evaluate the major biological functions of DEGs influenced by the Aki, BL, and BF treatments. These biological functions are classified into three categories: biological process, cellular component (CC), and molecular function (MF). Upregulated GO terms according to the GO analysis were identified in 34.4, 25.8 and 25.0% of DEGs after the Aki, BL and BF treatments, respectively, whereas 35.3, 32.9 and 41.4% were downregulated BP (**Table 2**).

**Table 2.** Gene Ontology (GO) terms influenced by treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG compared to the non-treated control (NTC) on cv. Garnacha Blanca grapevine leaves. UP: upregulated, DOWN: downregulated. Criteria:  $\text{Log}_2(\text{FC}) \geq |1.4|$  (FDR significant) and four or more genes per GO term.

GO Category	Aki_UP	Aki_DOWN	BL_UP	BL_DOWN	BF_UP	BF_DOWN
Biological Process	184/497	225/616	262/932	329/999	220/798	340/804
Cell Component	17/90	44/135	39/209	86/260	40/188	62/191
Molecular Function	135/390	139/404	151/613	204/624	121/538	225/518
<b>Total Categories</b>	<b>336/977</b>	<b>408/1155</b>	<b>452/1754</b>	<b>619/1883</b>	<b>381/1524</b>	<b>627/1513</b>

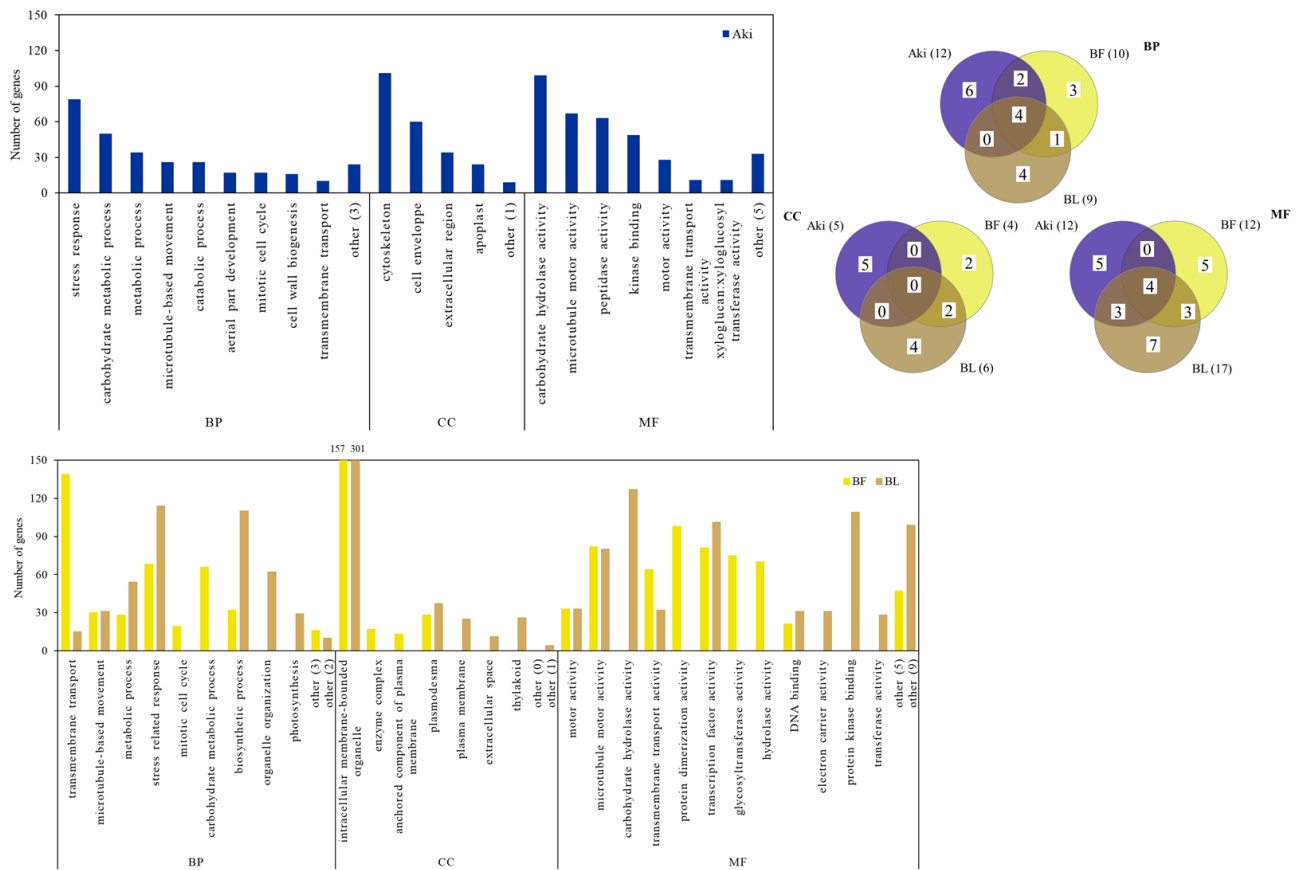
Selected GO terms according to the criteria / total significant GO terms

**Figure 2** shows the upregulated GO term clusters obtained by REVIGO analysis. Three biological processes associated with upregulated genes, namely “transmembrane transport”, “stress response”, and “regulation of defense response” were shared by the three treatments. The GO term clusters “Phosphorylation”, “biosynthetic process”, “cell differentiation”, and “recognition of pollen” were exclusively enriched by Aki treatment, whereas “protein catabolic process”, “organelle organization”, “protein folding”, and “developmental process”, “RNA modification”, and “protein refolding” were related to by BL and/or BF treatments.



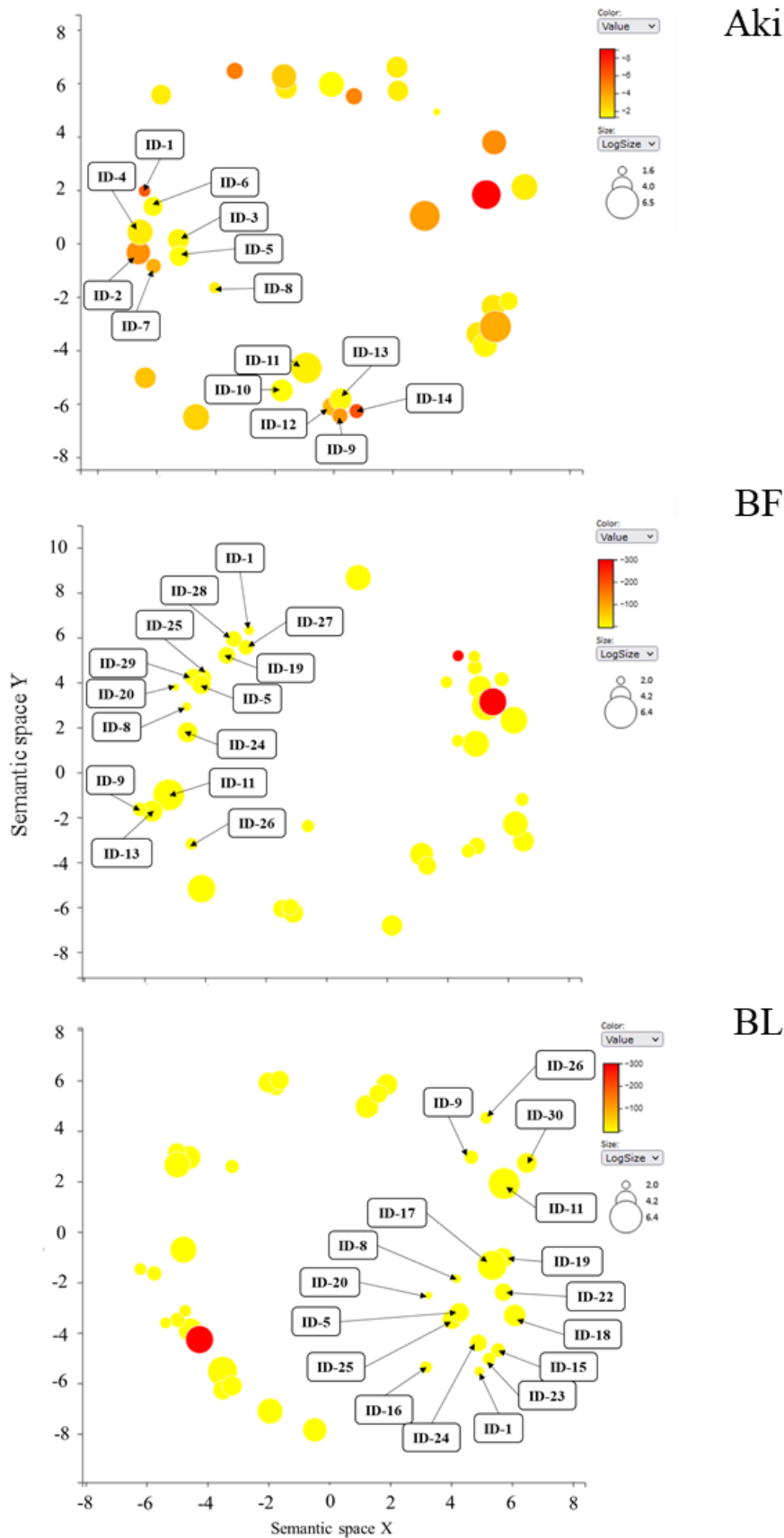
**Figure 2.** Upregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). Bar graphs show the number of upregulated DEGs in each GO term cluster. Clusters that showed less than 10 DEGs were included under the term “other”, indicating in parenthesis the number of clusters that represent. Venn diagrams show the total upregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

**Figure 3** shows the downregulated GO term clusters obtained by REVIGO analysis. Four biological processes associated with downregulated genes, namely “metabolic process”, “microtubule-based movement”, “stress response” and “transmembrane transport” were shared by the three treatments. “Catabolic process”, “aerial part development”, and “cell wall biogenesis” were exclusively reduced by Aki treatment. Whereas “carbohydrate metabolic process” and “mitotic cell cycle” were reduced by both Aki and BF treatments, “organelle organization”, “photosynthesis”, and “biosynthetic process” were related to BF and/or BL treatments.



**Figure 3.** Downregulated genes according to Gene Ontology (GO) enrichment and REVIGO analysis in cv. Garnacha Blanca grapevine after treatment with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, compared to the non-treated control (NTC). Bar graphs show the number of downregulated genes in each GO term cluster. Clusters that showed less than 10 DEGs (BP and CC for Aki, BF and BL and MF for Aki) or 20 DEGs (MF for BF and BL) were included under the term “other”, indicating in parenthesis the number of clusters that represent. Venn diagrams show the total downregulated GO term clusters. Categories of processes: biological (BP), cellular component (CC), and molecular function (MF).

It is worth to mention that some of the upregulated GO terms from biological processes that were arranged in two well-defined clusters are related to plant defense response, namely “stress response” and “regulation of defense response” (**Figure 4**). These two clusters include 30 GO terms (**Table 3**). In general, only 6 out of 30 GO terms were shared by Aki and *Bacillus* (BF and/or BL) treatments. Five GO terms were shared by the two *Bacillus* treatments (BF and BL), while eight, three, and eight GO terms were unique for Aki, BF and BL, respectively.



**Figure 4.** REVIGO graphs of upregulated GO term clusters (regulation of defense response and stress response) in cv. Garnacha Blanca grapevine leaves included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with **Table 3**.

**Table 3.** Representative groups (clusters) of upregulated GO terms of biological processes obtained with REVIGO and associated to plant defence responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Representative group	ID	GO ID: GO Term	Uniqueness*		
			Aki	BF	BL
Regulation of defense response	9	GO:2000022: regulation of jasmonic acid mediated signalling pathway	Orange	Yellow	Yellow
	13	GO:0031347: regulation of defense response	Orange	Yellow	White
	26	GO:0051096: positive regulation of helicase activity	White	Yellow	Yellow
	14	GO:0010112: regulation of systemic acquired resistance	Orange	White	White
	10	GO:0045454: cell redox homeostasis	Yellow	White	White
	12	GO:0010469: regulation of signalling receptor activity	Orange	White	White
	30	GO:0006879: cellular iron ion homeostasis	White	White	Yellow
Stress response	1	GO:0010200: response to chitin	Yellow	Orange	Orange
	8	GO:0061408: positive regulation of transcription from RNA polymerase II promoter in response to heat stress	Orange	Orange	Dark red
	5	GO:0034605: cellular response to heat	Orange	Orange	Dark red
	11	GO:0006355: regulation of transcription, DNA-templated	Yellow	Yellow	Orange
	19	GO:0000165: MAPK cascade	White	Orange	Orange
	25	GO:0006970: response to osmotic stress	White	Orange	Orange
	20	GO:0080167: response to karrikin	White	Orange	Orange
	24	GO:0042542: response to hydrogen peroxide	White	Orange	Dark red
	2	GO:0006955: immune response	Orange	White	White
	3	GO:0009611: response to wounding	Orange	White	White
	4	GO:0009607: response to biotic stimulus	Orange	White	White
	6	GO:0009723: response to ethylene	Orange	White	White
	7	GO:0009626: plant-type hypersensitive response	Orange	White	White
	27	GO:0046686: response to cadmium ion	White	Orange	White
	28	GO:0009739: response to gibberellin	White	Orange	White
	29	GO:0009651: response to salt stress	White	Orange	White
	15	GO:0010039: response to iron ion	White	White	Orange
	16	GO:0070413: trehalose metabolism in response to stress	White	White	Dark red
	17	GO:0035556: intracellular signal transduction	White	White	Dark red
	18	GO:0009617: response to bacterium	White	White	Orange
	21	GO:0006073: cellular glucan metabolic process	White	White	Orange
	22	GO:0009738: abscisic acid-activated signalling pathway	White	White	Dark red
	23	GO:0010167: response to nitrate	White	White	Dark red

Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments

ID: GO term assigned identifier

White space means not GO term

\* Smaller values denote higher uniqueness. Dark red (0.7-0.8), orange (0.8-0.9), yellow (0.9-1.0)

From the cluster named “regulation of defense response”, the GO term regulation of jasmonic acid mediated signalling pathway was shared by all treatments. Whereas the GO term regulation of defense response was shared by Aki and BF, regulation of systemic acquired resistance was unique for Aki. From the cluster named “stress response”, the GO terms response to osmotic stress, response to karrikin, and response to hydrogen peroxide were unique for *Bacillus* (BF and BL), while the GO terms immune response and plant-type hypersensitive response, and response to wounding, biotic stimulus and ethylene were unique for Aki.

The upregulated genes ( $\text{Log}_2(\text{FC}) > 1.4$ ) related to the GO terms included in “regulation of defense response” and “stress response” clusters are shown in **Table 4 (Supplementary Tables 5 and 6)**. Interestingly, some upregulated DEGs were also unique for each treatment (Aki and *Bacillus*). After *Bacillus* treatment, one gene related to the regulation of jasmonic acid, several genes related to transcription factors, chaperones, enzymes as catalase, PR protein with antimicrobial activity, abscisic acid receptor, and cold induced protein were upregulated after *Bacillus* treatment. However, after Akivi treatment, two genes related to the regulation of SAR, one defense response related gene, three genes related to the response to chitin, and one gene related to PR protein with antimicrobial activity were upregulated.

**Table 4.** Upregulated genes included in the GO terms that belong to regulation and stress response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

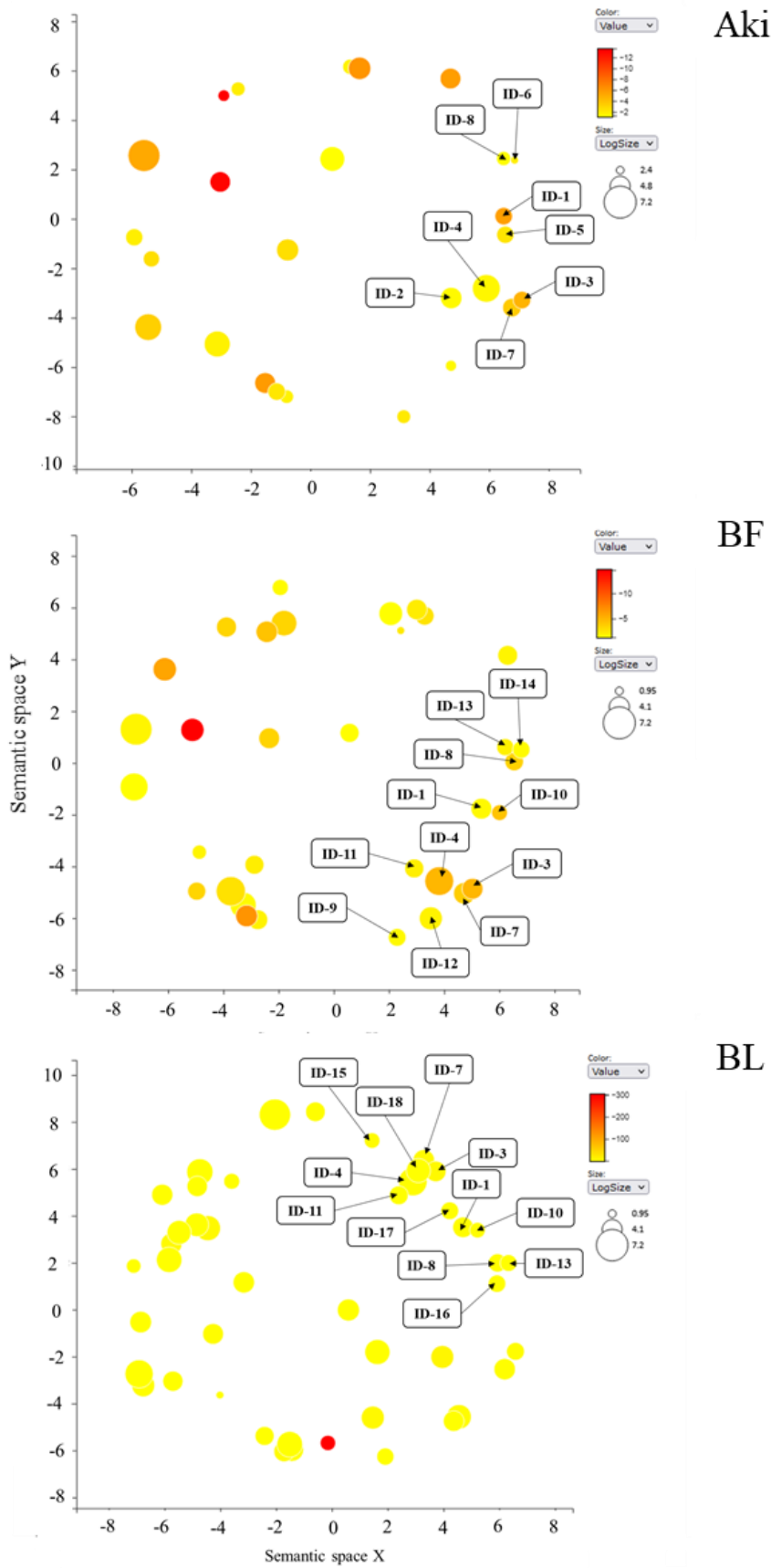
Description	Gene ID	GO ID	Aki	BF	BL
<b>Enzymes</b>					
Leucoanthocyanidin dioxygenase	VIT_13s0067g01020	9			
Trehalose 6-phosphate synthase	VIT_17s0000g08010;	16			
	VIT_01s0026g00280				
Trehalose-phosphatase	VIT_12s0028g01670	16			
Xyloglucan endotransglucosylase/hydrolase	VIT_11s0052g01280;	21			
	VIT_05s0062g00250;				
	VIT_01s0026g00200				
Catalase	VIT_00s0698g00010	27			
<b>Proteins that mediate the attachment of integral membrane proteins to the cytoskeleton</b>					
Ankyrin repeat	VIT_14s0081g00370	13			
Ankyrin repeat	VIT_05s0165g00010;	13			
	VIT_14s0081g00360				
Continue					



