FORMULATION OF NANOPARTICLES WITH APPLICATION IN AGRICULTURE

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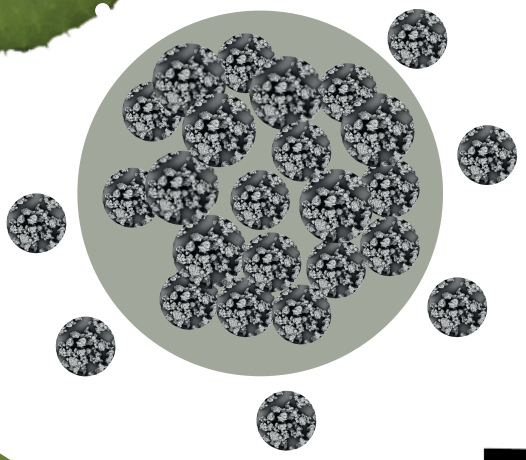

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UNIVERSITAT JAUME·I

Programa de Doctorado en Ciencias

Escuela de Doctorado de la Universitat Jaume I

Formulation of nanoparticles with application in agriculture

Memoria presentada por Jimmy Andres Sampedro Guerrero para optar al grado de doctor/a por la Universitat Jaume I

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Firmado por JIMMY ANDRES SAMPEDRO GUERRERO - NIF:****7415* el día 17/05/2024 con un certificado emitido por ACCVCA-120

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Castellón de la Plana, Mayo, 2024

AGRADECIMIENTOS INSTITUCIONALES

Agencias financiadoras del doctorando:

- Generalitat Valenciana, a través de una subvención del programa Santiago Grisolía (GRISOLIAP/2020/043).
- Universitat Jaume I, a través de la concesión de una ayuda para la realización de estancias cortas en otros centros de investigación.

Agencias financiadoras del proyecto de investigación o de los recursos materiales específicos del grupo de investigación:

- Ministerio de Ciencia e Innovación (MCIN), a través de la concesión del proyecto (PID2022-137825OB-100), financiado por MCIN/AEI/10.13039/501100011033 and ERDF A way of making Europe.
- Agencia Estatal de Investigación, a través de la concesión del proyecto European Union Next Generation (TED2021-129795B-I00).
- Programa AGROALNEXT (AGROALNEXT/2022/010), parcialmente financiado por el MCIN con fondos NextGenerationEU de la Unión Europea (PRTR-C17.I1) y por la Generalitat Valenciana
- INNEST/2023/122 financiado por la Unión Europea en el marco del Programa Fondo Europeo de Desarrollo Regional (FEDER) Comunitat Valenciana 2021-2027

MODALIDAD DE LA TESIS DOCTORAL

La tesis se encuentra enmarcada en el Area de Biología y Ciencias del Medio Natural, y se presenta por compendio de las siguientes publicaciones:

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2023). Efficient strategies for controlled release of nanoencapsulated phytohormones to improve plant stress tolerance. Plant Methods, 19(47). Impact factor 5.1, https://doi.org/10.1186/s13007-023-01025-x

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Improvement of salicylic acid biological effect through its encapsulation with silica or chitosan. International Journal of Biological Macromolecules, 199:108-120. Impact factor 8.2, https://doi.org/10.1016/j.ijbiomac.2021.12.124

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Encapsulation Reduces the Deleterious Effects of Salicylic Acid Treatments on Root Growth and Gravitropic Response. International Journal of Molecular Sciences, 23(22):14019. Impact factor 5.6, https://doi.org/10.3390/ijms232214019

Esta tesis dispone de la aceptación de los coautores de las publicaciones que el doctorando/a presenta como tesis y su renuncia expresa a presentarlas como parte de otra tesis doctoral.

AGRADECIMIENTOS

En primer lugar, quiero expresar mi más profunda gratitud a mis supervisores: Carola y Aurelio. Estoy infinitamente agradecido por la oportunidad de formar parte de este grupo multidisciplinar, por acogerme y hacerme sentir como en casa. Mil gracias por la invaluable guía y apoyo durante todo el proceso de mi tesis. Agradezco en gran manera sus comentarios y sugerencias, que ayudaron a mejorar mi trabajo, y que a su vez me permitieron consolidarme como investigador.

También quiero agradecer a Tico, por ser un pilar en mis primeros años de tesis, por sus ideas, enseñanzas, y apoyo, que fueron fundamentales para completar esta investigación. Estoy agradecido con las personas que conforman el Grupo de Ecofisiología y Biotecnología + Ingeniería Química, porque de todos y todas me llevo un invaluable aprendizaje.

Asimismo, quiero expresar mi agradecimiento al Dr. Mariano Perales por brindarme la oportunidad de realizar una breve estancia de investigación en su laboratorio. Agradezco a Mariano por compartir conmigo su invaluable conocimiento y experiencia, lo cual ha sido fundamental para continuar con mi desarrollo profesional.

Un agradecimiento especial a mi familia, y en especial a mis padres y mi hermana, gracias a ellos hoy estoy donde estoy, gracias a su apoyo incondicional a lo largo de toda mi carrera académica y de toda mi vida.

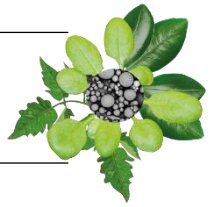
Finalmente, y más importante, gracias a mi esposa Vane, por haber confiado en este sueño que parecía muchas veces inalcanzable. Por acompañarme cada día, y por haber celebrado y llorado juntos cada momento que nos ha regalado la vida. Gracias por quedarte y creer en mí.

Gracias, Dios, por no dejarme caer.

“Toda persona debe decidir una vez en su vida,
si se lanza a conquistar sus sueños arriesgándolo todo,
o se sienta a contemplar el paso de los triunfadores”

Juan Manuel Marquez

CONTENTS



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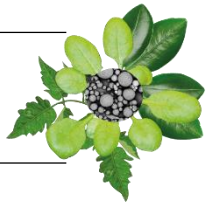

Introduction

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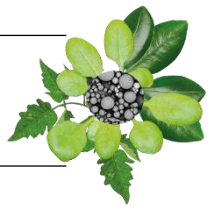
ABBREVIATIONS



PHs - phytohormones

SA - salicylic acid

JA - jasmonic acid

ABA - abscisic acid

IAA - indole-3-acetic acid

Et - ethylene

GAs - gibberellins

CKs - cytokinins

BRs - brassinosteroids

SL - strigolactones

OPDA - 12-oxophytodienoic acid

MeJA - methyl jasmonate

JA-Ile - jasmonoyl-isoleucine

JAs - jasmonates

NPR1 - NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1

COI1 - CORONATINE INSENSITIVE 1

PYR/PYL - PYRABACTIN RESISTANCE1/PYR1-LIKE

TIR1 - TRANSPORT INHIBITOR RESPONSE 1

ETR1 - ETHYLENE RECEPTOR 1

GID1 - GIBBERELLIN-INSENSITIVE DWARF 1

CRE1 - CYTOKININ RESPONSE 1

BRI1 - BRASSINOSTEROID INSENSITIVE 1

D14 - DWARF 14

TPP - tripolyphosphate

SGC - soft gelatine capsule

HGC - hard gelatine capsule

Phe - phenylalanine

t-CA - trans-cinnamic acid

BA - benzoic acid

IC - isochorismate

PDA - potato dextrose agar

DCM - Dichloromethane

Si - Silica

Ch - chitosan

Si:SA - encapsulated salicylic acid in silica

Ch:SA - encapsulated salicylic acid in chitosan

SEM - scanning electron microscopy

EDX/EDS - semi-quantitative energy dispersive X-ray

EE - encapsulation efficiency

S_{BET}/Se - specific surface area

TGA - thermogravimetric analysis

TG - weight losses

DTG - derivative weight losses

DTA - differential thermal curves

SC - solid content

$D_{10, 50, 90}$ - particle diameter

PGRs - plant growth regulators

MS - Murashige & Skoog Medium

DHJA - dehydro jasmonic acid

GFP - green fluorescent protein

ACT - actin

TUB - tubulin

PP2A - protein phosphatase 2A

HS - heat stress

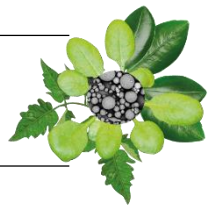
EDS1 - ENHANCED DISEADE SUCEPTIBILITY 1

ICS1 – ISOCHORISMATE SYNTHASE 1

PAL1 – PHENILALANINE AMMONIA-LYASE 1

PBS3 – avrPphB SUSCEPTIBLE 3

SUMMARY



Plants are exposed to extreme weather conditions throughout their entire life cycle. Climate change aggravates these unfavourable conditions, leading to a decrease in both the quality and quantity of crop production. Plants have developed several defensive strategies to face with abiotic stresses. These responses are coordinated and involve physiological, biochemical, and morphological changes that allow them to adapt to new conditions. Phytohormones play a vital role in controlling the defense response while maintaining normal plant growth and development.