**Table 4.** (continued)

Description	Gene ID	GO ID	Aki	BF	BL
<b>Transcriptional regulators / Transcriptional factors</b>					
Jasmonate ZIM domain-containing protein 8	VIT_10s0003g03790	9, 13		■	
Cold induced protein	VIT_17s0000g08010	27		■	
Zinc finger (C2H2 type) family	VIT_13s0019g00480	1	■		
Myb domain protein 14	VIT_05s0049g01020	1, 27, 29		■	■
Salt tolerance homolog2	VIT_03s0038g00340	1, 20	■	■	■
WRKY DNA-binding protein 33	VIT_08s0058g00690	1, 5, 25, 29		■	■
Heat shock transcription factor C1	VIT_11s0016g03940	5, 8		■	■
<b>Modulators and regulators of related defence responses and cell death program</b>					
NIM1	VIT_07s0005g02070; VIT_01s0011g03430	14	■		
NSL1 (necrotic spotted lesions 1)	VIT_01s0011g05950	2, 7	■		
Abscisic acid receptor PYL1 RCAR12	VIT_13s0067g01940	22			■
<b>Plant peptide growth factors.</b>					
Phytosulfokines PSK1	VIT_08s0007g03870	12	■		
<b>DNA replication</b>					
DNA mismatch repair protein MSH3	VIT_00s0388g00030	26		■	■
<b>Iron storage and transport proteins</b>					
Ferritin	VIT_08s0058g00440, VIT_08s0058g00430, VIT_08s0058g00410	15, 18, 24, 30			■
Metal-nicotianamine transporter YSL1	VIT_02s0025g02510	15			■
<b>Chaperones (HSP)</b>					
Heat shock protein 18.2 kDa class II	VIT_12s0035g01910	24, 29		■	
Heat shock protein 17.6 kDa class I	VIT_13s0019g03160	24, 29		■	
HSP (HSP26.5-P) 26.5 kDa class P	VIT_00s0992g00020	24, 29		■	■
<b>Pathogenesis related proteins</b>					
Pathogenesis protein 10	VIT_05s0077g01570	22			■
Pathogenesis protein 10	VIT_05s0077g01600	4	■		
<b>Unknown</b>					
unknown	VIT_09s0002g03340	27		■	

GO ID: GO term assigned identifier (Table 3)



**Figure 5.** REVIGO graphs of downregulated GO term clusters (regulation of defense response and stress response) in cv. Garnacha Blanca grapevine leaves included in biological process category after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG. ID: identification of GO terms associated with **Table 6**.

Some of the downregulated GO terms from biological processes were arranged a cluster related to plant defense response, namely “stress-related response” (**Figure 5**). This cluster include 18 GO terms related to regulation of cellular cycle and cell population proliferation, plant development, metabolic processes and their regulation, stress response, defense and response to stimuli and signal transduction (**Table 5**). The GO terms related to cellular cycle, stress and stimuli response, metabolic processes regulation and signal transduction were shared by the three treatments. However, GO terms related to plant development, defense response and metabolic processes were unique for *Bacillus* treatments.

**Table 5.** Representative groups (clusters) of downregulated GO terms of biological processes obtained with REVIGO and associated to plant defence responses, after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Representative group	ID	GO ID: GO Term	Uniqueness*		
			Aki	BF	BL
Stress-related response	3	GO:0045787: positive regulation of cell cycle	Orange	Orange	Orange
	4	GO:0006355: regulation of transcription	Orange	Orange	Orange
	8	GO:0009414: response to water deprivation	Orange	Orange	Orange
	7	GO:0008284: positive regulation of cell population proliferation	Orange	Orange	Yellow
	1	GO:0009734: auxin-activated signaling pathway	Dark red	Orange	Orange
	10	GO:0010017: red or far-red light signaling pathway	White	Orange	Orange
	13	GO:0009744: response to sucrose	White	Orange	Yellow
	11	GO:0045910: negative regulation of DNA recombination	White	Orange	Yellow
	2	GO:0043086: negative regulation of catalytic activity	Orange	White	White
	5	GO:0007178: transmembrane receptor protein serine/threonine kinase signaling pathway	Orange	White	White
	6	GO:0071249: cellular response to nitrate	Orange	White	White
	9	GO:0009909: regulation of flower development	White	Yellow	White
	12	GO:0043085: positive regulation of catalytic activity	White	Orange	White
	14	GO:0046686: response to cadmium ion	White	Yellow	White
	15	GO:0010112: regulation of systemic acquired resistance	White	White	Yellow
	16	GO:0009627: systemic acquired resistance	White	White	Yellow
	17	GO:0000076: DNA replication checkpoint signaling	White	White	Orange
	18	GO:0045893: positive regulation of transcription	White	White	Orange

Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments

ID: GO term assigned identifier

White space means not GO term

\* Smaller values denote higher uniqueness. Dark red (0.7-0.8), orange (0.8-0.9), yellow (0.9-1.0)

**Table 6.** Downregulated genes included in the GO terms that belong to stress-related response groups after treatments of cv. Garnacha Blanca grapevine leaves with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

Gene description	Gene ID	GO ID	Aki	BF	BL
<b>Transcription factor related to auxin signalling pathway</b>					
IAA31	VIT_05s0020g01070	1			
<b>Proteins that control the cell cycle by activating cyclin-dependent kinase (CDK) / Cycle regulators</b>					
Cyclin delta-3 (CYCD3_1)	VIT_18s0001g09920				
<i>Cyclin D3_2</i>	VIT_03s0180g00040	2, 3, 6, 7			
<i>Cyclin CYCB1_2</i>	VIT_06s0009g02090	2, 3, 6, 7			
<i>Cyclin B-type</i>	VIT_08s0040g00930	2, 3, 6, 7			
<i>Cyclin 1b (CYC1b)</i>	VIT_13s0067g01420	2, 3, 6, 7			
<i>Cyclin-dependent protein kinase regulator CYCB2_4</i>	VIT_18s0001g14170	2, 3, 6, 7			
Cyclin B2;4	VIT_03s0038g02800	3, 7			
Cyclin delta-2	VIT_03s0091g01060	3, 7			
Cyclin A1	VIT_18s0001g02060	3, 7			
Cyclin-dependent protein kinase CYCB3	VIT_19s0085g00690	3, 7			
Annexin ANN4	VIT_00s0131g00080	3,8			
Protein kinase WEE1	VIT_07s0104g01740	17			
<b>Proteins that join DNA to form nucleosomes</b>					
Histone H4	VIT_06s0004g04370; VIT_13s0019g00780; VIT_13s0019g00800	8			
	VIT_07s0005g01060; VIT_07s0141g00730; VIT_14s0081g00500		11		
<b>Receptors like-Kinases (RLK)</b>					
Proline extensin-like receptor kinase 1 (PERK1)	VIT_01s0127g00670	3			
Receptor protein kinase	VIT_05s0020g01690	3			
<b>DNA replication and repair</b>					
ATP-dependent DNA helicase RecQ	VIT_01s0010g02590	3, 7			
Origin recognition complex subunit 5	VIT_01s0011g04400	13			
Origin recognition complex subunit 4	VIT_17s0000g01960	13			
DNA mismatch repair protein	VIT_01s0011g03440	15			
<b>B ZipDNA binding proteins /Transcription factors/ Zinc finger proteins</b>					
BZIP protein HY5 (HY5)	VIT_04s0008g05210	10			
BZIP protein HY5 (HY5)	VIT_05s0020g01090	10			
BZIP transcription factor BZIP6	VIT_00s0541g00020	18			
AP2/ERF domain containing protein	VIT_08s0007g08150	18			

Continue

**Table 6.** (continued)

Gene description	Gene ID	GO ID	Aki	BF	BL
NAC Secondary wall thickening promoting factor1	VIT_02s0025g02710	18			
Late meristem identity1 HB51/LMI1	VIT_08s0007g04200	18			
Homeodomain leucine zipper protein HB-1	VIT_01s0026g01550	18			
Homeobox-leucine zipper protein HB-7	VIT_15s0048g02870	18			
Constans 2 (COL2)	VIT_14s0083g00640	9			
Zinc knucle	VIT_01s0010g01670	17			
<b>Lipid Transfer Proteins (LTP)</b>					
DIR1 (defective IN induced resistance 1)	VIT_00s0333g00050	16			
Protease inhibitor/seed storage/lipid transfer protein (LTP)	VIT_08s0007g01370	16			
<b>Unknown</b>					
unknown	VIT_04s0008g04200	5			
unknown	VIT_04s0023g03760	5			
unknown	VIT_07s0129g00200	3, 7			
unknown	VIT_13s0067g02560	18			

Go ID: GO term assigned identifier (**Table 4**)

The downregulated genes ( $\text{Log}_2(\text{FC}) < 1.4$ ) related to the GO terms included in “stress-related response” cluster are shown in **Table 6 (Supplementary Table 7)**. Interestingly, after both Aki and *Bacillus* treatments, some DEGs related to cellular cycle were downregulated. In particular, six cyclin proteins and one annexin protein were downregulated for Aki treatment. While four cyclin proteins were downregulated for *Bacillus* treatments.

Moreover, genes related to plant growth and development, such as transcriptional factors and zinc finger proteins, DNA replication, and two lipid transfer protein (LTP) that intervene in systemic acquired resistance SAR were downregulated after *Bacillus* treatments.

Considering different stress responses, after Aki treatment two genes connected with receptor like kinases that intervene in plant innate immunity were downregulated, while after *Bacillus* treatments transcription factors to several stresses and abiotic stresses were downregulated.

### 3.3.2. KEGG Pathway analysis of DEGs

KEGG pathway analysis was performed using a threshold value ( $p\text{-Value} < 0.05$ ) to evaluate the biological mechanisms influenced by the Aki, BL, and BF treatments. As shown in **Table 7**, a few pathways were associated with DEGs affected by the treatments and none are shared between Akivi and *Bacillus* treatments.

**Table 7.** KEGG pathways influenced by Akiwi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments compared to the non-treated control (NTC).

Pathway ID	Pathway description	Number of DEGs	Corrected p-Value
<b>Upregulated</b>			
<b>Aki vs NTC</b>			
vvi00480	Glutathione metabolism	23	2.05E-05
vvi00591	Linoleic acid metabolism	7	1.95E-03
vvi00592	alpha-Linolenic acid metabolism	11	8.75E-03
vvi00900	Terpenoid backbone biosynthesis	10	9.82E-03
vvi00410	beta-Alanine metabolism	10	9.82E-03
vvi00071	Fatty acid degradation	8	4.35E-02
<b>BL vs NTC</b>			
vvi00500	Starch and sucrose metabolism	32	9.80E-04
<b>BF vs NTC</b>			
vvi00500	Starch and sucrose metabolism	25	2.37E-02
vvi03008	Ribosome biogenesis in eukaryotes	17	2.44E-02
<b>Downregulated</b>			
<b>Aki vs NTC</b>			
vvi00860	Porphyrin and chlorophyll metabolism	11	2.37E-03
<b>BL vs NTC</b>			
vvi03030	DNA replication	17	2.60E-04
vvi00270	Cysteine and methionine metabolism	21	2.06E-02
<b>BF vs NTC</b>			
vvi00051	Fructose and mannose metabolism	12	2.56E-02
vvi00940	Phenylpropanoid biosynthesis	24	3.10E-02
vvi03030	DNA replication	10	3.10E-02
vvi00500	Starch and sucrose metabolism	19	3.64E-02

“Glutathione metabolism”, “Linoleic acid metabolism”, “alpha-Linolenic acid metabolism”, “Terpenoid backbone biosynthesis”, “beta-Alanine metabolism”, and “Fatty acid degradation” were triggered after Aki treatment, whereas “Starch and sucrose metabolism” was triggered by both BL and BF treatments, “Ribosome biogenesis in eukaryotes” was exclusively triggered by BF treatment.

“Porphyrin and chlorophyll metabolism” were reduced after Aki treatment; “DNA replication” was reduced after both BL and BF treatments. “Cysteine and methionine metabolism” was reduced after BL treatment; while “Fructose and mannose metabolism”, “Phenylpropanoid biosynthesis”, and “Starch and sucrose metabolism” were reduced after BF treatment.

### 3.4. Gene Marker Candidates on Grapevine

#### 3.4.1. Selection of DEGs

Genes whose expression level was modified by Aki, BL and BF treatments on cv. Garnacha Blanca were chosen to identify treatment-effect related markers. A total of 27 DEGs were selected (**Table 8**).

**Table 8.** Selected Differentially Expressed Genes (DEGs) on cv. Garnacha Blanca grapevine leaves after treatment with Akivi (Aki) or lyophilized (BL) *Bacillus* UdG.

Code	Gene ID	Log <sub>2</sub> (FC)	FC	FDR	vCOST Description
<b>Akivi</b>					
A1	VIT_12s0059g02600	4.96	31.18	5.01E-15	Receptor protein kinase RK20-1
A2	VIT_06s0004g03350	3.46	11.03	9.18E-24	Lateral organ boundaries protein 1
A3	VIT_05s0077g00520	3.17	8.99	7.76E-11	Gibberellin 2-oxidase
A4	VIT_17s0000g00200	3.23	9.40	1.58E-17	Ethylene-responsive transcription factor ERF114
A5	VIT_08s0058g00970	2.39	5.23	1.67E-17	Cationic peroxidase
A6	VIT_12s0055g01010	3.04	8.23	1.38E-28	Peroxidase
A7	VIT_00s0372g00040	2.74	6.68	1.68E-08	1,8-cineole synthase, chloroplast
A8	VIT_04s0023g02240	2.83	7.11	7.56E-56	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase
A9	VIT_12s0034g01140	2.07	4.21	5.28E-21	Plastocyanin domain-containing protein
A10	VIT_19s0090g00660	2.01	4.03	1.03E-32	Lipase GDSL
A11	VIT_03s0088g00810	1.88	3.67	3.94E-16	Pathogenesis-related protein 1 precursor (PRP 1)
A12	VIT_07s0005g06090	1.67	3.19	9.06E-19	Pore-forming toxin-like protein Hfr-2
<b>Bacillus</b>					
B1	VIT_16s0022g00860	5.23	37.41	1.25E-28	Invertase/pectin methylesterase inhibitor
B2	VIT_06s0004g07210	5.45	43.58	4.78E-65	CCT motif constans-like
B3	VIT_16s0100g00740	4.26	19.16	2.49E-15	unknown
B4	VIT_14s0068g01160	2.91	7.53	5.22E-12	Cytokinin-repressed protein CR9
B5	VIT_00s1490g00010	-2.46	0.18	9.93E-38	5'-adenylylsulfate reductase (APR1)
B6	VIT_13s0064g01370	3.08	8.43	4.30E-07	Polygalacturonase inhibiting protein 1 PGIP1
B7	VIT_09s0002g04280	3.14	8.81	2.33E-47	Dynein light chain LC6, flagellar outer arm
B8	VIT_03s0091g00310	2.96	7.80	6.23E-16	Indole-3-acetic acid-amido synthetase GH3.8
B9	VIT_01s0011g01980	2.47	5.52	3.91E-22	fasciclin arabinogalactan-protein (FLA21)
B10	VIT_01s0026g02740	2.64	6.25	9.54E-28	unknown
B11	VIT_08s0058g00430	1.82	3.52	1.32E-02	ferritin
B12	VIT_10s0116g00530	1.96	3.89	1.07E-30	Thiazole biosynthetic enzyme, chloroplast (ARA6)
B13	VIT_00s0480g00060	1.49	2.81	7.91E-20	Polyphenol oxidase [Vitis vinifera]
B14	VIT_07s0031g02610	2.98	7.92	3.48E-14	NAC domain containing protein 2
B15	VIT_13s0067g02130	2.50	5.64	6.67E-10	Dehydration-induced protein (ERD15)

FC, fold change

FDR, false discovery rate

From the 12 DEGs highly triggered by Aki treatment, eight genes are related to defence response (A1, A3, A4, A5, A6, A7, A11, and A12); from them, two genes are involved in detoxification of reactive oxidative species (A5 and A6); two genes are related to hormone signalling pathway (A3, and A4), one gene was involved in biosynthesis of secondary metabolites (A7), and one gene is a marker of SAR response (A11). From the 15 DEGs highly triggered by BL and BF treatments, four genes are involved in defence response (B1, B6, B8, and B14).

### **3.4.2. Validation of Selected DEGs by RT-qPCR**

The expression data of the 27 DEGs selected according to the results of RNA-seq analysis on the ‘Garnacha Blanca’ experiment was validated by RT-qPCR analysis.

Standard curves of the 27 DEGs showed R-squared values above 0.99 and, in general, amplification efficiencies above 90%, except for three DEGs (A1, A3 and A4) that showed slightly lower efficiencies above 80% (**Supplementary Table 2**). In addition, relative quantification was allowed because similar amplification efficiencies were confirmed between the selected DEGs and the endogenous gene (UBQ).

The expression levels of the 27 DEGs within the NTC samples on the ‘Garnacha Blanca’ experiment were stable showing FC values close to 1 (**Table 9**). The selected DEGs were upregulated after Aki (12) and BL treatments (14) with significant differences in comparison with the NTC, with the exception of B5 gene that was downregulated.

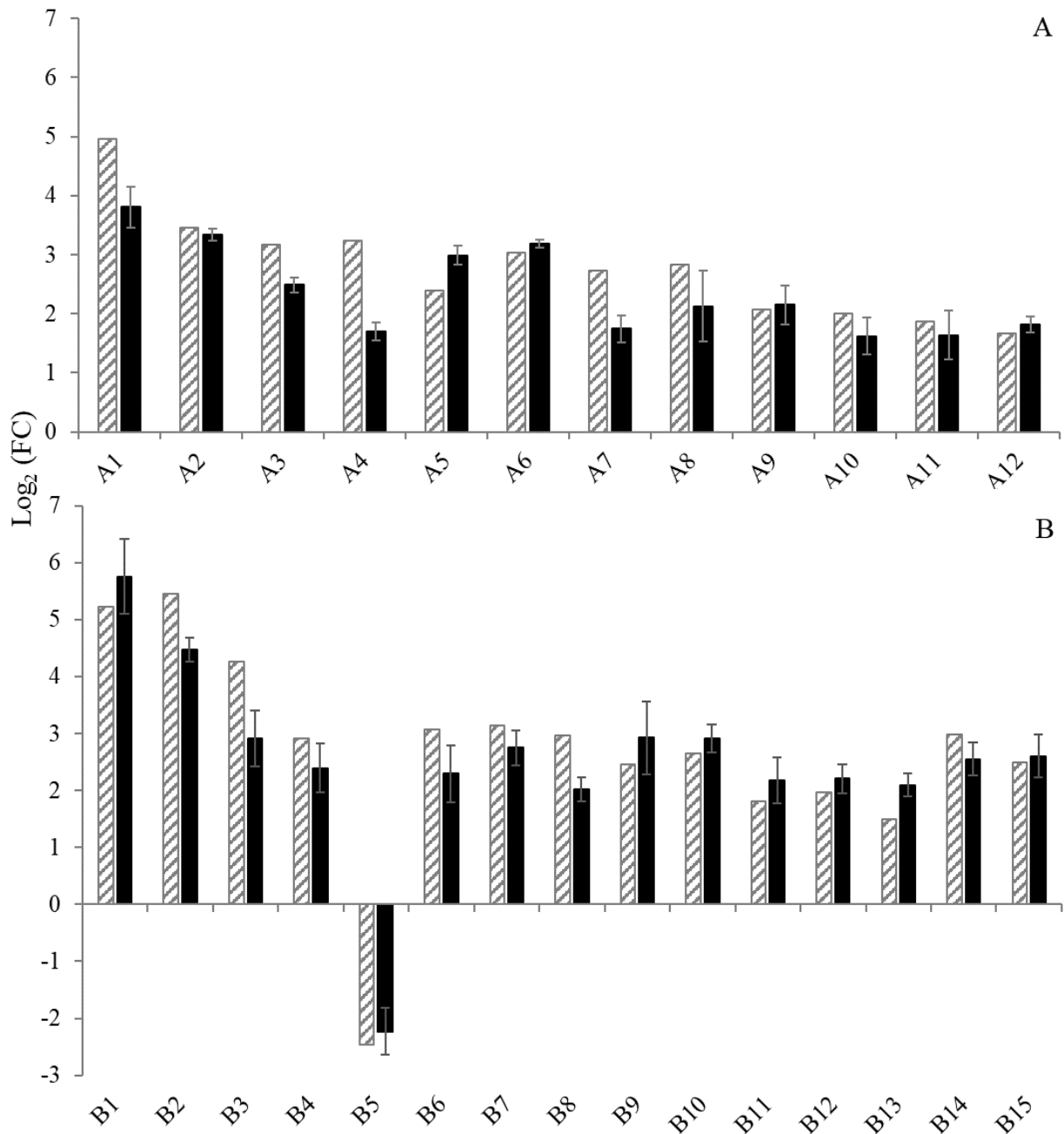
Moreover, the relative expression levels of the 27 DEGs on cv. Garnacha Blanca obtained by RT-qPCR and RNA-seq analysis were highly consistent for both Aki and BL treatments (**Figure 6**). That was confirmed by Pearson correlation test that showed high correlation coefficient values, 0.729 and 0.938 for Aki and BL, respectively, and statistical significances with p-values<0.05 (**Supplementary Figure 5**). Therefore, the 27 DEGs that were previously selected by RNA-seq analysis were validated by RT-qPCR on grapevine cv. Garnacha Blanca.



**Table 9.** Expression levels in fold change of the selected DEGs influenced by treatments of cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi (Aki) and lyophilized *Bacillus* UdG (BL). Data correspond to RT-qPCR. The relative expression level of each gene was calculated by the comparative critical threshold ( $2^{-\Delta\Delta Ct}$ ) method using the non-treated control samples (NTC) as the calibrator and UQB gene as internal control for data normalization. Data mean the fold change ( $2^{-\Delta\Delta Ct}$ ) and significant differences are represented by \*.

DEGs	'Garnacha Blanca'			'Garnacha Tinta'			'Macabeo'		
	NTC	Aki		NTC	Aki		NTC	Aki	
A1	1.29	14.37	*	1.29	4.86		1.11	4.46	*
A2	1.08	10.12	*	1.00	1.88	*	1.02	1.58	*
A3	1.05	5.64	*	1.03	1.43	*	1.04	1.74	*
A4	1.12	3.27	*	1.02	2.22	*	1.02	3.20	*
A5	1.05	8.01	*	1.02	1.55	*	0.99	1.68	
A6	1.05	9.11	*	1.09	1.39		1.05	1.16	
A7	1.06	3.39	*	1.06	1.65	*	1.10	1.10	
A8	1.08	4.71	*	1.05	2.94	*	1.16	1.78	
A9	1.06	4.56	*	1.01	2.79	*	1.01	0.44	*
A10	1.01	3.15	*	1.00	1.69	*	1.01	0.92	
A11	1.01	3.24	*	1.12	1.44		1.02	0.69	
A12	1.10	3.54	*	1.01	9.96	*	1.01	2.23	*
DEGs	NTC	BL		NTC	BL		NTC	BL	
B1	1.16	58.79	*	1.06	14.51	*	1.01	4.07	*
B2	1.05	22.43	*	1.03	3.19	*	1.02	4.05	*
B3	1.06	7.98	*	1.00	2.21	*	1.00	2.02	*
B4	1.15	5.49	*	1.09	0.77		0.90	1.63	
B5	1.04	0.22	*	1.02	0.50	*	1.09	3.03	*
B6	1.01	5.17	*	1.01	1.48	*	1.04	0.54	*
B7	1.29	6.86	*	1.04	1.39	*	1.07	0.71	*
B8	1.03	4.10	*	1.01	1.38		1.08	2.43	*
B9	1.09	8.30	*	1.01	5.07	*	1.06	2.76	*
B10	1.07	7.66	*	1.01	3.17	*	1.01	1.26	
B11	1.15	4.71	*	1.02	1.16		1.07	7.18	*
B12	1.01	4.68	*	1.01	3.76	*	1.03	8.59	*
B13	1.00	4.32	*	1.02	2.84	*	1.02	2.07	*
B14	1.12	5.96	*	1.04	3.15	*	1.22	2.50	*
B15	1.03	6.26	*	1.04	2.66	*	1.03	0.92	

DEG functions are indicated in **Table 1**.



**Figure 6.** Expression levels of twenty-seven genes selected for validation of the RNA-Seq data by RT-qPCR. The gene expression was analysed after treatments with Akivi (A) and lyophilized *Bacillus UdG*. RNA-seq (stripped bars) and RT-qPCR (black bars) analysis. Gene functions are indicated in Table 1. RT-qPCR data are shown as the mean of  $\text{Log}_2(\text{FC})$  of three biological replicates, where FC is the fold-change value and was calculated as  $2^{-\Delta\Delta\text{Ct}}$  using non-treated control (NTC) samples as the calibrator and UBQ gene for data normalization. Error bars mean confidence interval of three biological replicates.

### 3.4.3. Expression of Validated DEGs in the Three Grapevine Cultivars

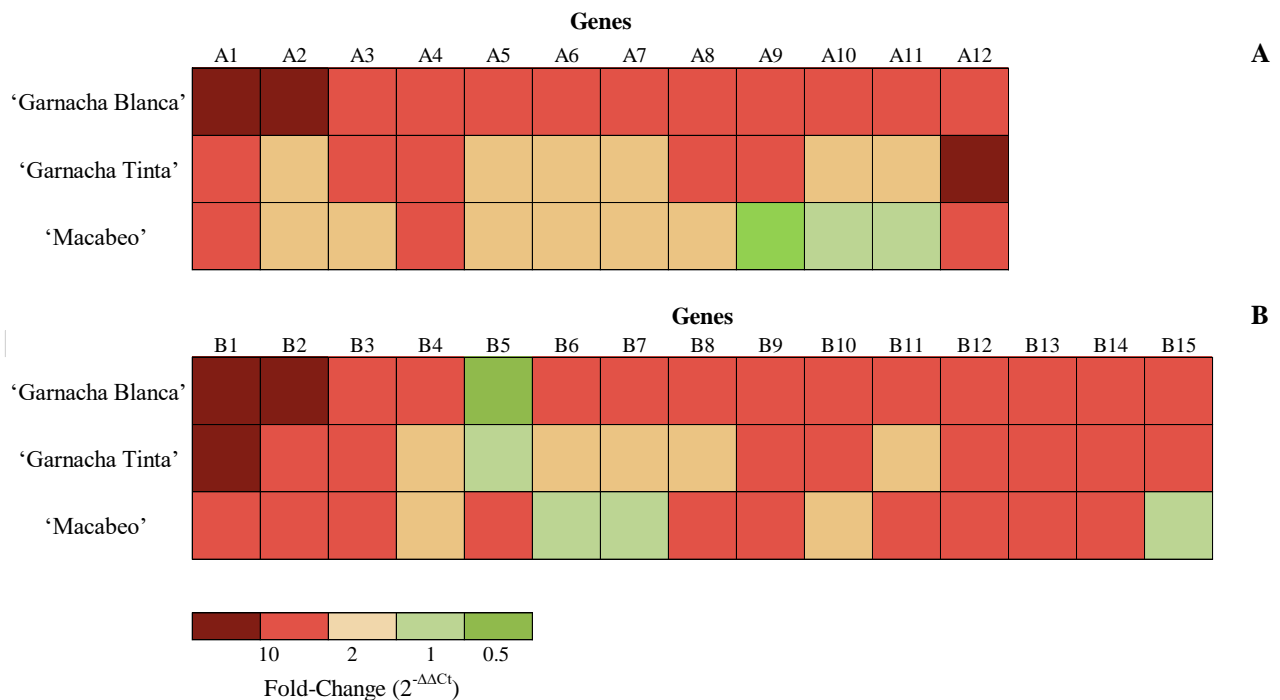
The expression levels of the 27 DEGs were further subjected to RT-qPCR using samples from experiments performed with two other grapevine cultivars, namely ‘Garnacha Tinta’ and ‘Macabeo’. The expression levels of the 27 DEGs within the NTC samples of the ‘Garnacha Tinta’ and ‘Macabeo’ experiments were stable showing fold change values close to 1 (**Table 9**).

Concerning the 12 selected DEGs by Akivi treatment, nine (A2, A3, A4, A5, A7, A8, A9, A10, and A12) and six genes (A1, A2, A3, A4, A9, and A12) respectively in ‘Garnacha Tinta’ and ‘Macabeo’, showed differential expression levels compared to the NTC (**Table 9**). Twenty-four hours after Akivi treatment, the A1, A4, and A12 genes were upregulated on the three grapevine cultivars (Figure 7), whereas A9 gene was upregulated on ‘Garnacha Blanca’ and ‘Garnacha Tinta’, this gene was downregulated on ‘Macabeo’. In the case of A3 and A8 genes, despite they were upregulated on ‘Garnacha Blanca’ and ‘Garnacha Tinta’, these genes expression were not affected on ‘Macabeo’. Six genes, namely, A2, A5, A6, A7, A10 and A11, were only upregulated on ‘Garnacha Blanca’, while the expression pattern of these genes was not affected on ‘Garnacha Tinta’ and ‘Macabeo’. Therefore, the expression pattern at 24 hours after Akivi treatment was quite similar in the ‘Garnacha Blanca’ and ‘Garnacha Tinta’ (6 out of 12 genes were upregulated). However, the expression pattern obtained on ‘Macabeo’ differed from ‘Garnacha Blanca’ and ‘Garnacha Tinta’ since only 3 out of 12 (A1, A4, A12) genes were upregulated on all cultivars tested. In particular, three genes (A9, A10 and A11) showed FC below 1 only on ‘Macabeo’, being A9 downregulated.

In relation to the 15 selected DEGs by BL treatment, twelve genes showed significant differential expression levels comparing with the NTC within ‘Garnacha Tinta’ (B1, B2, B3, B5, B6, B7, B9, B10, B12, B13, B14, and B15) and ‘Macabeo’ (B1, B2, B3, B5, B6, B7, B8, B9, B11, B12, B13, and B14) (**Table 9**). Twenty-four hours after BL treatment, B1, B2, B3, B9, B12, B13, and B14 genes were upregulated on the three grapevine cultivars (**Figure 7**). The only gene that was downregulated on ‘Garnacha Blanca’ was B5, which was unaltered and upregulated on ‘Garnacha Tinta’ and ‘Macabeo’, respectively. Three genes, namely B4, B6, and B7, were only upregulated on ‘Garnacha Blanca’, while they were unaltered (FC between 0.5-2) on ‘Garnacha Tinta’ and ‘Macabeo’. In the case of B8 and B11 genes, their expressions were upregulated on both ‘Garnacha Blanca’ and ‘Macabeo’, while their expressions were not affected on ‘Garnacha Tinta’. Two genes, namely B10 and B15, were upregulated on both ‘Garnacha Blanca’ and ‘Garnacha Tinta’, while their expressions were not affected on ‘Macabeo’. Therefore, the expression pattern corresponding to 24 hours after BL treatment was quite similar in the ‘Garnacha Blanca’ and ‘Garnacha Tinta’ (9 out of 15 genes were upregulated). Similar results were also observed comparing expression patterns in ‘Garnacha

Blanca' and 'Macabeo' (9 out of 15 genes were upregulated) despite B5 gene was clearly upregulated on 'Macabeo' and downregulated on 'Garnacha Blanca'. However, the expression pattern obtained on 'Macabeo' differed from 'Garnacha Tinta' since a smaller number of genes (7 out of 15) shared the same upregulation transcriptional pattern.

Given these results, A1, A4 and A12 genes could be appropriate markers of Akivi treatment; and B1, B2, B3, B9, B12, B13, and B14 genes could be appropriate markers of *Bacillus* treatment because they showed the same expression pattern (upregulation) 24 hours after the treatment on the three grapevine cultivars.



**Figure 7.** Transcriptional pattern of selected DEGs after treatments of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo with Akivi (A) or lyophilized *Bacillus* UdG (B). The fold change was assessed by the  $\Delta\Delta C_t$  method. The UBQ gene was used as the internal control for data normalization. The  $\Delta C_t$  of the non-treated control (NTC) samples was defined as the calibrator. Three independent biological replicated were performed. Gene functions are indicated in **Table 1**.

### 3.5. Metabolite Concentrations

Macronutrient and micronutrient concentrations from fully developed leaves were analysed. Foliar-applied biocontrol products to grapevine plants slightly influenced some of the mineral nutrient concentrations in the leaves, but not enough to affect plant development in any of the three cultivars (**Supplementary Table 8**).

Phytohormones, organic acids (OA) and total phenolic compounds concentrations were compared for each treatment (Aki and BL) with the NTC (**Table 10**).

**Table 10.** Phytohormone, organic acids, and total phenolic contents in leaves of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo treated with Akivi (Aki) and lyophilized *Bacillus* UdG (BL) and water (NTC). The following concentrations correspond to phytohormone (ng/g FW), organic acid (mg/g FW, except for oxoglutaric acid in  $\mu\text{g/g}$  FW), and total phenolic ( $\mu\text{g}$  gallic acid equivalent/g PF). Results are means (n=3 biological replicates). Significant differences between treatment (Aki or BL) and NTC are represented by asterisks (\*).

	'Garnacha Blanca'			'Garnacha Tinta'			'Macabeo'		
	NTC	Aki	BL	NTC	Aki	BL	NTC	Aki	BL
<b>Phytohormone</b>									
<b>JA</b>	4.39	4.83	6.15	4.18	5.54	10.68*	5.75	6.95	5.85
<b>MeJA</b>	3.71	4.95 *	5.12*	4.98	4.76	4.31	4.41	4.13	4.00
<b>SA</b>	375	553	321	215	654*	375	131	218	159
<b>ACC</b>	8.86	10.44	10.65	11.06	10.23	11.36	12.69	11.54	12.13
<b>ABA<sup>1</sup></b>	1.51	0.94	1.54	6.15	7.37	5.71	21.25	21.55	17.96
<b>GA1&amp;4</b>	8.39	31.05*	18.29	9.49	6.02	6.70	27.34	4.13*	7.85*
<b>Organic acid</b>									
<b>Oxalic</b>	4.47	3.75	3.28	3.38	3.54	5.35	2.33	1.76	2.80
<b>Tartaric</b>	15.51	15.23	16.30	19.45	17.48	17.20*	17.13	16.43	15.02
<b>Malic</b>	1.48	1.87	1.78	1.34	2.02	1.39	0.92	0.88	1.18
<b>Oxoglutaric</b>	346	413	631*	667	674	671	155	88*	78*
<b>Total phenolic</b>	200	359	395*	876	912	808	656	679	984*

JA: jasmonic acid; MeJA: methyl jasmonate; SA: salicylic acid; ACC: ethylene precursor; ABA: abscisic acid; and GA1&4: Gibberellins A1 and A4.

<sup>1</sup> ABA concentration values in 'Garnacha Blanca' was at the limit of detection and only one value was detected for the treatment modalities (Aki and BL), thus, they were not included in statistical analysis.

Regarding Aki treatment, no phytohormones were significantly influenced in the same way among the three grapevine cultivars. SA tended to present higher levels 24h after Aki treatment in 'Garnacha Blanca' and 'Macabeo' and was significantly enhanced in 'Garnacha Tinta'. GAs showed an opposite pattern, their levels being significantly increased in 'Garnacha Blanca', but reduced in 'Macabeo'. MeJA in 'Garnacha Blanca' was significantly enhanced after Aki treatment. Even if in 'Garnacha Blanca' all the studied phytohormones excepted JA tended to be enhanced after Aki treatment; JA, ACC, and ABA were not significantly influenced by Aki treatment in any of the three cultivars.