Salicylic acid coordinates the systemic plant defense response by altering the endogenous levels of other phytohormones, triggering the nitrogen metabolism, the antioxidant system, the expression of defense-related genes, and the production of phytocompounds. Several studies have shown that applying salicylic acid (SA) as an exogenous treatment can alleviate the stress. Encapsulating SA in sheltering materials can enhance its potential palliative effect. Controlled release techniques allow for the simple management of bioactive compounds, enabling dosage control and protection against external conditions as pH and temperature.

The current doctoral thesis is structured into four chapters. The first chapter focuses on the formulation and characterization of the physico-chemical, and biological properties of SA encapsulated on silica and chitosan. Our results demonstrate that SA is successfully encapsulated and exhibits controlled release at the lower ratios of 1:0.25 and 1:0.5 for Si:SA and Ch:SA, respectively. These encapsulated samples evidence a promising antifungal effect, reducing the mycelial growth of pathogenic fungi. Furthermore, the encapsulated SA samples alleviate the toxic effect on Arabidopsis development due to the controlled release of SA.

In second chapter, the encapsulation of SA is optimized, centring the study on the principal variables process. This chapter demonstrate that SA is not degraded during spray drying, avoiding the use of acetone as a solvent. Optimization evidences that solid content, milling speed, and milling time are decisive variables to correctly raw material homogenization, and subsequently atomization drying. These results support an environmentally friendly atomization, preventing wear and tear on raw materials and energy consumption.

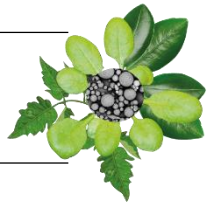
The third chapter is about the comparative effect of free SA vs encapsulated SA treatments on the morphological and physiological parameters of *Arabidopsis thaliana* plants. Results show that encapsulated SA samples are able to revert the free SA negative effects on Arabidopsis treated plants.

Encapsulated SA treatment decreases the SA available in the medium, and treated plants maintain the root length, rosette size and gravitropism similar to control plants. Additionally, encapsulation prevents the uncontrolled release of SA, thereby hindering its accumulation on the root and preventing a decrease of IAA in the root tip.

Finally, the fourth chapter focuses on determining the mechanism by which encapsulated SA promotes tolerance in Arabidopsis plants under simple and double stress conditions. The results demonstrate that encapsulated SA enhances plant tolerance against salt or mannitol stress, as well as their combination with HS. This positive effect is due to the maintenance of a proper equilibrium between endogenous levels of SA and IAA in plants treated with encapsulated SA (in contrast with those treated with free SA). The tight regulation of EDS1, PAL1, and NPR1 expression avoids excess SA synthesis and its non-physiological over-accumulation.

This work provides a novel tool for plant stress resilience. The knowledge gained about the mechanism by which encapsulated SA protects plants from salt, mannitol, heat stress, and their combinations offers a valuable starting point for encapsulating other phytocompounds with highly protective characteristics.

RESUMEN



Las plantas están expuestas a condiciones climáticas extremas a lo largo de todo su ciclo vital. El cambio climático agrava estas condiciones desfavorables, provocando una disminución tanto cualitativa como cuantitativa de la producción vegetal. Las plantas han desarrollado varias estrategias de defensa para hacer frente al estrés abiótico. Estas respuestas están coordinadas e implican cambios fisiológicos, bioquímicos y morfológicos que les permite a las plantas adaptarse a las nuevas condiciones. Las fitohormonas desempeñan un papel vital en el control de la respuesta de defensa al mismo tiempo que mantienen el crecimiento y desarrollo normal de la planta.

El ácido salicílico coordina la respuesta de defensa sistémica de la planta alterando los niveles endógenos de otras fitohormonas, activando el metabolismo del nitrógeno, el sistema antioxidante, la expresión de genes relacionados con la defensa y la producción de fitocompuestos. Varios estudios han demostrado que la aplicación de ácido salicílico (SA) como tratamiento exógeno puede aliviar el estrés. La encapsulación de SA en materiales de recubrimiento puede potenciar su efecto paliativo. Las técnicas de liberación controlada permiten un manejo sencillo de los compuestos bioactivos, posibilitando el control de la dosis y la protección frente a condiciones externas como el pH y la temperatura.

La presente tesis doctoral se estructura en cuatro capítulos. El primer capítulo se centra en la formulación y caracterización de las propiedades físico-químicas y biológicas del SA encapsulado en sílice y quitosano. Nuestros resultados demuestran que el SA se encapsula con éxito y presenta una liberación controlada en las proporciones más bajas de 1:0.25 y 1:0.5 para Si:SA y Ch:SA, respectivamente. Estas muestras encapsuladas evidencian un prometedor efecto antifúngico, reduciendo el crecimiento micelial de hongos patógenos. Además, las muestras encapsuladas de SA alivian el efecto tóxico sobre el desarrollo de Arabidopsis debido a la liberación controlada del SA.

En el segundo capítulo, se optimiza la encapsulación del SA, centrando el estudio en las principales variables del proceso. En este capítulo se demuestra que el SA no se degrada durante el secado por atomización, evitando así el uso de acetona como disolvente. La optimización evidencia que el contenido en sólidos, la velocidad de molienda y el tiempo de molienda son variables decisivas para la correcta homogeneización de la materia prima y, posteriormente, el secado por atomización. Estos resultados apoyan una atomización respetuosa con el medio ambiente, evitando el desgaste de las materias primas y el consumo de energía.

El tercer capítulo compara el efecto de los tratamientos con SA libre y SA encapsulado sobre los parámetros morfológicos y fisiológicos de las plantas de *Arabidopsis thaliana*. Los resultados evidencian que las muestras de SA encapsulado son capaces de revertir los efectos negativos del SA

libre sobre las plantas de Arabidopsis. El tratamiento con SA encapsulado disminuye el SA disponible en el medio, y las plantas tratadas mantienen la longitud de la raíz, el tamaño de la roseta y el gravitropismo similares a las plantas control. Además, la encapsulación impide la liberación incontrolada de SA, dificultando así su acumulación en la raíz y evitando la disminución de IAA en la punta de la misma.

Finalmente, el cuarto capítulo se centra en determinar el mecanismo por el cual el SA encapsulado promueve la tolerancia en plantas de Arabidopsis bajo condiciones de estrés simple y doble. Los resultados demuestran que el SA encapsulado mejora la tolerancia de las plantas frente al estrés por sal o manitol, así como su combinación con SA libre. Este efecto positivo se debe al mantenimiento de un equilibrio adecuado entre los niveles endógenos de SA e IAA en las plantas tratadas con SA encapsulado (en contraste con las tratadas con SA libre). La regulación estricta de la expresión de EDS1, PAL1 y NPR1 evita el exceso de síntesis de SA y su sobreacumulación no fisiológica.

Este trabajo proporciona una herramienta novedosa para potenciar la resistencia de las plantas al estrés. Los conocimientos adquiridos sobre el mecanismo por el cual el SA encapsulado protege a las plantas de la sal, el manitol, el estrés térmico y sus combinaciones, ofrecen un valioso punto de partida para encapsular otros fitocompuestos con características protectoras.

RESUM

Les plantes estan exposades a condicions climàtiques extremes al llarg de tot el seu cicle vital. El canvi climàtic agreuja estes condicions desfavorables, provocant una disminució tant qualitativa com quantitativa de la producció vegetal. Les plantes han desenvolupat diverses estratègies de defensa per a fer front a l'estrés abiòtic. Estes respostes estan coordinades i impliquen canvis fisiològics, bioquímics i morfològics que els permet a les plantes adaptar-se a les noves condicions. Les fitohormones exercixen un paper vital en el control de la resposta de defensa al mateix temps que mantenen el creixement i desenvolupament normal de la planta.

L'àcid salicílic coordina la resposta de defensa sistèmica de la planta alterant els nivells endògens d'altres fitohormones, activant el metabolisme del nitrogen, el sistema antioxidant, l'expressió de gens relacionats amb la defensa i la producció de fitocompuestos. Diversos estudis han demostrat que l'aplicació d'àcid salicílic (SA) com a tractament exogen pot alleujar l'estrés. L'encapsulació de SA en materials de recobriment pot potenciar el seu efecte pal·liatiu. Les tècniques d'alliberament controlat permeten un maneig senzill dels compostos bioactivos, possibilitant el control de la dosi i la protecció enfront de condicions externes com el pH i la temperatura.

La present tesi doctoral s'estructura en quatre capítols. El primer capítol se centra en la formulació i caracterització de les propietats fisicoquímiques i biològiques del SA encapsulat en sílice i *quitosano. Els nostres resultats demostren que el SA s'encapsula amb èxit i presenta un alliberament controlat en les proporcions més baixes de 1:0.25 i 1:0.5 per a Si:SA i Ch:SA, respectivament. Estes mostres encapsulades evidencien un prometedor efecte antifúngic, reduint el creixement micelial de fongs patògens. A més, les mostres encapsulades de SA alleugen l'efecte tòxic sobre el desenvolupament de Arabidopsis a causa de l'alliberament controlat del SA.

En el segon capítol, s'optimitza l'encapsulació del SA, centrant l'estudi en les principals variables del procés. En este capítol es demostra que el SA no es degrada durant l'assecat per atomització, evitant així l'ús d'acetona com a dissolvent. L'optimització evidencia que el contingut en sòlids, la velocitat de molta i el temps de molta són variables decisives per a la correcta homogeneïtzació de la matèria primera i, posteriorment, l'assecat per atomització. Estos resultats donen suport a una atomització respectuosa amb el medi ambient, evitant el desgast de les matèries primeres i el consum d'energia.

El tercer capítol compara l'efecte dels tractaments amb SA lliure i SA encapsulat sobre els paràmetres morfològics i fisiològics de les plantes de *Arabidopsis thaliana*. Els resultats evidencien que les mostres de SA encapsulat són capaces de revertir els efectes negatius del SA lliure sobre les plantes de Arabidopsis. El tractament amb SA encapsulat disminuïx el SA disponible en el mitjà, i les plantes

tractades mantenen la longitud de l'arrel, la grandària de la roseta i el gravitropismo similars a les plantes control. A més, l'encapsulació impedix l'alliberament incontrolat de SA, dificultant així la seua acumulació en l'arrel i evitant la disminució de IAA en la punta d'esta.

Finalment, el quart capítol se centra en determinar el mecanisme pel qual el SA encapsulat promou la tolerància en plantes de Arabidopsis sota condicions d'estrés simple i doble. Els resultats demostren que el SA encapsulat millora la tolerància de les plantes enfront de l'estrés per sal o mannitol, així com la seua combinació amb SA lliure. Este efecte positiu es deu al manteniment d'un equilibri adequat entre els nivells endògens de SA i IAA en les plantes tractades amb SA encapsulat (en contrast amb les tractades amb SA lliure). La regulació estricta de l'expressió d'EDS1, PAL1 i NPR1 evita l'excés de síntesi de SA i la seua sobreacumulación no fisiològica.

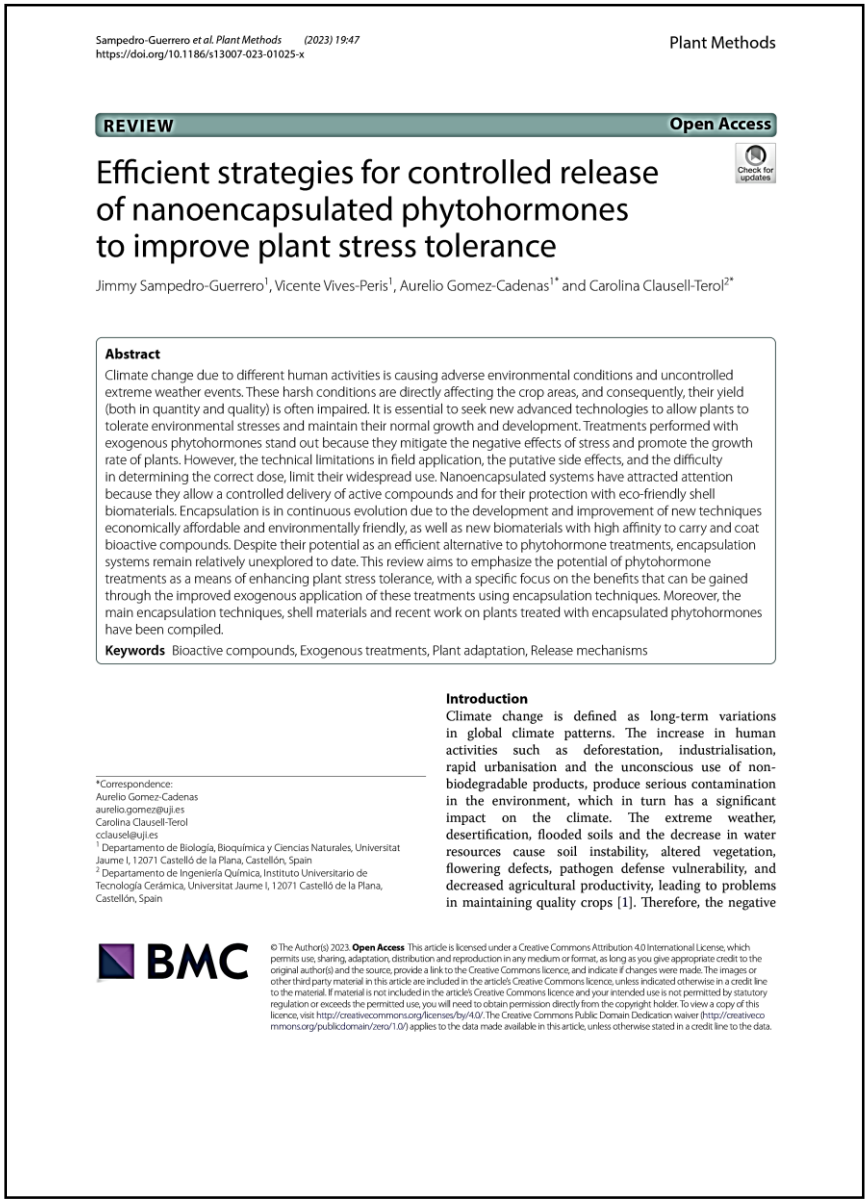
Este treball proporciona una ferramenta nova per a potenciar la resistència de les plantes a l'estrés. Els coneixements adquirits sobre el mecanisme pel qual el SA encapsulat protegix les plantes de la sal, el mannitol, l'estrés tèrmic i les seues combinacions, oferixen un valuós punt de partida per a encapsular altres fitocompuestos amb característiques protectores.