BL treatment caused a significant enhancement of JA in ‘Garnacha Tinta’ while MeJA was significantly enhanced and ACC and GAs tended to be increased by BL treatment in ‘Garnacha Blanca’. Oppositely, GAs were significantly reduced after BL treatment in ‘Macabeo’. However, neither SA, ACC, nor ABA were significantly influenced by BL treatment in any of the three cultivars.

Phytohormones, after 24 h of BL or Aki treatment, changed without a clear pattern to allow the establishment of a defense signaling triggering mechanism.

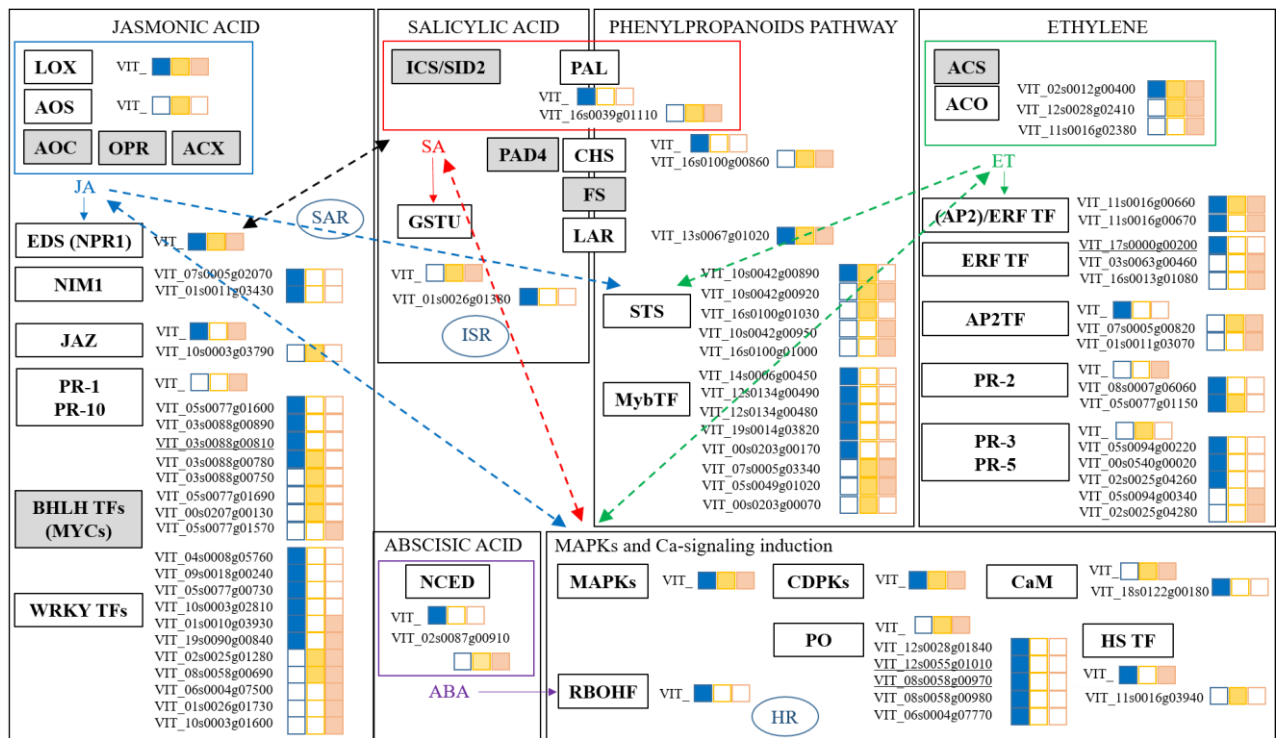
It is worth to mention that ABA global values detected in ‘Macabeo’ are higher than the values detected in the two ‘Garnacha’ varieties.

Four OA were identified in the leaves of the three grapevine cultivars: oxalic, tartaric, malic, and oxoglutaric (Table 10). Aki and BL treatments caused a significant reduction in oxoglutaric acid in ‘Macabeo’, but instead BL significantly increased the amount of this organic acid in ‘Garnacha Blanca’. BL treatment also reduced the level of tartaric acid in ‘Garnacha Tinta’. The rest of organic acids were not altered by these BPs application.

Total phenolic compounds concentration was significantly enhanced in ‘Macabeo’ and in ‘Garnacha Blanca’ after BL treatment. Aki treatment only tend to increase the level of total phenolic compounds.

## 4. DISCUSSION

In this study, we investigated the response of grapevine to the treatments with the botanical extract Akivi (Aki) and the beneficial microorganism *Bacillus* UdG (fresh, BF or lyophilized, BL). The GO analysis enlightens several upregulated GO terms that can be grouped in clusters related to stress response and plant defense stimulation; but KEGG analysis did not reveal similar results since no pathways related to stress response were enhanced after *Bacillus* treatments, and only the pathways of glutathione metabolism and terpenoid backbone biosynthesis related to stress response were enriched after Aki treatment. **Figure 8** shows a summary scheme of the genes related to the main plant defense response pathways whose expression levels were influenced by the treatments. Interestingly, BF triggered the same pathways as the BL treatment. However, the two *Bacillus* UdG treatments did not always trigger the same genes of the above mentioned pathways. Only Aki and BL treatments will be discussed hereafter. This result underlines the importance of the product formulation and conditioning on their efficacy and mode of action.



**Figure 8.** Scheme of main pathways related to plant defense response: Jasmonic Acid (JA); Salicylic Acid (SA); Ethylene (ET); Abscisic Acid (ABA); phenylpropanoids pathway; and mitogen activated protein kinases, Ca<sup>2+</sup> signaling induction (MAPKs). DEGs results are presented from RNA-seq analysis of grapevine leaves treated with the botanical extract (Aki, blue) and the microbial product (BF, yellow; or BL, orange). Complete DEGs transcript codes are written when the differential expression is above Log (FC) > 1.4; only VIT\_ is written otherwise. DEGs highly impacted by one of the treatments are underlined. Gene groups from the different pathways are indicated, the box is white colored when transcripts related to the genes' groups were found, the box is grey colored otherwise. JA and ET interactions with other pathways are represented with arrows. Black arrow represents JA and SA crosstalk.

LOX, LipOxygenase; AOS, Allene Oxide Synthases; AOC, Allene Oxide Cyclase; OPR, OPDA Reductase; ACX, Acetyl-CoA oXidase; EDS1/ NPR1, Enhanced Disease Susceptibility/ Non-expressor of Pathogen Related genes 1; NIM1, Non-Inducible Immunity 1; SAR, Systemic Acquired Resistance; JAZ, JAsonate-Zim domain; PR, Pathogenesis Related proteins; BHLH TFs, Helix Loop Helix TFs, WRKY TFs, Transcription Factors with domain WRKY.

ICS/SID2, IsoChorismate Synthase; PAL, Phenylalanine Ammonia Lyase; PAD4, PhytoAlexin Deficient 4; FS, Flavonoid Synthase; LAR, LeucoAnthocyanin Dioxygenase; GSTs, Glutathione-S-Tranferase; ISR, Induced Systemic Resistance; STS, STilbene Synthase; MyB TF, MyB Transcription Factors.

ACS: 1-Aminocyclopropane-1-Carboxylate Synthase; ACO, 1- Aminocyclopropane-1-Carboxylate Oxidase; ERF TF, (AP2)/ERF TF/AP2TF; Ethylene Response Factors Transcription Factors; PR, Pathogenesis Related proteins.

NCED, 9-Cis-Epoxy-carotenoid Dioxygenase. MAPKs, Mitogen Activated Protein Kinases; CDPKs, Ca<sup>2+</sup> DePendent Kinases; CaM, CalModulin; RBOHF, Respiratory Burst Oxidase Homologue protein F; PO, PerOxidases; HS TF, Heat Shock Transcription Factors.



Gene transcription related to JA biosynthesis was slightly influenced by either Aki and BL treatments since lipoxygenases (LOX) related genes did not show overexpression with  $\text{Log}_2(\text{FC})$  values higher than 1.4. However, phytohormone concentrations involved in JA pathway were affected in both cvs. Garnacha Tinta and Garnacha Blanca. Specifically, MeJA content was significantly higher after Aki and BL treatments in ‘Garnacha Blanca’ and JA content was doubled after BL treatment in ‘Garnacha Tinta’. These results were in agreement with the downstream genes regulated by the treatments. In fact, the expression levels of genes related to non-inducible immunity 1 (NIM1) were upregulated by Aki treatment and the expression of a gene related to enhanced disease susceptibility (EDS1) and to nonexpressor of pathogenesis-related genes 1 (NPR1) was slightly upregulated by both treatments. Moreover, Aki and BL treatments upregulated the expression of several genes involved in different transcription factors with domain WRKYs (WRKYs TFs) and pathogenesis related proteins (PR). Interestingly, one of the PR related genes (VIT\_03s0088g00810) was highly influenced by Aki treatment. Therefore, JA defensive pathway seemed to be triggered by both treatments. This is in agreement with the essential role of JA as a phytohormone in the regulation of defense gene expression (Rienth *et al.*, 2019). JA is able to regulate several genes involved in defense response, such as EDS1, NPR1, or NIM1 related genes. EDS1, NPR1 and NIM1 are involved in SAR modulation and they are essential for the expression of genes related to PR proteins (Ochsenbein *et al.*, 2006; Pajerowska-Mukhtar *et al.*, 2013; Backer *et al.*, 2019). PR genes are related to transcriptional regulators jasmonate-zim domain (JAZ) that target various transcription factors like helix loop helix transcription factors (BHLH TFs) and transcription factors containing WRKY domain (Kazan and Manners, 2012; Guerreiro *et al.*, 2016). Our results enlightened the link between PR upregulated genes and WRKYs TFs but they did not underline JAZ related genes intermediate.

The expression level of genes related to SA pathway were not clearly affected by neither Aki nor BL treatments. However, the measured content of SA phytohormone in grapevine leaves treated with Aki tended to be higher than leaves treated with NTC and BL on all three cultivars, especially in ‘Garnacha Tinta’ in which significant differences were observed. It could be explained by the upregulation of the expression of some genes related to EDS1, NIM1, and NPR1 already commented in JA part; in fact, they modulators that intervene in SA accumulation and they are produced in crosstalk between SA and JA pathways (Rustérucci *et al.*, 2001; Zhu *et al.*, 2011; Chen *et al.*, 2021). Interestingly, the expression level of the glutathione-S-transferase (GST) related gene was also upregulated by Aki treatment. It is reported that SA is able to regulate several genes from GST family that are upregulated through SA pathway in treated plants with beneficial microbials resulting in ISR priming (Gullner *et al.*, 2018). GST family enzymes are implied in detoxifying cytotoxic

compounds and the process involves transmembrane transport (Burdziej *et al.*, 2021), which is in agreement with our results since “transmembrane transport” GO term was found influenced by Aki and BL treatments.

The biosynthesis of ET seemed to be triggered by both treatments through the upregulation of the expression level of 1-aminocyclopropane-1-carboxylate oxidase (ACO) related genes. Moreover, the concentration of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) tended to present higher levels in ‘Garnacha Blanca’ leaves after Aki and BL treatments. These results were in agreement with the downstream ET response factors (ERF TF, (AP2)/ERF TF, AP2 TF) related genes which expression level was also upregulated by both treatments. In addition, the expression level of one of the genes related to ERF TF was highly influenced by Aki treatment (VIT\_17s0000g00200). Therefore, ET and JA defensive pathways seemed to be triggered by both treatments. ET response factors are key regulators of JA, ET, and ABA pathways in response to biotic and abiotic stresses, activating PR genes such as osmotins (PR-5), chitinases (PR-3) y  $\beta$ -1,3-glucanases (PR-2) (Mizoi *et al.*, 2012; Bahieldin *et al.*, 2016; Rienth *et al.*, 2019), which were indeed upregulated after both Aki and BL treatments.

The ABA biosynthesis was triggered by BL treatment through the upregulation of 9-cis-epoxycarotenoid dioxygenase (NCED) related gene expression. In fact, ABA biosynthesis starts with carotenoids and involves NCED enzyme that is strongly upregulated by stress (Xiong and Zhu, 2003). ABA is involved in the response to water stress and it particularly intervenes in stomatal closure (Catacchio *et al.*, 2019; Postiglione and Muday, 2020). It is expected to have variability in water stress response between these grapevine cultivars because the two ‘Garnacha’ are more resistant to drought than ‘Macabeo’. These results are consistent with ABA measured concentrations that were twice or three times higher in ‘Macabeo’ than in the two ‘Garnacha’; in fact, Macabeo cv. that is less resistant to drought is more likely to trigger water stress response involving ABA signaling. ABA is also involved in pathogen response signaling pathway and linked with SA, JA, and ET related genes regulation (Nishad *et al.*, 2020); for instance, ABA biosynthesis induction by laminarin treatment triggered JA production in grapevine (Balestrini *et al.*, 2020). However, ABA relation with JA-dependent related genes are closely linked with MYCs TF (Pieterse *et al.*, 2014) that were not influenced by any of the treatments in the present study.

The present study also underlined that the expression of a Phenylalanine Ammonia Lyase (PAL) related gene and a Chalcone Synthase (CHS) related gene were upregulated after BL treatment, whereas the expression of one Leucoanthocyanidin dioxygenase (LAR) related gene was upregulated after both treatments (Aki or BL). Stilbene biosynthesis was also

triggered by both treatments through the upregulation of Stilbene Synthase (STS) and Myb TF related genes expression. The transcriptomic results are in accordance with the total phenolic concentration in leaves, which tended to be higher after Aki and BL treatments in 'Garnacha Blanca' and statistically higher in 'Macabeo' after BL treatment. PAL, CHS, LAR, and flavonol synthase (FS) are key enzymes for the biosynthesis of the secondary metabolites phenylpropanoids, anthocyanins, flavonoids, and phytoalexins isoflavonoids (Campos *et al.*, 2003; Yonekura-Sakakibara *et al.*, 2019). These enzymes are related to SA biosynthesis as they share PAL enzyme that is in agreement with our results. Phytoalexins are produced and accumulated in the plant after a pathogen infection but it can also be triggered by PTI (Ahuja *et al.*, 2012; Piasecka *et al.*, 2015). From phytoalexins, several modifications lead to stilbene biosynthesis that require STS presence. It is worth to mention that the phytohormones JA, MeJA, SA, ET, and ABA positively regulate stilbene biosynthesis (Dubrovina and Kiselev, 2017). Particularly, JA and ET strongly trigger phenylpropanoids pathway, notably stilbene biosynthesis (Belhadj *et al.*, 2008; Rienth *et al.*, 2019) that is in agreement with our results.

The expression level of several genes related to Mitogen-Activated Protein Kinases (MAPKs) and Calcium ion ( $\text{Ca}^{2+}$ ) signaling pathways were slightly upregulated after Aki treatment and some of them after BL treatment as well; such as the expression of genes related to  $\text{Ca}^{2+}$  Dependent Kinases (CDPKs), Calmodulin (CaM), Respiratory Burst Oxydase Protein (RBOHF) and Heat Shock Transcription Factors (HS TFs) with an upregulation lower than  $\text{Log}_2(\text{FC}) > 1.4$ . In addition, one CaM and several peroxidases (PO) related genes were clearly upregulated after Aki treatment, and two of them were highly affected by Aki treatment (VIT\_12s0055g01010; VIT\_08s0058g00970) and involved in hypersensitive response (HR). As no phytotoxicity was observed after the treatments, Aki treatment may prime HR to be faster in case of pathogen infection. A crosstalk is described between MAPKs, JA, SA, and ET pathways (Rasmussen *et al.*, 2012; Jagodzik *et al.*, 2018; Nishad *et al.*, 2020) that is consistent with our results as all these pathways are upregulated by the treatments.

In addition, Aki and BL treatments had an effect on other metabolites, including the Oxoglutaric acid (2-OG) that showed higher content in 'Garnacha Blanca' leaves after BL treatment and lower content in 'Macabeo' leaves after Aki or BL treatments. The 2-OG is one of the intermediates of the tricarboxylic acid (TCA) cycle, that is one of the main metabolic cycles in living beings. The 2-OG is also involved in gibberellin (GA), alkaloid and flavonoid biosynthesis. In fact, the dioxygenase enzyme that intervene in both biosynthesis needs 2-OG as a co-substrate (Araújo *et al.*, 2014). Moreover, it was reported that treating grapevine with a structural mimic of 2-OG (prohexadione-Ca) inhibited the enzyme and altered flavonoid biosynthesis (high amount of unusual flavonoids) (Puhl *et al.*, 2008). This is in agreement with our results since phenylpropanoids pathway was triggered by either Aki and BL treatments.

Moreover, some authors point out that a reduction in the enzymatic activity of the TCA cycle leads to a reduction in the total levels of GA in tomato roots. The synthesis of GA from 2-OG and the activity of the involved enzymes suggests that it is the levels of this organic acid that control the rate of GA biosynthesis, but evidence at the molecular level is needed to establish and assess that interaction (Araújo *et al.*, 2012). In fact, the GA content in leaves was also affected in the present study, being GA1 and GA4 contents higher after treatments (Aki or BL) in ‘Garnacha Blanca’ and lower in ‘Macabeo’. However, the link with 2-OG concentration variations was not clear, that reinforce the hypothesis linking the 2-OG with flavonoid biosynthesis. It is known that GA is involved in the regulation of flowering, bunch morphology and berry size (Giacomelli *et al.*, 2013) that could be affected by the treatments, but further investigations will be necessary.

The concentration of Tartaric acid in grapevine leaves was also affected since lower concentrations were detected in ‘Garnacha Tinta’ leaves treated with BL. The explanation of this fact could be because tartaric acid was more used or its synthesis was negatively affected by the treatment. Grapevine presents a high concentration on tartaric acid and its biosynthesis occurs in leaves and berries. Tartaric acid was shown to be involved in various processes like apoplastic redox state, ROS signaling, cell wall softening of berries, antioxidant metabolism, oxidative burst, and stress tolerance. More particularly, abrupt changes in tartaric acid biosynthesis was linked with oxidative burst as well as ascorbate/glutathione redox state in berries (Burbidge *et al.*, 2021). More insight on this matter could give interesting results like a kinetics study of tartaric acid after BL treatment.

Grapevine response to BPs treatments at transcripts and metabolic level gave strong hints on the ability of the products to trigger the plant defense response. In fact, as shown in **Figure 8**, many transcripts related to defense responses were detected during the RNA-seq analysis of leaves after the treatments to the grapevine plants. Some of the transcripts did not present differential expression, but other transcripts were highly affected by Aki treatment (VIT\_17s0000g00200, VIT\_08s0058g00970, VIT\_12s0055g01010, and VIT\_03s0088g00810) and were selected as DEGs markers candidate’s analysis (A4, A5, A6, and A11, respectively). Considering all the upregulated transcripts in JA, ET, SA, and ABA pathways (**Figure 8**) and the contents of phytohormones; we can assess that the application of Aki and BL treatments to grapevine can stimulate several processes related to plant defense immune system like SAR. Particularly, the treatments upregulated JA, ET, and phenylpropanoid pathways. Moreover, Aki treatment seemed to trigger several genes involved with HR. Further investigations are needed to identify the mode of action of the two BPs candidates (Aki and BL).

These results also indicate that the treatments with the botanical extract (Aki) and with the beneficial microorganism (BL) might prime a defense response through ISR. However, the study was designed to investigate the interaction between the BPs and grapevine without infection of grapevine with a pathogen. If the mode of action is priming ISR, the effect can be seen only with the presence of the pathogen attack (Van Wees *et al.*, 2008; Pieterse *et al.*, 2014; Esmaeel *et al.*, 2020). In fact, a complex effect acting in two steps was observed on various BPs, such as the *Rheum palmatum* plant extract (Godard *et al.*, 2009), *Trichoderma harzianum* T39 (Perazzolli *et al.*, 2011), and sulphated laminarin (Trouvelot *et al.*, 2008), this last one already used in vineyards against downy mildew. These products presented stimulation of defence response through the induction of some genes directly after the treatment and the reinforcement of the modulation of defense response through other genes after pathogen inoculation. The infection may trigger BPs' activity and different grapevine response as observed using transcriptomics in watermelon (*Citrullus lanatus*) roots treated with the beneficial microbial candidate *B. velezensis* against *Fusarium oxysporum* fungal pathogen (Jiang *et al.*, 2019). More insights on Aki and BL possible modes of action could be revealed through another investigation introducing pathogen inoculation in the study like *P. viticola*, *E. necator*, or *B. cinerea* and analyzing grapevine response to the treatment after pathogen infection. This new investigation could be more accurate by doing a sampling kinetics to study the plant response along time to both treatment and pathogen inoculation as both transcriptional response and metabolic content response show interesting evolution along time (Jiang *et al.*, 2019).

Highly influenced DEGs were selected for both treatments and, as it was mentioned before, several of them are related to plant defense response (**Table 1**). In fact, from the twelve DEGs selected for Aki treatment eight are related to plant defense (A1, A3, A4, A5, A6, A7, A11, and A12). The principal ones are involved in the main pathways related to plant defense response (**Figure 8**) and were discussed above (A4, A5, A6, and A11). From the fifteen DEGs selected for BL treatment, four of them are related to plant defense (B1, B6, B8, and B14). In addition, another gene could be related to defense response (B12-VIT\_10s0116g00530) as it is involved in thiazole biosynthesis and thiazole is a precursor of thiamine that has been showed to be able to stimulate defense response. In fact, thiamine (vitamin B1) treatment activates resistance in tobacco against tobacco mosaic virus through PR-1 gene activation and SA pathway. It also triggered SAR defense response in several plants like in *Arabidopsis* against *Pseudomonas syringae* by activating PR-1 gene, callose deposition and oxidative burst hypersensitive response (Boubakri *et al.*, 2012). Focusing on grapevine, thiamine is able to induce resistance to downy mildew defense response elicitation in leaves of 'Chardonnay' cultivated in greenhouse-controlled conditions. The elicited defense response included

accumulation of hydrogen peroxide ( $H_2O_2$ ), callose deposition in stomata cells, phenylpropanoid compounds accumulation (stilbenes, phenolic compounds, flavonoids and lignin) and hypersensitive response. Thiamine triggered several genes involved in defense response like PR genes (glucanase, chitinases, serine protease inhibitor, glutathione-S-transferase) and lipoxygenases pathway involved in JA biosynthesis (Boubakri *et al.*, 2012, 2013) that is consistent with our results. This high rate of DEGs highly impacted by the treatments and related to defense response is consistent with the transcripts analysis previously mentioned, and with the hypothesis that Aki and BL treatments are able to induce resistance on grapevine.

Several DEGs marker candidates presented stable overexpression after the treatments (Aki or BL) in the three grapevine cultivars. They could be used as markers of activity of the products to test different doses of treatment, or different formulations in greenhouse-controlled conditions. In fact, doses and formulation are crucial steps on a product development and highly impact its efficacy. As observed in the present study and described in others (Bota *et al.*, 2015; Catacchio *et al.*, 2019; Fasoli *et al.*, 2019; Balestrini *et al.*, 2020), grapevine response to the treatment is variable according to the studied cultivar, so the identified markers are robust for the three tested cultivars and should be tested to extend their use on other cultivars. The markers could also be tested in field conditions, but it is difficult to detect an impact on transcriptome in field conditions due to vineyard biological variability (Balestrini *et al.*, 2020).

## **5. CONCLUSIONS**

Grapevine response to the treatments (Aki or BL) at transcripts and metabolites levels gave insights on modes of action of these BPs under development. RNA sequencing gave data about the different gene expression following treatments with BPs compared with the NTC in Garnacha Blanca cv. Furthermore, RT-qPCR enabled a quantification of several selected genes' response in three different cultivars. This information was complemented with metabolic analysis (phytohormones, phenols, and organic acids). Considering all the upregulated transcripts and enhanced metabolites concentrations related to JA, ET, and phenylpropanoids pathways; strong hints were found on grapevine defense induction by the treatments, but further investigations will be necessary to confirm these first results. In addition, several DEGs markers were identified presenting a stable overexpression after the treatments (Aki or BL) in the three grapevine cultivars. They could be used as markers of activity of the products for further investigations.

## **CONFLICT OF INTEREST**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## **AUTHOR CONTRIBUTIONS**

ML, EM, and EB obtained the financial support. MR, ND, ML, EM, and EB conceived and designed the research. MR, ND, ML, RT and EB conducted and performed the experiments. MR, ML, and EB analyzed the data. MR and ND wrote the first draft of the manuscript. ML, EM, and EB revised and edited the manuscript. All authors read, reviewed, and approved the final manuscript.

## **FUNDING**

This work was supported by PALVIP project that has been 65% co-financed by the European Regional Development Fund (ERDF) through the Interreg V-A Spain France Andorra programme ([POCTEFA 2014-2020](#)). POCTEFA aims to reinforce the economic and social integration of the French-Spanish-Andorran borders. Its support is focused on developing economic, social and environmental cross-border activities through joint strategies favouring sustainable territorial development.

## **ACKNOWLEDGMENTS**

The authors would like to thank Gemma Roselló, Héctor Naún Saravia, and Josep Pereda for the technical support in the greenhouse experiments and for growing up the grapevine stocks, and Rosa Padilla for her help in the laboratory. We are grateful to Anna Bonaterra for her help and expertise in cultivating Bacillus UdG strain. We are also thankful towards Anna Bonaterra and Luis Alejandro Moll for their help during the intense days of grapevine leaves samplings.





Appendix B. Supplementary material for:

## **GRAPEVINE RESPONSE TO PLANT EXTRACT AND MICROBIAL BIOCONTROL PRODUCTS**

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**Supplementary Table 1.** Concentration ( $\text{ng } \mu\text{L}^{-1}$ ), purity ( $\text{Abs}_{260/280}$  and  $\text{Abs}_{260/230}$  ratios) and integrity (RIN) of total RNA in samples of cv. Garnacha grapevine leaves obtained with five different protocols. Total RNA samples treated using the Spectrum™ Plant Total RNA Kit fulfilled the quantity and quality requirements to carry on RNA-sequencing (RNA-seq) analysis (RNA concentration above  $200 \text{ ng } \mu\text{L}^{-1}$ ,  $\text{Abs}_{260/280}$  ratio from 1.9 to 2.1,  $\text{Abs}_{260/230}$  ratio from 1.8 to 2.2, and RIN above 7).

Spectrophotometric measurements <sup>6</sup>					
RNA isolation protocol	Replicate	RNA concentration ( $\text{ng } \mu\text{L}^{-1}$ )	$\text{Abs}_{260/280}$	$\text{Abs}_{260/230}$	RIN <sup>7</sup>
PureLink® Plant RNA Reagent <sup>1</sup>	R1	1138.0	2.01	1.85	8.10
	R2	553.1	1.99	1.59	3.40
	R3	454.4	1.92	1.52	2.80
TRIzol™ Reagent <sup>2</sup>	R1	85.4	0.79	0.14	-
	R2	62.8	0.76	0.13	-
	R3	75.6	0.78	0.13	-
Spectrum™ Plant Total RNA Kit <sup>3</sup>	R1	507.0	2.06	1.97	8.80
	R2	473.8	2.05	1.94	8.80
	R3	483.0	2.00	1.97	8.60
CTAB 2-day extraction <sup>4</sup>	R1	222.4	2.07	1.87	4.80
	R2	624.0	2.09	2.1	3.70
	R3	326.9	2.07	1.97	4.10
CTAB 1-day extraction <sup>5</sup>	R1	32.1	1.69	0.64	-
	R2	58.3	1.87	1.16	-
	R3	42.6	1.75	0.95	-

<sup>1, 2</sup> Invitrogen™, Life Technologies, Carlsbad, CA

<sup>3</sup> Sigma-Aldrich, Merck KGaA, Darmstadt, Germany

<sup>4</sup> Iandolino *et al.*, 2004

<sup>5</sup> Mu *et al.*, 2017

<sup>6</sup> Spectrophotometric measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA)

<sup>7</sup> RNA Integrity Number (RIN). RIN measurement was performed using an Agilent 2100 Bioanalyzer (Agilent technologies, USA)

**Supplementary Table 2.** Standard curves and amplification efficiencies of selected DEGs and endogenous genes. Efficiency was calculated using the following formula:  $E = (10^{(-1/a)} - 1) \times 100$ ; where “a” is the slope of the curve.

Gene code	Slope	Linearity (R <sup>2</sup> )	Efficiency (%)
<b>Endogenous genes for expression data normalization</b>			
UBQ	-3.1975	0.9982	94.5319
VAG	-3.1984	0.9980	94.5736
<b>Selected DEGs impacted by <i>Bacillus</i> treatment</b>			
B1	-3.3549	0.9990	98.6422
B2	-3.2400	0.9992	96.4637
B3	-3.1767	0.9984	93.5608
B4	-3.2187	0.9995	95.5042
B5	-3.3248	0.9965	99.8803
B6	-3.1607	0.9958	92.8020
B7	-3.1415	0.9956	91.8774
B8	-3.2086	0.9991	95.0432
B9	-3.2204	0.9968	95.5814
B10	-3.2200	0.9996	95.5632
B11	-3.1472	0.9992	92.1535
B12	-3.1988	0.9995	94.5921
B13	-3.1957	0.9995	94.4486
B14	-3.3611	0.9990	98.3909
B15	-3.1398	0.9997	91.7948
<b>Selected DEGs impacted by Akivi treatment</b>			
A1	-2.9448	0.9922	81.4345
A2	-3.2068	0.9923	94.9606
A3	-2.9788	0.9977	83.3765
A4	-2.9636	0.9920	82.5159
A5	-3.2492	0.9984	96.8728
A6	-3.2250	0.9988	95.7898
A7	-3.2345	0.9990	96.2175
A8	-3.1898	0.9991	94.1745
A9	-3.2784	0.9997	98.1509
A10	-3.1796	0.9997	93.6973
A11	-3.1849	0.9994	93.9457
A12	-3.2421	0.9996	96.5573

**Supplementary Table 3.** Trimming of the total reads obtained by RNA-seq of each sample: Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, and non-treated control (NTC). Four biological replicates (R1, R2, R3 and R4).

<b>Sample</b>	<b>Before Trimming</b>	<b>After Trimming</b>
<b>Aki_R1</b>	38,686,326	32,735,444 (84.62%)
<b>Aki_R2</b>	31,168,690	26,280,264 (84.32%)
<b>Aki_R3</b>	45,077,410	38,346,848 (85.07%)
<b>Aki_R4</b>	42,671,630	36,470,692 (85.47%)
<b>BF_R1</b>	35,932,354	30,911,962 (86.03%)
<b>BF_R2</b>	39,632,304	34,292,640 (86.53%)
<b>BF_R3</b>	30,960,456	26,219,334 (84.69%)
<b>BF_R4</b>	37,725,956	32,086,976 (85.05%)
<b>BL_R1</b>	44,957,978	38,463,562 (85.55%)
<b>BL_R2</b>	33,369,744	28,770,018 (86.22%)
<b>BL_R3</b>	33,800,350	29,312,548 (86.72%)
<b>BL_R4</b>	33,260,154	28,436,820 (85.50%)
<b>NTC_R1</b>	43,724,406	36,870,722 (84.33%)
<b>NTC_R2</b>	35,748,642	30,338,146 (84.87%)
<b>NTC_R3</b>	38,252,254	32,522,744 (85.02%)
<b>NTC_R4</b>	38,341,052	32,965,052 (85.98%)

**Supplementary Table 4.** Mapping of the paired reads (fragments) obtained by RNA-seq of each sample: Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG, and non-treated control (NTC). Four biological replicates (R1, R2, R3 and R4).

Sample	Input paired reads	Unique Mapping	Multi Mapping	Unmapped	Assigned to genes
<b>Aki_R1</b>	16,367,722	15,513,853 (94.78%)	366,741 (2.24%)	487,128 (2.98%)	13,934,098 (85.13%)
<b>Aki_R2</b>	13,140,132	12,443,289 (94.70%)	310,816 (2.37%)	386,027 (2.94%)	11,078,501 (84.31%)
<b>Aki_R3</b>	19,173,424	18,135,605 (94.59%)	452,430 (2.36%)	585,389 (3.05%)	16,330,627 (85.17%)
<b>Aki_R4</b>	18,235,346	17,297,878 (94.86%)	396,665 (2.18%)	540,803 (2.97%)	15,920,944 (87.31%)
<b>BF_R1</b>	15,455,981	14,719,273 (95.23%)	314,501 (2.03%)	422,207 (2.73%)	13,665,530 (88.42%)
<b>BF_R2</b>	17,146,320	16,289,530 (95.00%)	355,456 (2.07%)	501,334 (2.92%)	14,754,625 (86.05%)
<b>BF_R3</b>	13,109,667	12,479,648 (95.19%)	277,620 (2.12%)	352,399 (2.69%)	11,255,908 (85.86%)
<b>BF_R4</b>	16,043,488	15,137,197 (94.35%)	325,128 (2.03%)	581,163 (3.62%)	13,828,936 (86.20%)
<b>BL_R1</b>	19,231,781	18,296,618 (95.14%)	395,252 (2.06%)	539,911 (2.81%)	16,991,936 (88.35%)
<b>BL_R2</b>	14,385,009	13,609,023 (94.61%)	301,182 (2.09%)	474,804 (3.30%)	12,282,573 (85.38%)
<b>BL_R3</b>	14,656,274	13,857,455 (94.55%)	314,967 (2.15%)	483,852 (3.30%)	12,522,586 (85.44%)
<b>BL_R4</b>	14,218,410	13,540,312 (95.23%)	291,038 (2.05%)	387,060 (2.72%)	12,471,903 (87.72%)
<b>NTC_R1</b>	18,435,361	17,494,387 (94.90%)	377,416 (2.05%)	563,558 (3.06%)	16,124,887 (87.47%)
<b>NTC_R2</b>	15,169,073	14,419,641 (95.06%)	325,207 (2.14%)	424,225 (2.80%)	13,078,392 (86.22%)
<b>NTC_R3</b>	16,261,372	15,380,534 (94.58%)	364,828 (2.24%)	516,010 (3.17%)	13,780,451 (84.74%)
<b>NTC_R4</b>	16,482,526	15,546,674 (94.32%)	340,108 (2.06%)	595,744 (3.61%)	14,006,571 (84.98%)

**Supplementary Table 5.** Genes included in the GO terms that belongs to the group of defense response regulation and in which their expression pattern shows upregulation ( $\text{Log}_2(\text{FC}) \geq 1.4$ ) after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR		
30	GO:0006879	cellular ion iron homeostasis	VIT_08s0058g00440					1,96	8,77E-04	ferritin	15, 18, 24
			VIT_08s0058g00430					1,82	1,32E-02	ferritin	15, 18, 24
			VIT_08s0058g00410					1,60	3,84E-03	ferritin 1 (FER1)	15, 18, 24
9	GO:2000022	regulation of JA mediated signalling pathway	VIT_13s0067g01020	1,98	5,40E-04	2,84	4,03E-11	2,32	1,49E-07	Leucoanthocyanidin dioxygenase	
			VIT_10s0003g03790			1,83	2,44E-03			Jasmonate domain-containing protein 8	ZIM 13
12	GO:0010469	regulation of signalling receptor activity	VIT_18s0001g08760	1,81	1,42E-11					<i>Phytosulfokines</i>	
13	GO:0031347	regulation of defense response	VIT_14s0081g00370	1,42	9,94E-06	1,85	3,59E-09			Ankyrin repeat	
			VIT_05s0165g00010			5,41	9,83E-06			Ankyrin repeat	
			VIT_10s0003g03790			1,83	2,44E-03			Jasmonate domain-containing protein 8	ZIM 9
			VIT_14s0081g00360			1,53	6,02E-13			Ankyrin repeat	
14	GO:0010112	regulation of SAR	VIT_07s0005g02070	1,86	4,11E-24					<i>NIMIN-1 ortologue</i>	
			VIT_01s0011g03430	1,76	3,64E-21					<i>NIM-1-Intracting 2 like</i>	
26	GO:0051096	positive regulation of helixase activity	VIT_00s0388g00030			1,45	3,85E-01	2,32	8,58E-04	DNA mismatch repair protein MSH3	

Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments

ID: GO term assigned identifier. (\*) the gene was also upregulated in this ID. Description in cursive means that other databases were used instead of vCOST.