INTRODUCTION

EFFICIENT STRATEGIES FOR CONTROLLED RELEASE OF NANOENCAPSULATED PHYTOHORMONES TO IMPROVE PLANT STRESS TOLERANCE

Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., and Clausell-Terol, C. (2023). *Plant Methods* **19**, 47. https://doi.org/10.1186/s13007-023-01025-x

Key words for the tittle: Bioactive compounds – Exogenous treatments – Plant adaptation – Release mechanisms



Sampedro-Guerrero *et al. Plant Methods* (2023) 19:47
https://doi.org/10.1186/s13007-023-01025-x

Plant Methods

REVIEW Open Access

Efficient strategies for controlled release of nanoencapsulated phytohormones to improve plant stress tolerance

Jimmy Sampedro-Guerrero[1], Vicente Vives-Peris[1], Aurelio Gomez-Cadenas[1*] and Carolina Clausell-Terol[2*]

Abstract

Climate change due to different human activities is causing adverse environmental conditions and uncontrolled extreme weather events. These harsh conditions are directly affecting the crop areas, and consequently, their yield (both in quantity and quality) is often impaired. It is essential to seek new advanced technologies to allow plants to tolerate environmental stresses and maintain their normal growth and development. Treatments performed with exogenous phytohormones stand out because they mitigate the negative effects of stress and promote the growth rate of plants. However, the technical limitations in field application, the putative side effects, and the difficulty in determining the correct dose, limit their widespread use. Nanoencapsulated systems have attracted attention because they allow a controlled delivery of active compounds and for their protection with eco-friendly shell biomaterials. Encapsulation is in continuous evolution due to the development and improvement of new techniques economically affordable and environmentally friendly, as well as new biomaterials with high affinity to carry and coat bioactive compounds. Despite their potential as an efficient alternative to phytohormone treatments, encapsulation systems remain relatively unexplored to date. This review aims to emphasize the potential of phytohormone treatments as a means of enhancing plant stress tolerance, with a specific focus on the benefits that can be gained through the improved exogenous application of these treatments using encapsulation techniques. Moreover, the main encapsulation techniques, shell materials and recent work on plants treated with encapsulated phytohormones have been compiled.

Keywords Bioactive compounds, Exogenous treatments, Plant adaptation, Release mechanisms

Introduction

Climate change is defined as long-term variations in global climate patterns. The increase in human activities such as deforestation, industrialisation, rapid urbanisation and the unconscious use of non-biodegradable products, produce serious contamination in the environment, which in turn has a significant impact on the climate. The extreme weather, desertification, flooded soils and the decrease in water resources cause soil instability, altered vegetation, flowering defects, pathogen defense vulnerability, and decreased agricultural productivity, leading to problems in maintaining quality crops [1]. Therefore, the negative

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Abstract

Climate change due to different human activities is causing adverse environmental conditions and uncontrolled extreme weather events. These harsh conditions are directly affecting the crop areas, and consequently, their yield (both in quantity and quality) is often impaired. It is essential to seek new advanced technologies to allow plants to tolerate environmental stresses and maintain their normal growth and development. Treatments performed with exogenous phytohormones stand out because they mitigate the negative effects of stress and promote the growth rate of plants. However, the technical limitations in field application, the putative side effects, and the difficulty in determining the correct dose, limit their widespread use. Nanoencapsulated systems have attracted attention because they allow a controlled delivery of active compounds and for their protection with eco-friendly shell biomaterials. Encapsulation is in continuous evolution due to the development and improvement of new techniques economically affordable and environmentally friendly, as well as new biomaterials with high affinity to carry and coat bioactive compounds. Although encapsulation systems are an efficient alternative to phytohormone treatments, they have been little explored up to date. This review is focused on highlighting phytohormone treatments as an alternative to improve plant stress tolerance, particularly if their exogenous application is improved through encapsulation. Moreover, the main encapsulation techniques, shell materials and recent work on plants treated with encapsulated phytohormones have been compiled.

1. Introduction

Climate change is defined as long-term variations in global climate patterns. The increase in human activities such as deforestation, industrialisation, rapid urbanisation and the unconscious use of non-biodegradable products, produce serious contamination in the environment, which in turn has a significant impact on the climate. The extreme weather, desertification, flooded soils and the decrease in water resources cause soil instability, altered vegetation, flowering defects, pathogen defence vulnerability, and decreased agricultural productivity, leading to problems in maintaining quality crops [1]. Therefore, the negative effect of these changes decreases the capacity to meet the high food demand of the world population [2,3].

Biotic and abiotic stresses caused by climate change increase pressure for plants [4]. Plants respond to stresses in different ways: change in gene expression, variation of growth rates, alteration in cellular metabolism, production of molecular chaperones and reactive species scavengers, etc [5]. Among these responses, increased biosynthesis of secondary metabolites with a protective function has an important role [6]. These compounds help the plant to tolerate the adverse condition as an adaptive defence but, if the magnitude of the stress is too high or it appears too fast, they may not be enough to protect the plant completely. These metabolites are produced by plants as a defense mechanism; however, they can also be chemically synthesized or obtained from microbial sources [7,8], and their exogenous

application (via foliar or soil) can become a tool for mitigating the adverse effects of environmental stresses on plants [9]. These compounds include different acids, flavonoids and carotenoids and unsaturated fatty acids, among others (Supplementary Figure 1).

It is important to highlight the role of phytohormones (PHs) as regulators of plant development and plastic growth [10]. PHs also modulate several physiological processes in plants subjected to stress conditions, and their interactions allow reconfiguring plant architecture, enhancing its capacity to adapt to negative scenarios [11]. This review emphasizes the importance of PHs in environmental stress tolerance and the benefits of exogenous hormonal treatments on plants, especially when PHs are encapsulated.

2. Phytohormone modulate plant tolerance to several stresses

PHs are signalling molecules with a controlled homeostasis that mediate plant responses to internal and external stimuli [12]. They can act at their synthesis site or be transported to different parts of the plant. PHs regulate cell division, root and shoot elongation and differentiation, seed germination, dormancy, sex determination, and flowering and fruiting differentiation. Actually, the existence of different hormonal groups has been widely reported, including salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), indole-3-acetic acid (IAA), ethylene (ET), gibberellins (GAs), cytokinins (CKs), brassinosteroids (BRs), strigolactones (SL), etc. [13,14]. Undoubtedly, SA, JA, ABA, IAA, GA and CKs have a key role in the modulation of physiological and molecular responses to environmental stresses. The effects of phytohormones on plant development and growth, as well as their interactions under various stress conditions, are discussed below and illustrated in Figure 1: i) SA is a phenolic compound that is principally synthesized by the phenylalanine pathway and secondarily by the isochorismate route [15]. SA promotes defense responses against pathogenic organisms and abiotic stresses such as chilling, drought, heat, heavy metals and salinity. SA controls several aspects of plant development, including: seed germination, root differentiation and growth, photosynthesis, stomatal closure, senescence, flowering and fruit yield [16]. Interestingly, SA enhances plant antioxidant capacity at low concentrations but causes pleiotropic effects and susceptibility to abiotic stresses at highest ones [17,18].