FDR, false discovery rate. Upregulated gene:  $\text{log}_2(\text{FC}) \geq 1.4$

**Supplementary Table 6.** Genes included in the GO terms that belongs to the group of stress response regulation and in which their expression pattern shows upregulation ( $\text{Log}_2(\text{FC}) \geq 1.4$ ) after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR		
1	GO:0010200		VIT_13s0019g00480	1,87	4,63E-05					Zinc finger (C2H2 type) family	
		Response to chitin	VIT_03s0038g00340	1,77	5,41E-10	1,86	8,79E-06	1,53	6,21E-07	Salt tolerance homolog2	20
			VIT_05s0049g01020			1,69	9,99E-04	1,73	1,37E-03	Myb domain protein 14	27, 29
			VIT_08s0058g00690			1,68	7,00E-07	2,12	1,08E-06	WRKY DNA-binding protein 33	5, 25, 29
2	GO:0006955		Immune response	VIT_01s0011g05950	1,71	2,33E-06					NSL1 (necrotic spotted lesions 1)
4	GO:0009607	Response to biotic stimulus	VIT_05s0077g01600	1,61	1,21E-03					Pathogenesis protein 10 [Vitis vinifera]	
5	GO:0034605	Celular response to heat	VIT_08s0058g00690			1,68	7,00E-07	2,12	1,08E-06	WRKY DNA-binding protein 33	1, 25, 29
			VIT_11s0016g03940			1,49	1,73E-04			Heat shock transcription factor C1	8
7	GO:0009626	Plant-type hypersensitive response	VIT_01s0011g05950	1,71	2,33E-06					NSL1 (necrotic spotted lesions 1)	2
8	GO:0061408	Positive regulation of transcription from RNA polymerase II promoter in response to heat stress	VIT_11s0016g03940			1,49	1,73E-04			Heat shock transcription factor C1	5
15	GO:0010039	Response to iron ion	VIT_08s0058g00440					1,96	8,77E-04	ferritin	18, 24, 30
			VIT_08s0058g00430					1,82	1,32E-02	ferritin	18, 24, 30
			VIT_08s0058g00410					1,60	3,84E-03	ferritin 1 (FER1)	18, 24, 30
			VIT_02s0025g02510					1,52	2,54E-05	Metal-nicotianamine transporter YSL1	
16	GO:0070413	Trealose metabolism in respons to stress	VIT_17s0000g08010					2,06	4,40E-05	Trehalose 6-phosphate synthase	
			VIT_12s0028g01670					1,93	6,43E-06	Trehalose-phosphatase	
			VIT_01s0026g00280					1,72	3,72E-18	Trehalose 6-phosphate synthase	

Continue



**Supplementary Table 6.** (continued)

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR		
18	GO:0009617		VIT_08s0058g00410					1,60	3,84E-03	ferritin 1 (FER1)	15, 24, 30
		Response to bacterium	VIT_08s0058g00430					1,82	1,32E-02	ferritin	15, 24, 30
			VIT_08s0058g00440					1,96	8,77E-04	ferritin	15, 24, 30
20	GO:0080167	Response to karritin	VIT_03s0038g00340			1,86	8,79E-06	1,53	6,21E-07	Salt tolerance homolog2	1
21	GO:0006073		VIT_11s0052g01280					2,68	9,56E-05	<i>Xyloglucan endotransglucosylase/hydrolase</i>	
		Cellular glucan metabolic process	VIT_05s0062g00250					1,91	2,28E-28	Xyloglucan endotransglucosylase/hydrolase 15	
			VIT_01s0026g00200					1,50	3,19E-13	Xyloglucan endotransglucosylase/hydrolase 28	
22	GO:0009738	Abscisic acid activated signalling pathway	VIT_13s0067g01940					1,66	4,14E-07	Abscisic acid receptor PYL4 RCAR10	
			VIT_05s0077g01570					1,60	3,19E-06	<i>PR10</i>	
24	GO:0042542		VIT_12s0035g01910			2,32	1,82E-03			Heat shock protein 18.2 kDa class II	29
			VIT_13s0019g03160			1,75	8,91E-04			Heat shock protein 17.6 kDa class I	29
			VIT_00s0992g00020			1,63	1,11E-22	1,44	2,48E-17	Heat shock protein (HSP26.5-P) 26.5 kDa class P	29
		Response to hydrogen peroxide	VIT_08s0058g00440					1,96	8,77E-04	ferritin	15, 18, 30
			VIT_08s0058g00410					1,60	3,84E-03	ferritin 1 (FER1)	15, 18, 30
			VIT_00s0698g00010					1,60	9,15E-11	Catalase	
			VIT_08s0058g00430					1,82	1,32E-02	ferritin	15, 18
25	GO:0006970	Response to osmotic stress	VIT_08s0058g00690			1,68	7,00E-07	2,12	1,08E-06	WRKY DNA-binding protein 33	1, 5, 29

Continue

**Supplementary Table 6.** (continued)

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR		
27	GO:0046686	Response to cadmium ion	VIT_09s0002g03340			2,76	9,79E-04			unknown	
			VIT_04s0079g00810			1,89	7,11E-03			Cold induced protein	
			VIT_05s0049g01020			1,69	9,99E-04			Myb domain protein 14	1, 5, 25
29	GO:0009651:	Response to salt stress	VIT_12s0035g01910			2,32	1,82E-03			Heat shock protein 18.2 kDa class II	24
			VIT_13s0019g03160			1,75	8,91E-04			Heat shock protein 17.6 kDa class I	24
			VIT_05s0049g01020			1,69	9,99E-04			Myb domain protein 14	1, 27
			VIT_08s0058g00690			1,68	7,00E-07			WRKY DNA-binding protein 33	1, 5, 25
			VIT_00s0992g00020			1,63	1,11E-22			Heat shock protein (HSP26.5-P) 26.5 kDa class P	24

Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments

ID: GO term assigned identifier. (\*) the gene was also upregulated in this ID. Description in cursive means that other databases were used instead of vCOST.

FDR, false discovery rate. Upregulated gene: Log<sub>2</sub>(FC) ≥ 1.4.

**Supplementary Table 7.** Genes included in the GO terms that belongs to the group of stress-related response regulation and in which their expression pattern shows downregulation ( $\text{Log}_2(\text{FC}) \geq -1.4$ ) after treatments with Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG.

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR	$\text{Log}_2(\text{FC})$	FDR		
1	GO:0009734	Auxin-activated signaling pathway	VIT_05s0020g01070					-2,04	8,48E-07	IAA31	
2	GO:0043086	Negative regulation of catalytic activity	VIT_18s0001g09920	-1,40	3,67E-07					Cyclin delta-3 (CYCD3_1)	
			VIT_03s0180g00040	-2,48	4,16E-08					Cyclin D3_2	
	GO:0043086,	Negative regulation of catalytic activity, Positive	VIT_06s0009g02090	-2,73	3,90E-03					Cyclin CYCB1_2	
2, 3, 6 and 7	GO:0045787,	regulation of cell cycle,	VIT_08s0040g00930	-1,55	1,49E-10					Cyclin B-type	
	GO:0071249	and Cellular response to nitrate, and Positive	VIT_13s0067g01420	-1,67	6,59E-06					Cyclin 1b (CYC1b)	
	GO:0008284	regulation of cell population proliferation	VIT_18s0001g14170	-1,51	2,61E-05					Cyclin-dependent protein kinase regulator CYCB2_4	
			VIT_03s0038g02800			-1,89	6,37E-09	-1,93	1,55E-12	Cyclin B2;4	7
			VIT_03s0091g01060					-1,49	1,85E-11	Cyclin delta-2	7
			VIT_07s0129g00200					-1,65	2,50E-02	unknown	7
3	GO:0045787	Positive regulation of cell cycle	VIT_18s0001g02060			-1,46	1,13E-04	-2,00	1,26E-10	Cyclin A1	7
			VIT_19s0085g00690			-1,40	8,44E-04	-1,42	2,16E-04	Cyclin-dependent protein kinase CYCB3	7
			VIT_01s0127g00670	-2,60	3,69E-07					Proline extensin-like receptor kinase 1 (PERK1)	
			VIT_05s0020g01690	-1,79	5,21E-06					Receptor protein kinase	
5	GO:0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway	VIT_04s0008g04200	-1,86	1,99E-05					unknown	
			VIT_04s0023g03760	-1,49	9,27E-05					unknown	
			VIT_01s0010g02590					-1,47	5,14E-03	ATP-dependent DNA helicase RecQ	
7	GO:0008284	Positive regulation of cell population proliferation	VIT_03s0038g02800			-1,89	6,37E-09	-1,93	1,55E-12	Cyclin B2;4	3
			VIT_03s0091g01060					-1,49	1,85E-11	Cyclin delta-2	3
			VIT_07s0129g00200					-1,65	2,50E-02	unknown	3

Continue

**Supplementary Table 7.** (continued)

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR		
7	GO:0008284	Positive regulation of cell population proliferation	VIT_18s0001g02060			-1,46	1,13E-04	-2,00	1,26E-10	Cyclin A1	3
			VIT_19s0085g00690					-1,42	2,16E-04	Cyclin-dependent protein kinase CYCB3	3
			VIT_00s0131g00080	-1,79	1,36E-03					Annexin ANN4	8
8	GO:0009414	Response to water deprivation	VIT_06s0004g04370					-1,56	1,07E-17	Histone H4	
			VIT_13s0019g00780					-1,87	6,94E-17	Histone H4	
			VIT_13s0019g00800					-1,46	7,02E-05	<i>Histone H4</i>	
9	GO:0009909	Regulation of flower development	VIT_00s0131g00080	-1,79	1,36E-03					Annexin ANN4	7
			VIT_14s0083g00640			-1,82	4,54E-32			Constans 2 (COL2)	
10	GO:0010017	Red or far-red light signaling pathway	VIT_04s0008g05210			-1,56	2,64E-05	-1,52	1,44E-02	BZIP protein HY5 (HY5)	
			VIT_05s0020g01090					-1,40	3,60E-13	BZIP protein HY5 (HY5)	
11	GO:0045910	Negative regulation of DNA recombination	VIT_07s0005g01060					-1,48	1,36E-05	Histone H1	
			VIT_07s0141g00730					-1,41	2,14E-12	Histone H1	
			VIT_14s0081g00500					-1,72	3,13E-17	Histone H1	
13	GO:0009744	Response to sucrose	VIT_01s0011g04400					-1,63	3,85E-04	Origin recognition complex subunit 4	
			VIT_17s0000g01960			-1,69	4,58E-02	-1,92	7,87E-03	<i>Origin recognition complex subunit 5</i>	
15	GO:0010112	Regulation of systemic acquired resistance	VIT_01s0011g03440					-1,53	1,47E-09	DNA mismatch repair protein	
16	GO:0009627	Systemic acquired resistance	VIT_00s0333g00050					-1,58	1,09E-13	DIR1 (defective IN induced resistance 1)	
			VIT_08s0007g01370					-1,77	4,42E-03	Protease inhibitor/seed storage/lipid transfer protein (LTP)	
17	GO:0000076	DNA replication checkpoint signaling	VIT_01s0010g01670					-1,45	4,68E-03	<i>Zinc knucle</i>	
			VIT_07s0104g01740					-1,87	6,15E-04	Protein kinase WEE1	

Continue

**Supplementary Table 7.** (continued)

ID	GO ID	GO Description	Gene ID	Aki		BF		BL		vCOST Description	Other ID*
				Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR	Log <sub>2</sub> (FC)	FDR		
			VIT_00s0541g00020					-1,71	1,21E-03	BZIP transcription factor BZIP6	
			VIT_01s0026g01550					-1,71	1,34E-08	Homeodomain leucine zipper protein HB-1	
			VIT_02s0025g02710					-1,50	4,89E-03	NAC Secondary wall thickening promoting factor1	
18	GO:0045893	Positive regulation of transcription	VIT_08s0007g04200					-2,12	5,45E-05	Late meristem identity1 HB51/LMI1	
			VIT_08s0007g08150					-1,45	5,00E-02	<i>AP2/ERF domain- containing protein</i>	
			VIT_13s0067g02560					-3,09	5,60E-21	unknown	
			VIT_15s0048g02870					-1,73	1,94E-07	Homeobox-leucine zipper protein HB-7	

Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG treatments

ID: GO term assigned identifier. (\*) the gene was also downregulated in this ID

FDR, false discovery rate. Upregulated gene: Log<sub>2</sub>(FC) ≥ -1.4

Genes in cursive are shared by ID 2, 3, 6 and 7

Description in cursive means that other databases were used instead of vCOST.

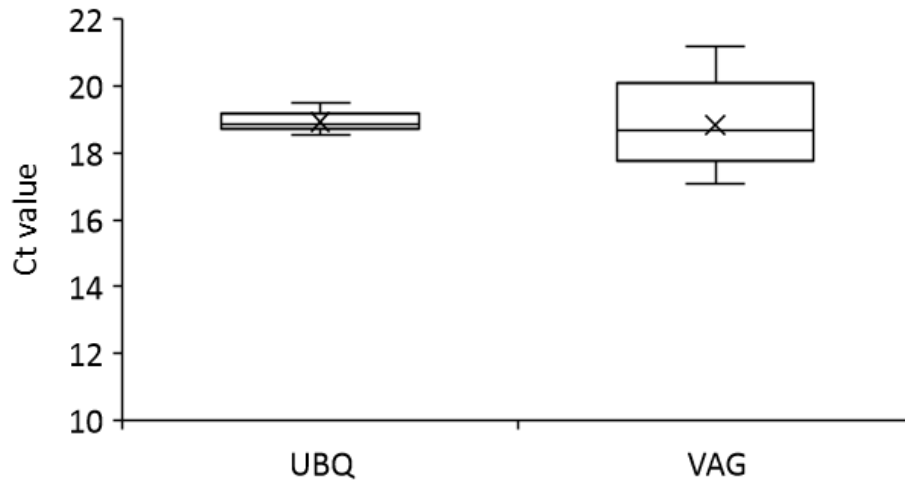
**Supplementary Table 8.** Mineral nutrient concentrations in leaves of grapevine cvs. Garnacha Blanca, Garnacha Tinta, and Macabeo treated with Akivi (Aki), lyophilized *Bacillus* UdG (BL), or water (NTC). Data show mean values of three biological replicates. Significant differences between treatment (Aki or BL) and NTC are represented by asterisks (\*).

Codes	'Garnacha Blanca'			'Garnacha Tinta'			'Macabeo'		
	NTC	Aki	BL	NTC	Aki	BL	NTC	Aki	BL
<b>Macronutrient (mg /g DW)</b>									
P	7.02	7.06	7.77	7.64	9.91	8.34	5.81	6.19	6.84
K	18.94	19.34	18.17	19.22	23.32	17.31	15.18	16.46	16.28
Ca	17.94	17.99	17.88	13.49	11.05	15.73	14.73	17.95	16.94
Mg	3.23	3.26	3.10	2.74	3.07	2.98	2.55	2.98	2.91
S	3.62	3.58	3.52	3.10	3.71	3.45	3.42	3.48	3.61
<b>Micronutrient (µg /g DW)</b>									
Fe	154.0	153.0	155.0	104.0	124.0	129.0	301.0	194.0*	165.0*
Mn	125.0	122.0	122.0	187.0	185.0	208.0	95.0	112.0	120.0
Na	171.0	91.0	211.0	132.0	161.0	276.0	375.0	359.0	617.0
B	37.8	34.5	35.6	35.0	39.7	34.2	27.5	36.1	31.1
Cu	16.2	13.1	10.7*	6.7	8.1	8.4	n.d.	n.d.	11.9
Zn	35.0	30.0	28.0	29.0	31.0	33.0	30.0	35.0	37.0

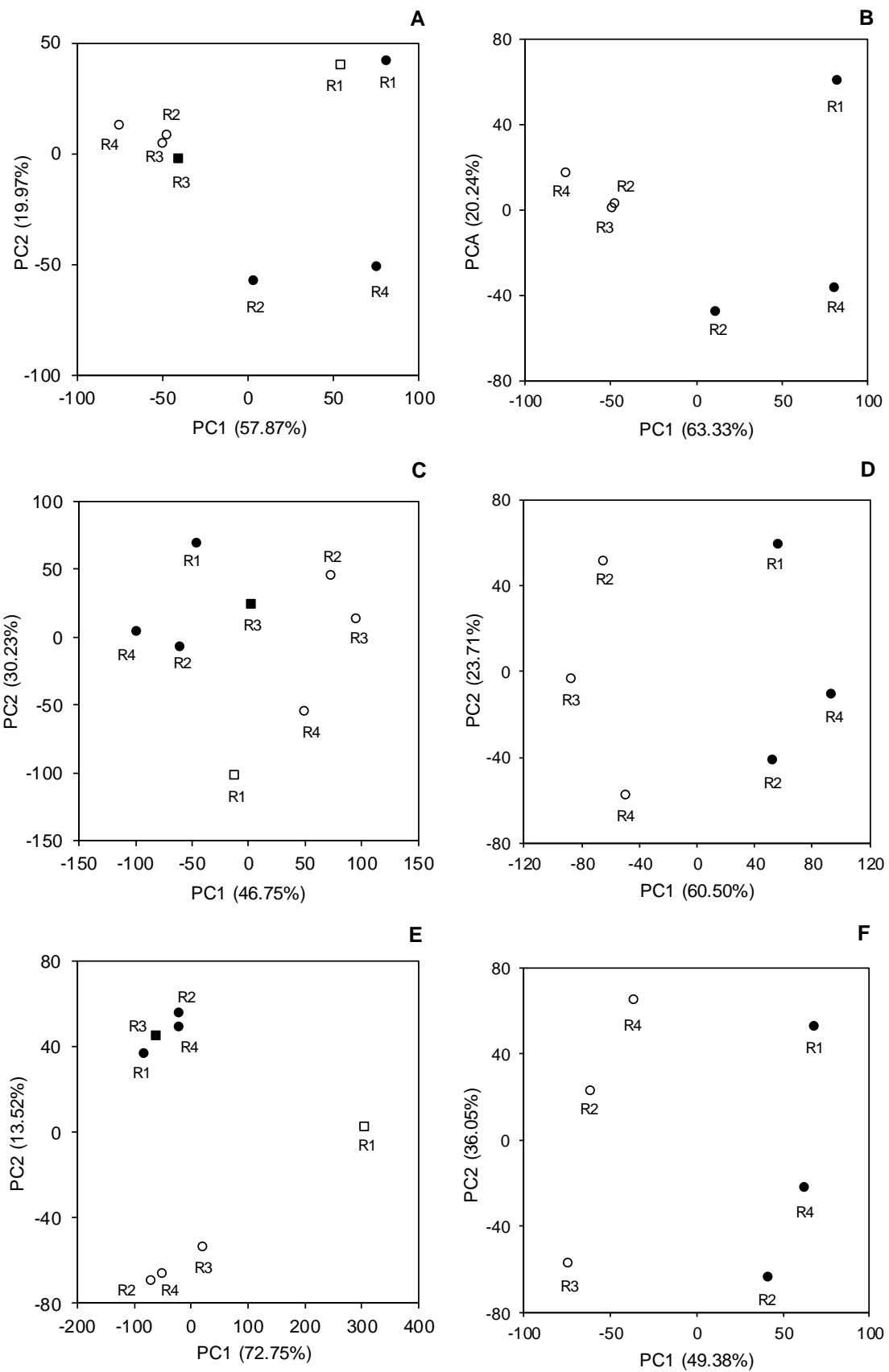
Macronutrient: P, phosphorus ; K, potassium ; Ca, calcium ; Mg, magnesium; S: sulphur

Micronutrient: Mn, manganese; Cu, copper; B, boron; Fe, iron; Na, sodium; Zn, zinc

n.d. no determined

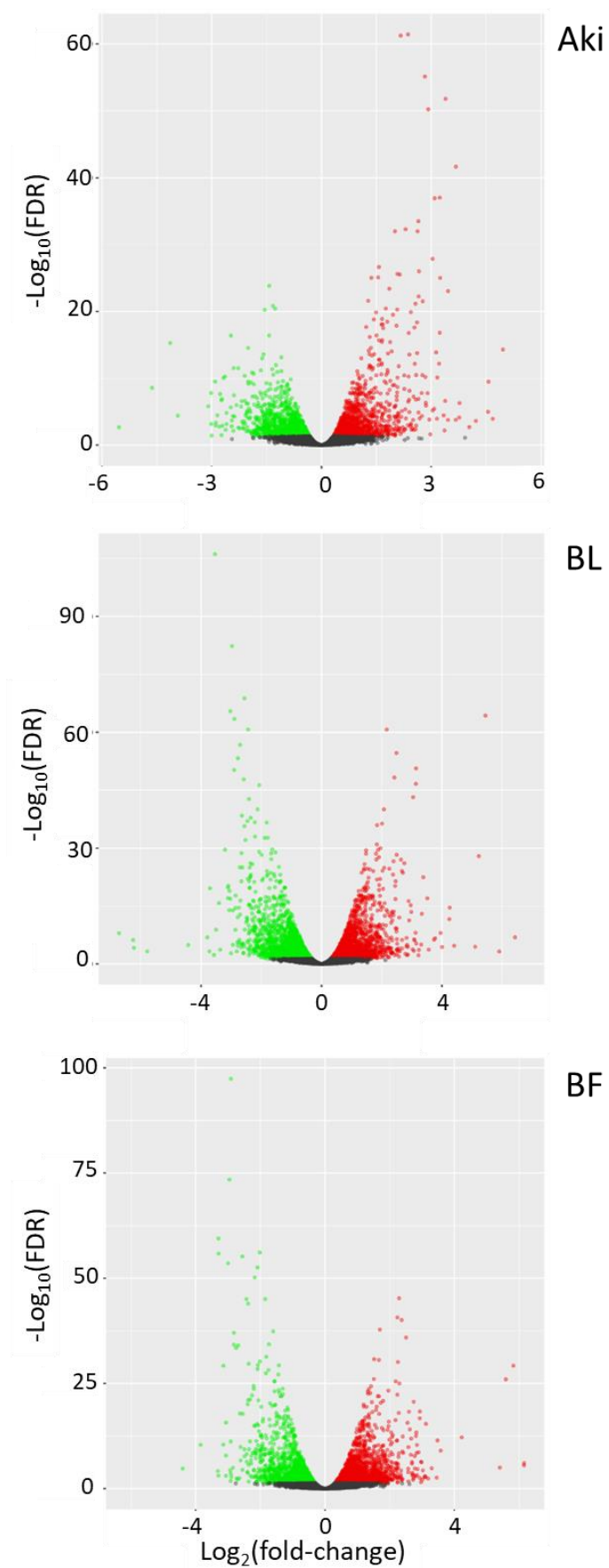


**Supplementary Figure 1.** Evaluation and validation of the most appropriate endogenous gene to normalize gene expression data according to the method described by Silver et al., (2006). VAG gene coding for the Vacuolar ATPase subunit G (GenBank accession number XM\_002281110.1) and UBQ gene coding for the Ubiquitin-conjugating enzyme (GenBank accession number EC922622) (Monteiro et al., 2013). Expression level stability of endogenous gene candidates between the four treatments (non-treated control, Akivi, lyophilized and fresh *Bacillus* UdG). Boxplots comparing Ct values of UBQ and VAG between treatments.

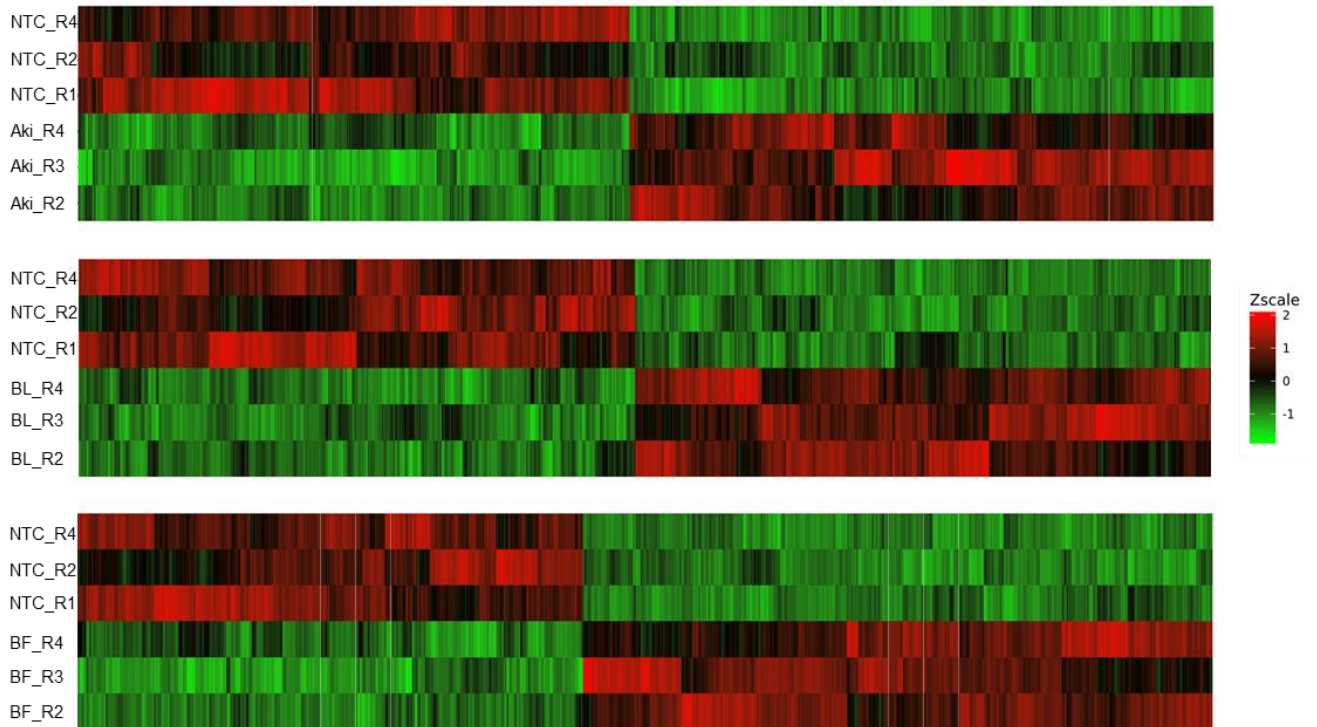




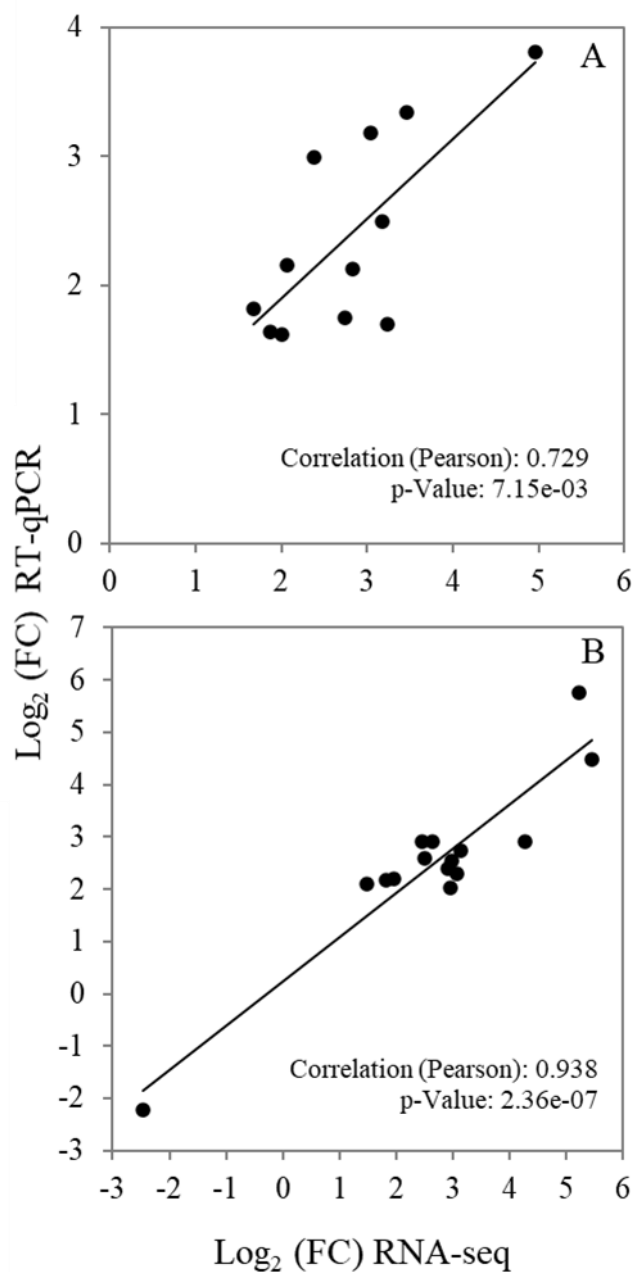
**Supplementary Figure 2.** Principal component analysis (PCA) of the biological replicates of Akivi (A and B), lyophilized (C and D), and fresh (E and F) *Bacillus* UdG treatments (white symbol) compared to the non-treated control (black symbol). In the left panels (A, C, and E) the four biological replicates (R1, R2, R3 and R4) are shown in the PCA for each modality. The three selected replicates for further analysis are represented by circles and the odd replicates are represented by squares. In the right panels (B, D, and F) shows the PCA of the filtered three replicates presenting less variability for each modality.



**Supplementary Figure 3.** Volcano Plots of differentially expressed genes (DEGs) in grapevine leaves comparing the treatments Akivi (Aki), lyophilized (BL) and fresh (BF) *Bacillus* UdG to the non-treated control (NTC). FDR: False discovery rate (adjusted P-values). Black dots represent the genes that are not significantly differentially expressed, while red and green dots are the genes that are significantly up and down regulated, respectively. The most upregulated genes are towards the right, the most downregulated genes are towards the left, and the most statistically significant genes are towards the top.



**Supplementary Figure 4.** Heatmaps of the differentially expressed genes (DEGs) (Z-scaled FPKM values) showing the results from the different treatments (Akivi, Aki; lyophilized, BL, and fresh, BF, *Bacillus UdG*) compared to the non-treated control (NTC). Changes in expression levels are displayed from green (down-expressed) to red (over-expressed). The order of the genes was established after hierarchical clustering using the Euclidean distance X.



**Supplementary Figure 5.** Relationship between RNA-seq and RT-qPCR transcriptomic data of selected genes in leaves of cv. Garnacha Blanca grapevine after treatment with Akivi (A) and lyophilized *Bacillus* UdG (B), respectively. Data shown as  $\text{Log}_2$  (FC), where FC means fold-change. The Pearson's correlation coefficient between relative expression levels is also shown.

**Supplementary information about mineral nutrient content**

The mineral nutrients were extracted with a classical extraction protocol. Briefly, 100 mg of fresh grapevine leave powder was dried and acid pre-digested overnight with 7 mL of solvent (HNO<sub>3</sub>:H<sub>2</sub>O<sub>2</sub> 69 %: 30 %, 5:2 v/v) and then digested in a hot-block digestion system (SC154-54-Well Hot Block™, Environmental Express, SC, USA) at 110 °C for 4 h. The digested samples were adjusted at 25mL with H<sub>2</sub>O milli-Q and filtrated at 45µm prior to nutrient element concentrations measurements by ICP-OES (Perkin Elmer Optima 8300, MA, USA). Blanks were included in each batch of samples for quality control (**Supplementary Table 8**).



## **Chapter 4.**

# **Discussion, Perspectives, and Conclusions**





## DISCUSSION AND PERSPECTIVES

The present PhD work aimed to develop new tools in order to monitor BPs fate in the treated plants and to give new insights on plant response to BPs in development. In fact, two BPs candidates evaluated within PALVIP project with promising efficacy results against fungal diseases on peach trees (*P. persica*) and grapevine (*V. vinifera*) were studied: Akivi, a botanical extract formulated prototype (*D. viscosa*, AkiNaO, (Tamm *et al.*, 2017)); and *Bacillus* UdG, a bacterial strain (*B. velezensis*, Universitat de Girona, (Montesinos *et al.*, 2018)). Akivi and *Bacillus* UdG are BPs able to preserve crops from fungal pathogens as claimed in the corresponding patents (Tamm *et al.*, 2017; Montesinos *et al.*, 2018). Their efficacies were evaluated in field experiments within the PALVIP project against fungal and oomycete pathogens like *M. fructigena* causal agent of brown rot in peach orchards; or *E. necator* and *P. viticola* respectively causal agents of powdery mildew and downy mildew in vineyards. The evaluation of the two BPs was conducted during four years from 2018 to 2021 and they gave promising results against these three pathogens.

In fact, Akivi presented intermediate efficacy in peach orchards experiments (2018 and 2019) against brown rot that is a postharvest disease impacting peach conservation. Particularly, during 2018 field experiments Akivi treated trees presented no rotten fruits; and during 2019 field experiments Akivi provided promising protection results with less than 25% of rotten fruits after 23 days of conservation, that was equivalent to the chemical reference. Moreover, Akivi presented promising results in grapevine in case of low powdery mildew (*E. necator*) pressure (2019, 2020, 2021) with an efficacy comparable to the sulphur reference treatment. In case of higher pressure (2018), Akivi presented intermediate efficacy. In addition, Akivi presented promising results in grapevine against downy mildew (*P. viticola*) at the beginning of the treatment campaign with an efficacy comparable to the copper reference treatment; when the disease pressure rise later on the treatment campaign, Akivi was no longer able to provide protection.

*Bacillus* UdG also provided promising protection results against brown rot on peach orchards (2018 and 2019) with an intermediate efficacy that was equivalent to another bacterial BP already registered (*Bacillus subtilis* QST 713). Moreover, *Bacillus* UdG presented intermediate efficacy against powdery mildew representing half the efficacy of the sulphur reference (2019). For that, *Bacillus* UdG was associated with half dose of sulphur in 2020 field experiments showing equivalent efficacy with the sulphur reference at low pressure of the disease, in the beginning of the treatment campaign.

Akivi and *Bacillus* UdG BPs efficacy results are in agreement with the EU objective to reduce the use of chemical PPPs (European Parliament and Council of the European Union, 2009a). BPs can indeed be used alone or associated with lower doses of chemical PPPs in case of low pathogen pressure that can last all the season some years. However, low pathogen pressure usually occurs at the beginning and the pressure rise along the treatment campaign; later, if the disease pressure is too high, BPs treatments can be substitute by classical PPPs treatments. Further treatment campaigns could be conducted to assess the promising results of both BPs.

In order to investigate BPs fate monitoring in the treated crops, samples from PALVIP field efficacy experiments were studied. Akivi was chosen as BP model to develop an innovative method based on untargeted metabolomics for the isolation and monitoring of BPs' residues (xenometabolites). In fact, residues dissipation study is mandatory for marketing authorization processes of PPPs (OECD, 2007b; European Parliament and Council of the European Union, 2009b). Current PPPs kinetics dissipation studies in plants are made through half-life approach (Fantke and Juraske, 2013) however this method is not suitable for complex BPs with unknown compounds like Akivi. That is why a new methodology was developed. It is based on the EMF (Environmental Metabolic Footprinting) approach that was firstly developed on soils and sediments in order to target the whole metabolome, *i.e.* the endometabolome and the xenometabolome [Chapter 1. - 3.3.] (Patil *et al.*, 2016; Salvia *et al.*, 2018).

The present PhD work mainly consisted in optimizing EMF approach in order to target only the xenometabolome with the aim of investigating residues dissipation. Also another kind of environmental matrix was studied as fruit matrices were investigated in my work. The approach was successfully applied on peach samples from field experiments conducted against brown rot (*M. fructigena*) and it was proven reliable for Akivi residues fate monitoring. Xenometabolites were successfully detected, isolated from peach endometabolites and tracked through the kinetics. Dissipation of Akivi residues was observed with different kinetics patterns: xenometabolites from the original BP more or less persistent that tend to dissipate between 7 days and 14 days after the treatment, but also xenometabolites showing by-products evolution pattern. The present study also enlightened that optimized EMF was able to monitor co-formulants besides the active substance (AS). We could make this hypothesis through our results. Indeed, one of the reference BP product used in the present study had an AS (potassium hydrogen carbonate salt,  $\text{KHCO}_3$ ) that presented too high solubility in water to be detected with our analytical

method but xenometabolites from the applied BP product were still detected that must be the co-formulants. Formulation is an important point for PPPs fate studies as it represents a high percentage of the final applied product and co-formulants can change the interaction between the AS and its environment by delaying AS release for example (encapsulation) or change the product efficacy. It was described for the chemical glyphosate AS that is specifically toxic to plants and that is not supposed to be toxic for animals but formulation compounds enhancing the AS's efficacy can make the final product toxic to many organisms (Marc *et al.*, 2002; Howe *et al.*, 2004).

On another hand, optimized EMF approach set up to target and monitor residues dissipation could also be used to determine the duration of the product efficacy. In fact, according to our results, total dissipation of Akivi xenometabolites is not reached 14 days after the treatment. On another hand, it was observed that xenometabolites from the original BP, that should represent Akivi efficacy, tend to dissipate 7 days after the treatment. Thus, the present work may conclude that crop protection provided by Akivi cannot be guaranteed beyond 7 days after the treatment but further experiments would be necessary to confirm that hypothesis. That is in agreement with the treatment campaign applied on peach tree against brown rot; treatments were indeed done every 7 days as classical interval for peach orchard fungicidal treatment campaign. It is worth to mention that, according to classical fungicidal treatment campaign in vineyards, the treatments were applied every 10 days during PALVIP grapevine field experiments against powdery mildew. Optimized EMF could be applied to grapevine matrix, in that case, dissipation of the original product xenometabolites must be carefully investigate around 10 days after the treatment to check if the product can still provide protection or if the treatments must be applied more frequently.