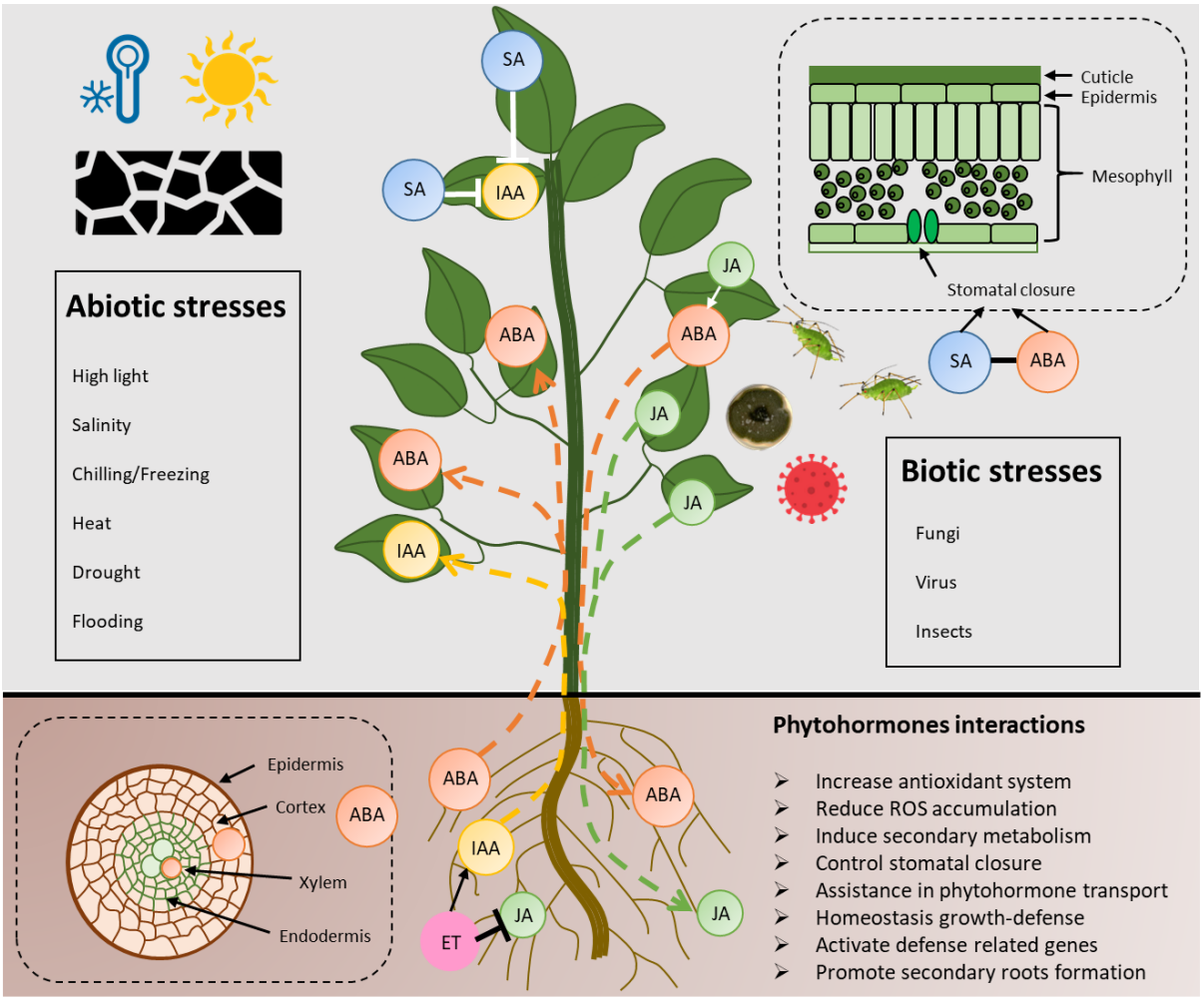


Figure 1. Phytohormone interactions play a crucial role in plant responses to biotic and abiotic stresses. Under biotic stress, the interaction between salicylic acid (SA) and abscisic acid (ABA) regulates stomata opening, while jasmonic acid (JA) induces ABA transport from leaves to roots. During abiotic stress, ABA is synthesized in roots and transported through the xylem, while SA blocks indole-3-acetic acid (IAA) to balance growth and defense, and ethylene (ET) inhibits JA to promote IAA synthesis and transport from roots to leaves.

Its principal role consists on the induction of the systemic acquired resistance to various pathogens, while in coordination with ABA regulate plant defense responses against pathogens and pests [19]. When defense responses are activated, SA levels and signalling increase, leading to a reduction in auxin biosynthesis and transport. This coordination between defense and growth trade-offs helps the plant to effectively manage its resources [20]. ii) JA, its precursor 12-oxophytodienoic acid (OPDA), and the conjugated molecules methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile), known as jasmonates (JAs), are crucial for plant development and can act directly or indirectly in defense

responses [21,22]. High concentrations of JAs are found on root tips, shoot apex, immature fruits and young leaves [23]. JAs are involved in physiological and molecular responses which protect plants against pathogenic attack, chilling, drought and high salinity. Some of the responses observed include an increase in the antioxidant system, the accumulation of amino acids such as methionine, and the regulation of stomatal closure [24]. The interaction between JAs and ABA can have both synergistic and antagonistic effects in inducing plant tolerance. Additionally, the interaction between JAs and ET is regulated through antagonism in response to abiotic stresses [22]. iii) ABA is an isoprenoid with a fundamental role in plant adaptation to abiotic stresses; among other roles it modulates stomatal opening to prevent water loosing when plant suffers drought stress [25]. ABA is synthesized via mevalonic acid-independent pathway and its biosynthesis starts in plastids and is carried in direction to the cytosol [26]. It also plays a role on seed dormancy and maturation, fruit ripening, root architecture organization [27]. It is well-known that ABA improves stress responses, activating stress-related pathways and modifying gene expression [28,29]. It also regulates cell turgor and restrict cell growth as adaptation mechanisms [30]. In plants exposed to abiotic stresses, ABA interacts with auxins to control root meristem activity and lateral root development [31]. iv) IAA is the most studied auxin and has been reported as a vital molecule for the proper development of plants [32]. It promotes cell division, differentiation and elongation, after plants exposure to stress. Auxins activate numerous genes in response to abiotic and biotic stress responses, although their role as a stress response regulator is still under study [33]. It is known that the crosstalk between IAA and SA mediates plant tolerance [34]. However, when plants are subjected to multiple stresses simultaneously, their homeostasis is altered, leading to changes in genes related to auxin transport, such as PIN1. This can result in the inhibition of IAA transport in the plant [35]. Excess IAA accumulation causes altered morphogenesis of principal root and avoids the formation of lateral roots, disrupting the nutrient uptake [36]. v) GAs are a group of molecules derived from a tetracyclic di-terpenoid carboxylic acid that have positive effects on tissue expansion, trichome initiation, and the development of flowers and fruits [37]. There is also evidence that GAs play a role in abiotic stress adaptation, where their antagonistic interaction with CKs helps control the elongation of the plant shoot apex and root tip [38]. vi) CKs control chloroplast differentiation, cell division and interaction with other organisms (especially pathogens) in the plant. Interestingly, plants alter their endogenous CK levels in response to abiotic stress (heat and chill) [39].

PHs have been extensively used as exogenous treatments for enhancing plant tolerance to both biotic and abiotic stresses, with numerous studies highlighting their potential to improve plant growth, development, and stress responses, as shown in Table 1.

Table 1. Representative list of phytohormones applied in treatments to improve the resistance of plants to abiotic and biotic stresses.

		Stresses											
		Abiotic								Biotic			
Phytohormones	Plants	Heat	Chilling	Drought	Salinity	Heavy metals (Cd, Cu, Ni, Pb)	Freezing	Osmotic	UV	Bacteria	Fungi	Insect, nematode or virus	References
Salicylic acid	Tomato	✓	✓	✓	✓						✓		[40], [41], [42]
	Bean	✓	✓	✓		✓							[40], [43]
	Maize	✓		✓	✓	✓							[44], [45], [46], [47]
	Barely		✓	✓		✓		✓					[48], [49], [50], [51]
	Wheat	✓	✓			✓							[52], [53], [54]
	Rice			✓		✓			✓	✓	✓		[55], [56], [57], [58], [59]
	Orange										✓		[60]
	Banana		✓								✓		[61], [62]
	Tobacco											✓	[63]
	Arabidopsis					✓					✓		[64], [65]
Jasmonic acid	Tomato	✓			✓								[66], [67]
	Maize			✓		✓							[68], [69]
	Rice	✓	✓	✓	✓							✓	[70], [71], [72], [73] [74]
	Orange		✓										[75]
	Banana		✓										[76]
	Soybean				✓	✓							[77], [78]
	Canola										✓		[79]
	Arabidopsis			✓			✓			✓	✓		[80], [81], [82], [83]
Abscisic acid	Tomato			✓									[84]
	Maize			✓									[85]
	Rice									✓	✓		[86], [87]
	Wheat			✓									[88]
	Cucumber		✓										[89]
	Bean										✓		[90]
	Arabidopsis									✓			[91]
Indole acetic acid	Tomato					✓							[92]
	Pea				✓								[93]
	Alfalfa			✓									[94]
	Maize				✓								[95]
	Wheat				✓								[96]
	Soybean			✓									[97]
	Potato				✓								[98]
Gibberellins	Tomato					✓						✓	[92], [99]
	Maize				✓								[95]
	Rice											✓	[100]
	Wheat				✓								[101]
	Potato				✓								[98]
	Fava bean					✓							[102]
	Soybean					✓							[103]
Cytokinins	Arabidopsis		✓							✓			[104], [105]
	Cucumber		✓										[106]
	Rice	✓								✓			[107], [108], [109]

Traditional methods to treat plants with PHs consist in either adding them to a nutrient solution for root absorption or spraying them to the aerial organs. Among them, the use of absorbent cotton to maintain the quantitative concentration of the phytohormone and promote correct absorption by the plant is one of the most popular [110]. Plants absorb PHs through leaf stomata or rhizodermis, to later transport them to the internal structures by ion channels and protein transporters, through phloem and xylem [111]. Inside plants, PHs are perceived by their specific protein receptors, for example: SA joins to NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1), JA joins to CORONATINE INSENSITIVE 1 (COI1), ABA joins to PYRABACTIN RESISTANCE1/PYR1-LIKE (PYR/PYL), IAA joins to TRANSPORT INHIBITOR RESPONSE 1 (TIR1), ET joins to ETHYLENE RECEPTOR 1 (ETR1), GAs join to GIBBERELLIN-INSENSITIVE DWARF 1 (GID1), CKs join to CYTOKININ RESPONSE 1 (CRE1), BRs join to BRASSINOSTEROID INSENSITIVE 1 (BRI1) and SL joins to DWARF 14 (D14) [112]. However, exogenous applications of free PHs have several problems such as the difficulty to define the correct dosage. Depending on the application purpose and chosen technique, the plant might need different doses, ranging from low quantities (at the nanomolar level) to much higher amounts, which is costly and inefficient. Furthermore, externally applied products are expected to maintain their initial concentration in PHs and be stable over time, but diverse environmental conditions and low stability of the molecules can affect the treatment. Even the structure of the molecule can be affected by light or temperature, modifying its behaviour and decreasing its efficiency [113].

Exogenous application of PHs can have negative biological impacts in plants. Firstly, hormonal imbalances may arise from excessive application, which can affect normal plant growth and development and increase plant susceptibility to pests [114]. Therefore, PHs can alter plant morphology by inducing the formation of adventitious roots or altering leaf shape; excessive use of GAs can lead to weakened stems and increased susceptibility to pests and diseases. In this sense, in citrus trees, over-saturation of uptake capacity due to GA applications can lead to the production of small fruits with poor flavour [115]. Secondly, long-term PHs treatments can cause plants to become dependent on external PH sources, leading to a loss of their natural ability to generate hormones, which can adversely affect growth rate and health [116]. From an ecological point of view, the application of PHs can have also some negative effects. In the case of treatments applied to the watering solution, a large amount of a free PH could affect the microbiological communities associated with the plant, changing soil ecosystem characteristics and even altering nutrient levels [117]. Moreover, some plant hormones, such as synthetic auxins, can have negative impacts on non-target organisms like pollinators. In this way, the herbicide 2,4-D, which contains a synthetic auxin, has been shown to harm bees and other beneficial insects [118]. Excessive or inappropriate use of plant hormones can lead to contamination of soil and water. In addition, the use of synthetic growth regulators like paclobutrazol in crop production has been shown to affect the health of organisms and ecological systems [119]. It is important to note that the ecological impacts of PHs applications depend on the specific hormone being used, the method and

timing of application, and the surrounding ecosystem. As such, it is important to carefully consider the potential risks and benefits of any plant hormone application. Encapsulation can help mitigate these issues by allowing for better management of PHs application and dosage.

3. Encapsulation can improve phytohormone biological effects in agriculture

Encapsulation has attracted attention due to the possibility of controlled release of most biologically active compounds and for the eco-friendly nature of the biomaterials used as coatings [120] Encapsulation produces particles with high hydrophilicity and lipophilicity, enhancing their ability to penetrate plant tissues [121]. This is a process where a bioactive compound or active agent, defined as core material, is packaged or coated in a carrier (protective material) to create capsules with enhanced biological characteristics (Figure 2A). The coating material is used to encapsulate the bioactive compounds forming a matrix capable to create a barrier for the core against important factors such as: heat, oxygen concentration, light, pH and shear [122]. Capsules are able to inhibit volatilization and protect the core versus extreme environmental conditions, reducing its sensitivity to degradation [123]. Encapsulation is an effective alternative to solve physical or chemical instability problems of PHs. These kind of compounds are encapsulated for increasing their durability and functionality, in addition to obtain a controlled release [124]. For a successful encapsulation, it is important to consider and correctly select three factors: a) the core, target active agent to encapsulate, b) the shell, coat or wall material used as coating and, c) the encapsulation method, depending on the nature of the materials and the final application [125]. Plant treatments performed with encapsulated PHs have increased in recent years due to their ability to promote plant growth and control the pathogen effects [126].