However, for further experiments, field experimental design needs to be improved to avoid contamination. As a matter of fact, the innovative method developed in the present work to monitor BPs residues was conducted on samples from PALVIP field efficacy experiments. However, we observed that this experimental design introduced an important risk of contamination of the control samples. In fact, field efficacy studies are usually designed in Fisher blocks mixing the replicates of every treatment modality in the agricultural plot to represent soil plot, and weather exposure variations. Untreated controls are also mixed within the Fisher block as showed **Chapter 2. – Figure S3**, therefore, untreated control crop replicates are close to the treated crop modalities and subjected to high risk of spray drift contaminations. The untreated control samples used

in the present study were contaminated by the chemical reference, and it should be avoided for further experiments. To avoid contaminations, crop field trials designed to study PPPs residues, like maximum residue limits settlements, are usually distinct from efficacy trials as they are conducted comparing distinct agricultural plots; treated plot on one side and untreated controls plot on the other side according to EU guidelines (OECD, 2021). More precisely, untreated control plot should be located near the treated plot to limit soil and weather variabilities between the plots, but control plots should be far enough to avoid spray drift (OECD, 2021). There is no settled minimum distance between the plots, but 20metres is commonly admitted as good distance. If the control plots are in close proximity to the treated plot, protective measures must be taken to prevent contamination like covering the control crop for instance. It is important to take into account the plots characteristics for field trial design, like major wind direction or plot slope to foresee spray drift and take preventive measures (OECD, 2021). Using a field experimental design specific to residues monitoring must be considered in order to monitor complex BPs in treated crops for marketing authorization purposes.

Another way to optimize the EMF methodology is through the analytical method. It could be enriched to rise the scope of EMF approach. As a matter of fact, in order to be used on a lot of samples and for national regulation application, a simple and reproducible extraction method was chosen in the present work. In fact, the approach was conducted using a classical extraction solvent, acetonitrile, used for PPPs studies (Carneiro *et al.*, 2013; Rajski *et al.*, 2014; Ferreira *et al.*, 2016; Rizzetti *et al.*, 2016; Machado *et al.*, 2017; Rutkowska *et al.*, 2018); together with a large polarity scale UHPLC column suitable for the study of most components (Polar C18 Reverse-Phase LC (RPLC) column). Optimized EMF to target residues was proven reliable for the monitoring of botanical extracts like Akivi BP. Another perspective of the present work would be to extend the approach to other types of BPs like beneficial microbials that will be a challenge as microorganisms are living organisms introducing more variability in the study. In addition, microorganisms produce specific compounds like cyclic lipopeptides (Mora *et al.*, 2015) that will need analysis optimizations in order to be extracted, separated, and detected. The used analysis method based on acetonitrile could be combined with other methods to detect and monitor special family compounds to rise the scope of the approach. For instance, small and polar components could be better extracted using different extraction solvents and different chromatography column types. In fact, acetonitrile is a middle range solvent in terms of relative polarity (0.460) in a scale from the most polar solvent, *i.e.* water (1.000) to the less polar solvent, *i.e.* tetramethylsilane (0.000) (Reichardt and Welton, 2010a).

Thus, acetonitrile is able to extract a large range of components especially components with middle range polarity that is why it was chosen, but components with extreme polarity close to 0 or close to 1 are not likely to be extracted. A different extraction method combining different solvents, taking into account solvents miscibility, could enrich the approach like acetone (0.355), 2-propanol (0.546), ethanol (0.654), methanol (0.762), and water (1.000) that are five miscible solvents along relative polarity scale excepted components presenting a very low polarity. Using a mix between these solvents as extraction solvent could extract more types of components and enrich the approach; that is currently considered in the lab. In addition, considering the environmental concerns that motivated the promotion of BPs, it could be interesting to integrate green chemistry principles to the approach like using green chemistry preferred solvents such as acetone, 2-propanol, ethanol, methanol or water. Acetonitrile is not part of them; it is not undesirable (red,) nor preferred (green), but usable (orange) (Reichardt and Welton, 2010b). Moreover, the samples were injected in a Polar C18 RPLC column, C18 RPLC columns are adapted to separate and allow the detection of middle range polarity components and, in addition, Polar C18 RPLC columns are adapted to separate some polar components that could be extracted with our extraction method using acetonitrile. If we increase the range of extracted components polarity by the above discussed extraction method, the extracts could be injected in different UHPLC column types to increase the number and properties of metabolites separated to allow their detection like the use of HILIC or ion chromatography type columns. However, as mentioned previously, one step extraction with acetonitrile was chosen because it is simple, reproducible, and has demonstrated good results for PPPs residues monitoring. If we want to improve the methodology with a multi-step extraction, the robustness of a more complex method will have to be evaluated to ensure that it does not show decreased repeatability on large amount of samples that would be an obstacle for the approach use at national regulation level like PPPs marketing authorizations.

Using a field experimental design specific to residues monitoring, EMF optimized approach developed in this PhD work to target residues dissipation could be a promising approach to monitor complex BPs in treated crops for marketing authorization purposes and to be adapted to other plant matrices. In fact, it is currently being adapted to grapevine leaves samples from field experiments conducted against powdery mildew (*E. necator*). The next step would be adapting the entire EMF approach (**Chapter 1. – Figure 11**) to plant matrices in order to monitor the impact of BPs on the treated crops; targeting the residues (xenometabolome) dissipation of the product, but also the product influence

on the studied matrix endometabolome. First, endometabolome study should be adapted to peach peel samples as xenometabolome study is already optimized for that matrix. Then, the entire approach could be adapted to other matrices as it is currently in progress for grapevine leaves samples; after that, more plant matrices could be tested like other fruits (apricots, cherries, or grapevine berries) or other plant parts (roots, stems, flowers). Entire EMF approach would be able to determine the resilience time of the global matrix metabolites (endometabolites and xenometabolites) after the treatment. The results could then be compared with the xenometabolome study (optimized approach) in order to evaluate if the treatment impact (xenometabolome and endometabolome monitoring) on the matrix last longer than residues dissipation. Entire EMF approach could also be valuable for marketing authorization purposes as investigating the environmental impact (plant, soil, and water) of the product is mandatory for PPPs approval, EMF approach could be used for plant and soil matrices (European Commission, 1994, 2013; European Parliament and Council of the European Union, 2009b).

To give more insights on the interaction between the selected BPs candidates and the treated plant, grapevine response to Akivi (botanical extract) and *Bacillus* UdG (microbial strain) was investigated. Studying this interaction is important as BPs are PPPs that present different modes of action (Villaverde *et al.*, 2016) that may need specific application field conditions to reach their optimum efficacy (Delaunois *et al.*, 2014). In fact, BPs candidates presenting interesting efficacy results in laboratory or greenhouse conditions may have difficulties to show an efficacy in vineyard conditions due to environmental variability (Delaunois *et al.*, 2014). BPs can be more fragile and sensible to degradation especially when they are applied on the phyllosphere; it has been pointed out for essential oil that are sensitive to humidity, light, heat, or oxygen degradation (Rienth *et al.*, 2019). It is also the case for beneficial microbials that can have colonization difficulties due to limited fitness on treated plant environment in field conditions (Bonaterra *et al.*, 2012), but it was not observed with *Bacillus* UdG that colonized the crop surface without difficulties during PALVIP field experiment. For that, BPs can present variable efficacy and need to be carefully applied considering their specific modes of action. Thus, grapevine response to Akivi or *Bacillus* UdG BPs candidate's treatment was studied at gene expression level and at metabolic content level using both transcriptomics and targeted metabolomics approaches. Natural extracts BPs like Akivi (botanical extract) or beneficial microbials BPs like *Bacillus* UdG (living bacteria strain) may present several modes of action that can be combined [Chapter 1. - 4.1.]. Such as direct action against the pathogen, plant defenses elicitation, and for microbials BPs, competition for space and

nutrients supplies (Bonaterra *et al.*, 2012; Perazzolli *et al.*, 2012; Mora *et al.*, 2015; Krzyzaniak *et al.*, 2018; Jiang *et al.*, 2019; Rienth *et al.*, 2019; Bodin *et al.*, 2020; Liu and Zhu, 2020; Burdziej *et al.*, 2021; González-Morales *et al.*, 2021).

Akivi was shown to have fungicidal activity due to its rich content on phenolic and terpenes compounds (Tamm *et al.*, 2017). As previously mentioned, field efficacy studies conducted within PALVIP project showed significant results of Akivi ability to protect grapevine against powdery mildew (*E. necator*) and downy mildew (*P. viticola*) that is concordant with previous studies and claims of Akivi patent (Tamm *et al.*, 2017), no phytotoxicity was observed. In addition, with its direct fungicidal action, the present study enlightens strong hints of plant defense stimulation (PDS) activity of Akivi treatment. In fact, transcriptomics study of grapevine ‘Garnacha Blanca’ cultivar leaves samples treated with Akivi candidate presented an upregulation of transcripts related to JA, ET, SA, and ABA pathways that are well known to be key actors of plant defense response and resistance induction against pathogen infection (Pieterse *et al.*, 2014; Rienth *et al.*, 2019; Abdul Malik *et al.*, 2020; Nishad *et al.*, 2020; Burdziej *et al.*, 2021). More especially, Akivi treatment triggered genes related to JA, ET, phenylpropanoid pathways, and genes related to peroxidases involved in HR; in fact, highly upregulated transcripts from ET and HR pathways were detected. The results of the present study seem to indicate that Akivi BP candidate present PDS activity. These results are in agreement with other studies on grapevine response to natural extracts presenting PDS activity such as laminarin upregulating a gene linked with ABA biosynthesis leading to JA production in grapevine (Balestrini *et al.*, 2020); chitosan triggering SA, JA, and ET defensive pathways in grapevine against mildews (Bodin *et al.*, 2020); or a confidential plant extract able to upregulate PR genes related to JA and ET defensive pathways, to upregulate phenylpropanoids pathway as well as enhance H<sub>2</sub>O<sub>2</sub> production related to peroxidases and HR leading to grapevine resistance against downy mildew (Krzyzaniak *et al.*, 2018). Transcriptomics results from the present study, conducted on grapevine from ‘Garnacha Blanca’ cultivar, were concordant with some of the results obtained by targeted metabolomics conducted on grapevine from ‘Garnacha Blanca’, ‘Garnacha Tinta’, and ‘Macabeo’.

*Bacillus* UdG was shown to have antagonistic activity against bacteria and fungi due to the production of cyclolipopeptides like surfactin, bacilomycin, iturin and fengycin, the lantibiotic subtilin, and the dipeptide bacilysin (Montesinos *et al.*, 2018). As previously mentioned, field efficacy studies conducted within PALVIP project showed promising results of *Bacillus* UdG ability to protect grapevine against powdery mildew (*E. necator*)



and downy mildew (*P. viticola*) that is concordant with the antifungal claims of the patent (Montesinos *et al.*, 2018), but rise the scope of target pathogens as neither mildews were mentioned; no phytotoxicity was observed. The present study enlightened strong hints of PDS activity of *Bacillus* UdG treatment that can be added to its direct antifungal activity. In fact, transcriptomics study of grapevine Garnacha Blanca cultivar leaves samples treated with *Bacillus* UdG BP candidate presented an upregulation of transcripts related to JA, ET, SA, and ABA pathways that are well known to be key actors of plant defense response and resistance induction against pathogen infection (Pieterse *et al.*, 2014; Rienth *et al.*, 2019; Abdul Malik *et al.*, 2020; Nishad *et al.*, 2020; Burdziej *et al.*, 2021). More specifically, *Bacillus* UdG treatment triggered genes related to JA, ET, and phenylpropanoids pathways. These results indicate that *Bacillus* UdG BP candidate seems to be able to induce defense response on the treated plant. These results are in agreement with other studies on grapevine response to beneficial microorganisms presenting PDS activity such as T39 (*Trichoderma harzianum*) able to trigger SA, JA, and ET defensive pathways leading to grapevine resistance against downy mildew (Bodin *et al.*, 2020) or cell wall derivatives from *Saccharomyces cerevisiae* that upregulate SA, JA, ET, PR proteins, and phenylpropanoids pathways related genes that was effective against downy mildew (De Miccolis Angelini *et al.*, 2019). Transcriptomics results of the present study, conducted on grapevine from Garnacha Blanca cultivar, were concordant with some of the results obtained by targeted metabolomics conducted on grapevine from the three cultivars.

However, the exact triggered pathways after both Akivi or *Bacillus* UdG treatments were unclear and the link with metabolic content was variable among cultivars and not always consistent with the hints revealed by upregulated transcripts. In fact, several of JA, ET and phenylpropanoids pathways related genes expression were upregulated after the treatments but only two JA biosynthesis related genes (LOX and AOS) were detected and was only slightly upregulated; one gene related to ET biosynthesis was found upregulated but only related to one of the enzyme implied in ET biosynthesis (ACO); some of the key enzymes related genes expression leading to phenylpropanoids and particularly stilbene biosynthesis were found upregulated but not all of them and two enzymes related genes expression were shown only slightly upregulated (**Chapter 3. – Figure 8**). Moreover, JA pathways downstream regulated genes expression were found upregulated but some intermediates were not clearly upregulated as EDS1, NPR1 and JAZ related genes. In addition, even if MeJA concentration was enhanced by both treatments in ‘Garnacha Blanca’, and higher level of JA was detected in ‘Garnacha Tinta’

after *Bacillus* UdG treatment; neither of JA or ACC concentrations were affected by Akivi treatment, and neither of ACC or SA concentrations were affected by *Bacillus* UdG treatment in the three cultivars. Moreover, SA concentration tended to be enhanced after Akivi treatment in the three cultivars even if very few SA related genes expression were found affected and most of them were only slightly upregulated.

Thus, further studies must be conducted to explore the hypothesis that Akivi and *Bacillus* UdG present a PDS activity, specifically in triple interaction BP/ pathogen/ plant host. These next studies should involve inoculation of grapevine with pathogens like *P. viticola* or *E. necator* to investigate plant response to the treatment in presence of pathogen as it was shown to be relevant in case of natural extracts (Trouvelot *et al.*, 2008; Godard *et al.*, 2009) and beneficial microbials (Van Wees *et al.*, 2008; Pieterse *et al.*, 2014) specific ISR defense type of priming. In addition, more information about grapevine response to both treatments could be investigate by rising the number of components targeted by the targeted metabolomics study. In fact, total phenolic content was measured but phenylpropanoids pathway and more specifically stilbene biosynthesis seems to be upregulated by both treatments; specific components of these pathways could be measured in further investigations like viniferins, or resveratrol that has been showed to be involved in grapevine resistance against downy mildew (Krzyzaniak *et al.*, 2018). Moreover, as TCA cycle seems to be affected by the treatment, other organic acids could be investigated. However, UV detection used in the present work for targeted metabolomics investigation was not sensitive enough to clearly detect and quantify succinic acid in our grapevine leaves samples. Mass spectrometry detection could be used to give more information on the metabolic contents.

To give more insights on Akivi and *Bacillus* UdG BP candidate interaction with the different grapevine cultivars, several highly influenced transcripts were selected and the expression of their related genes were studied in leaves samples from the three cultivars. Three transcripts upregulated by Akivi treatment and seven transcripts upregulated by *Bacillus* UdG treatment presented a stable expression response within the three cultivars and were identified as markers of respectively Akivi and *Bacillus* UdG treatments. These transcripts could be used as efficacy markers of the products to conduct experiments in controlled conditions on these cultivars to test doses of the product, or test different formulations of the product as it is an important part of the product composition and it can influence product efficacy. In fact, the present study showed that lyophilization of the bacteria was sufficient to modify some of the transcripts influenced by *Bacillus* treatments (*Bacillus* UdG before and after lyophilization, **Chapter 3. – Figure 8**). Moreover, the

efficacy markers could be used to test associations between the studied BPs with other products, as already discussed above, it can be necessary to combine products in order to increase field efficacy in case of high pathogen pressure. In addition, these efficacy markers could be used to track the duration of the plant protection provided by both products through a kinetics study. That kinetics study could be used together with EMF approach to track the impact of the Akivi on the treated plant, for that, markers must be tested in field conditions and the kinetics should last at least 7 days as most of Akivi compounds dissipates around this time point in peach peels. The kinetics study could also be used together with the monitoring of the bacteria. In addition, once EMF approach will be adapted to beneficial microbials BPs, these markers could be used together with EMF to track residues fate and impact of *Bacillus* UdG on the treated plant. However, for that, markers must be tested in field conditions.

The results of this PhD thesis give new insights on two BPs behavior in greenhouse and eventually in the field conditions, and during interaction with the plants. These products originated from natural sources are a promising complement of chemical PPPs in order to reduce the use of conventional chemicals, to rise the potential PPPs' modes of action and to reduce or at least delay the pathogens' resistance. To promote biocontrol products, it is necessary to accumulate knowledge about their action and interaction with the plants, the pathogen, and the whole environment. There is still work to do!

## CONCLUSIONS

- 1) An innovative approach allowing for monitoring complex BPs residues was developed. The approach was able to detect and monitor the evolution of all the different parts of the products: active substances, formulation compounds, and degradation by-products.
- 2) This approach was successfully applied and was proven reliable in order to monitor Akivi (a botanical extract formulated prototype) residues dissipation in peach fruit matrix in field conditions. Dissipation of most of the Akivi extract substances occurred between 7 days and 14 days after the last treatment but the sampling did not last long enough to reach complete dissipation of residues and degradation by-products.
- 3) Even if experimental design and field sampling strategies should be improved in order to overcome field-linked variabilities, the approach could be considered to complete BPs residues' study registration requirements.
- 4) The transcriptomics studies conducted on grapevine genes' expression response to two BPs, Akivi and *Bacillus* UdG (bacterial strain) showed strong hints of plant defense stimulation activity. Particularly, the treatments triggered JA, ET, and phenylpropanoids defense pathways in grapevine.
- 5) Three gene markers presenting a stable upregulated expression along the three grapevine cultivars were identified after Akivi treatment, they are coding for: a receptor protein kinase RK20-1, an ethylene-responsive transcription factor ERF114, and a pore-forming toxin-like protein Hfr-2.
- 6) Seven gene markers were identified after *Bacillus* UdG treatment, they are coding for: an invertase/pectin methylesterase inhibitor, a CCT motif constans-like, an unknown protein, a fasciclin arabinogalactan-protein (FLA21), a thiazole biosynthetic enzyme from chloroplast (ARA6), a polyphenol oxidase, and a nascent polypeptide-associated complex domain containing protein 2.
- 7) Further experiments should be conducted in order to give more insights on PDS activity of both treatments; particularly, the interaction BPs/ plant/ pathogen must be investigated and a kinetics study could be conducted to better link genes expression and metabolic content response as metabolites may take more than 24h to be biosynthesized in response to the treatment.



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