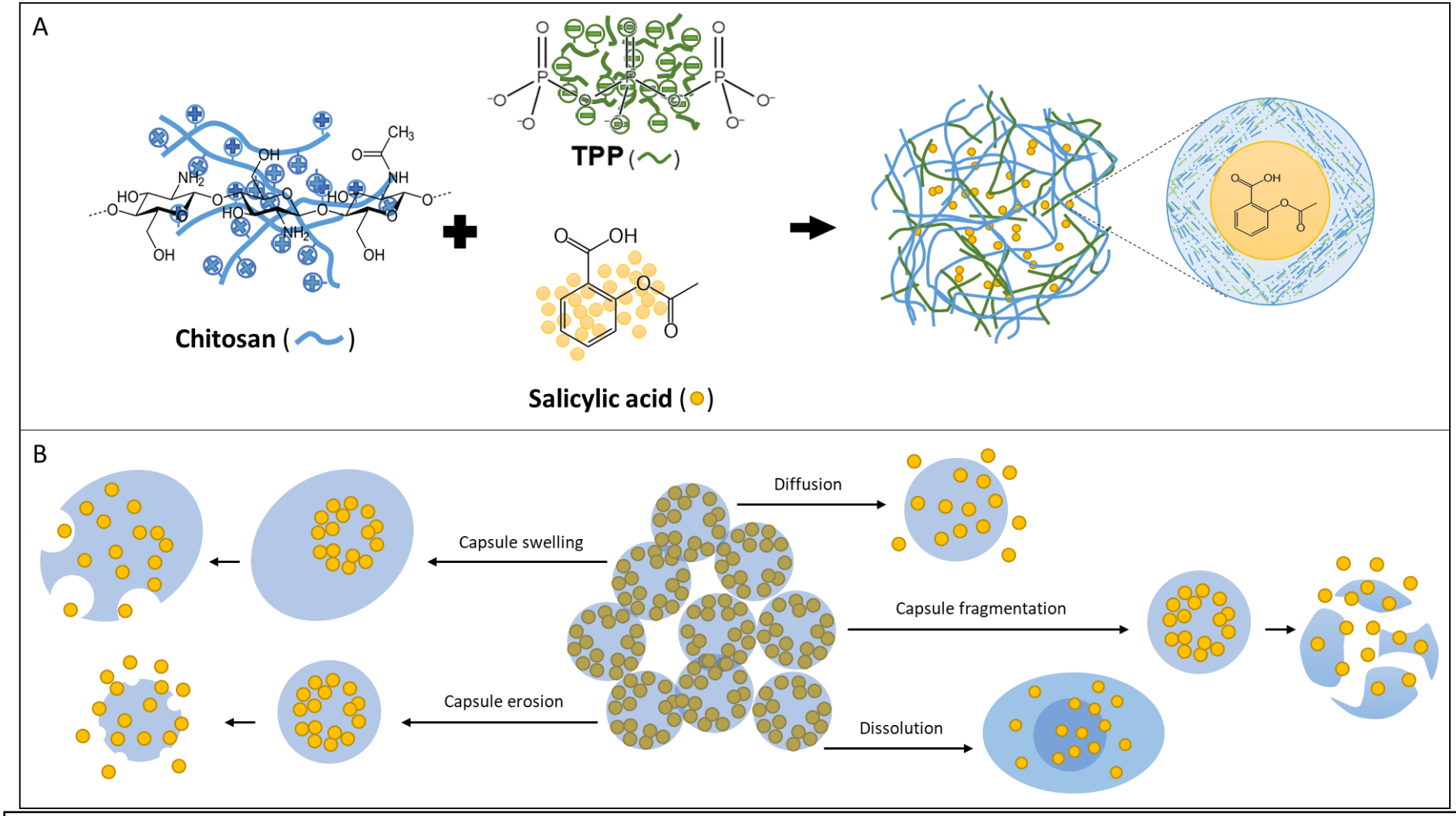


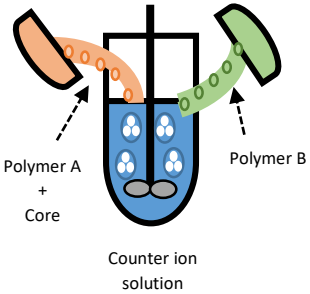
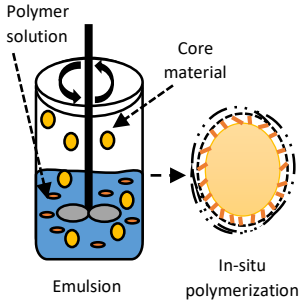
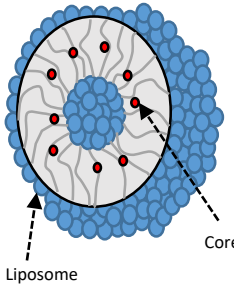
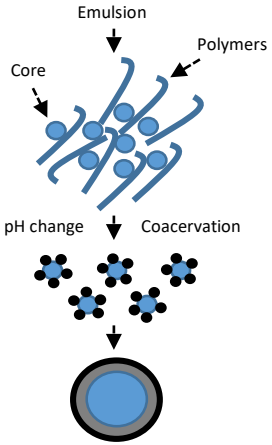
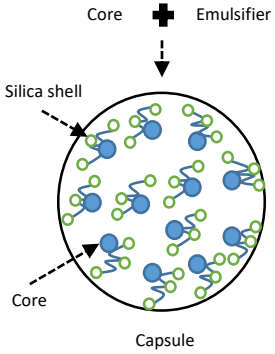
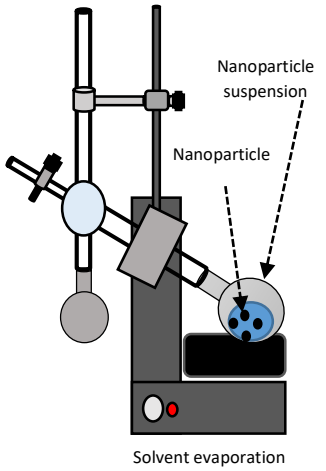
Figure 2. General processes of encapsulation and release of the active ingredient. Graph A represents SA encapsulation using chitosan as shelling material and tripolyphosphate (TPP) as bridge to form the nanocapsule. Graph B represents different mechanisms of PHs released from shell.

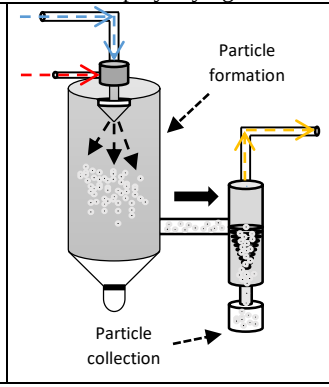
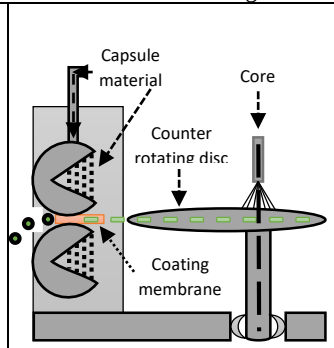
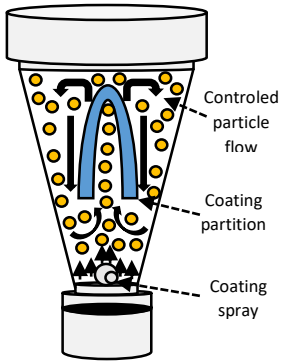
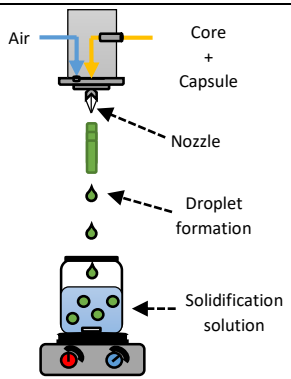
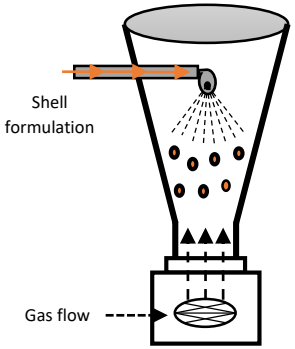
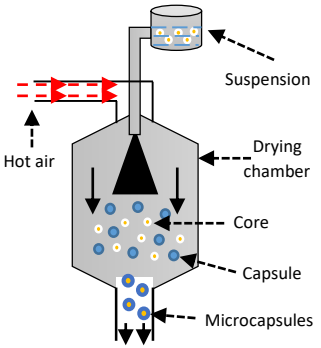
3.1 Principal encapsulation techniques used in agriculture

The selected encapsulation methodology depends on the core and shell characteristics, and their chemical and physical properties. The chosen technique has the challenge to achieve a high encapsulation efficiency and a controlled release capacity [127]. During the encapsulation process, the active agent has to remain intact, and the coating should not exhibit adherence or aggregation. The newly formed particles must have a homogeneous particle size distribution, with particles free of dents and/or holes [128]. Before starting the encapsulation process, the physical state of the core (solid or liquid) divides the fabrication process in either coating the solid particles with the shell material in a pan coater or fluidized bed, or forming droplets using an immiscible liquid or air, followed by droplet solidification [129]. The coat shell (in general, the capsules), can take numerous morphologies that could be classified based on the size of the encapsulation, into: nanocapsules (diameter < 0.2 μm), microcapsules (diameter between 0.2 to 5000 μm), and macrocapsules (diameter > 5000 μm) [130,131]. Moreover, capsules can be divided into microcapsules and microspheres, depending on their shape and construction. While microcapsules have a central inner core, which contains the active compound, in microspheres the core is heterogeneously dispersed in the encapsulation material. In general, encapsulation techniques fall into three categories: chemical, physical-chemical and physical-mechanical approaches. Table 2 shows the most important techniques used to encapsulate phytohormones, as well as their advantages and disadvantage.

The principal chemical techniques are: i) ionic gelation, which synthesizes particles from electrostatic interactions of ions with opposite charges. This technique requires a polymer (chitosan or alginate), a crosslinker, generally sodium triphosphate (TPP), and constant conditions of mechanical stirring [133]; ii) in-situ polymerization, consists in adding a biomolecule (core) to a polymer solution (shell material) and dispersed it until a certain size is obtained. Polymerization is performed in the continuous phase with no reactants added to the core material [134], and; iii) liposome entrapment, in which a lipid-based encapsulation system is used as a carrier for active compounds such as antioxidants, hormones, peptides, etc. This system is widely used due to its lipophilic/hydrophilic and compartmentalization properties [135]. In the case of physico-chemical techniques, there are mainly three: i) coacervation, a process that involves the electrostatic attraction between two polymers with opposite charges and coacervate formation by pH changes, which generally consists of four steps: a) suspension of the core in a liquid phase, b) addition of the polymer solution around the core, c) gelation, and d) solidification of the capsule wall [136]; ii) sol-gel encapsulation, in which an emulsion is produced from two immiscible phases prepared in the presence of a surfactant agent.

Table 2. Different encapsulation techniques used in agriculture to form capsule-core samples.

Process	Chemical			Physico-Chemical		
	Encapsulation techniques					
	Ionic gelation	In-situ polymerization	Liposome entrapment	Coacervation	Sol-gel encapsulation	Solvent evaporation
Diagram						
Advantages	• Simple process • High encapsulation efficiency	• Inexpensive materials • Simple manufacturing	• Stable	• Versatile operation • High encapsulation capacity	• Good thermal stability • Good encapsulation efficiency	• Simple procedure • Low cost
Disadvantages	• Limited polymers • Produced always in aqueous dispersion	• Complex procedure • Use a toxic precursor	• Difficult to scale	• Expensive process • Agglomeration • Difficult to scale up	• Long process time • Use of toxic organic solutions	• Low efficiency encapsulation • Restricted process
PHs encapsulated	SA, IAA, GAs	SA, ABA, GAs	SA, CKs	JAs	ABA	JAs
Particle size range	0.5-1000 µm	0.05-1100 µm	2-1200 nm	2-1200 µm	0.2-20 µm	0.5-1000 µm
Shelf life	Short	Short	Short	Short	Medium	Poor
Reliability	Poor	Poor	Poor	Poor	Good	Poor
References	[129,132,133]	[129,132,134]	[129,132,135]	[129,132,136]	[129,132,137]	[129,132,138]

Process	Physical-Mechanical					
	Spray drying	Multi-orifice centrifugation	Pan coating	Co-extrusion	Fluidized bed	Air suspension coating
Diagram						
Advantages	• Simple process • Easy to scale up • Adjustable cost	• Can use solid and liquid core materials	• Low cost	• Stability • High retention	• Extensive capsule materials	• Low cost
Disadvantages	• Limited capsule materials • High energy consumption	• High temperatures required	• Inconsistent encapsulation efficiency • Difficult to control • Time-consuming	• Limited capsules materials	• Restricted to solid particle coating • Agglomeration	• Restricted to solid cores • Complex process
PHs encapsulated	SA, ABA	ABA	SA	JAs, IAA	ABA	SA
Particle size range	5-5000 µm	5-1500 µm	600-5000 µm	500-3000 µm	20-1500 µm	0.1-1000 µm
Shelf life	Large	Medium	Short	Medium	Medium	Medium
Reliability	Good	Good	Poor	Good	Good	Poor
References	[129,132,139]	[129,132,140]	[129,132,141]	[129,132,142]	[129,132,143]	[129,132,144]

*Shell life: large (30-60 weeks), medium (10-30 weeks) and Short (<10 weeks)
*Reliability refers to degradation of encapsulated at temperature gradient (Thermogravimetric analysis): poor (>50% of mass lost) and good (<50% of mass lost)

Silica (Si) based particles are the most widely used because it is possible to obtain Si particles with a specific size and shape by changing the pH of sol-gel materials [137]; and iii) solvent evaporation, where a polymer is dissolved in an organic solvent, and then dispersed in an aqueous solution (with the core material) to form an emulsion, using a surfactant agent. Once the emulsion is formed, the organic solvent must be evaporated to obtain the final particles [138].

Concerning physical-mechanical techniques, the following are highlighted: i) spray drying, a fast and scalable process that allows obtaining dry powders from liquid suspensions [139]. Briefly, the suspension is sprayed through a nozzle, using a hot gas (either air or nitrogen), generating solid particles that move with the air stream and are collected by a cyclone [145]; ii) multi-orifice centrifugation, is a process that launches the core through a counter-rotating disk using centrifugal force [140]. The core passes through a membrane composed of the shell material, forming the encapsulated particles [146]; iii) pan coating, is a method in which a coating composition is added to a moving bed of core material using hot air to evaporate the solvent . The core material rotates on a pan while the coating material is applied at the same time [141]; iv) co-extrusion, consists of mixing the material of the core with that of the shell by means of a system of nozzles. The vibrations produced are capable of breaking the liquid phase and forming drops, which become capsules when falling into a solidification bath [142]; v) fluidized bed, this process is performed by spraying a shelling solution into a fluidized bed with the core, requiring numerous wetting–drying cycles to form a continuous film [143]; and vi) air suspension coating, in this technique the core is suspended in an upward draft and continuously coated with sprayed shell material [144]. The core passes through the coating-zone cyclically until it is encapsulated. The air stream allows in turn to dry the encapsulated particles [147].

3.2 Principal coating materials used in agriculture

The coating material influences the controlled release of bioactive molecules, also affecting their bioaccessibility. It is important that the shell or coating is not reactive or produces a non-specific conformation on its own. The chosen materials must provide, mainly, protective properties, in addition to others such as: flexible structure, stability, strength and permeability [148]. The initial core/shell ratio and the amount of shell are essential parameters during the encapsulation process since they directly affect the dispersion process and determine the particle surface area under specific conditions [149]. In relation to the environment, it is required that the coating material is also inert (does not react with the active principle). Its surface must be flexible to encapsulate and release different compounds, but also strong to protect against extreme conditions and, after use, it must be biodegradable to minimize environmental impact [150]. One of the main considerations to take into account is the shelling material structure, since it determines the capsule functional properties. The ideal shell material should have a stable emulsifying property and an easy handling during the encapsulation process. Furthermore, it must preserve its permeability and not react with the core during long-term storage conditions [151]. It must

be soluble in several solvents and, at high concentrations, the rheological properties under the influence of stresses must be stable, but with a desired flexibility that does not compromise its structure [152]. In some cases, to enhance the shell properties, the use of a combination of coating materials is necessary.

There are a large number of different materials used for the encapsulation process, for example: polysaccharides from different sources (plant, marine algae and fungi), lipids, proteins, synthetic or inorganic, and waxes, among others. The new trends in agriculture seek inert and/or biodegradable matrices for the encapsulation of plant extracts (flavonoids, fatty acids and main phytohormones) [153]. Polysaccharides are by far the most widely used shell material due to their structure, abundance and biodegradability [154]. Alginate, a natural hydrophilic compound isolated from algae cell wall, is widely used to formulate films, hydrogels, microspheres and microcapsules, since this material exhibits important shelling characteristics, such as: moisture absorption, gelation and biocompatibility [155,156]. Chitosan is undoubtedly the most popular coating material due to its superior characteristics, such as biocompatibility, biodegradability, resistance, non-toxicity and its ability to form films without relying on additives [157]. In agriculture, chitosan encapsulated molecules are used as an economical and ecological alternative to formulate biofertilizers, biopesticides, conditioners and growth promoters [158]. Among polysaccharides, starch has gained interest as a nanocarrier system, mainly due to its abundance, availability, biodegradability and competitive cost. In addition, starch can present different molecular structures, depending of its plant tissue origin as fruits, roots, seeds and tubers, and its structure can bring several shapes, sizes and granule composition [159,160]. Gum polysaccharides (arabic, carrageenan, xanthan, among others) are used as coating material due to their characteristics, such as good emulsification, high solubility, low viscosity and oxidation reaction inhibition [161].

Other interesting coating materials are amorphous silica, waxes and caseinates. Amorphous silica (SiO_2) is a non-toxic material whose use in the encapsulation process is inexpensive and its manufacture is safe and friendly to the environment [162]. This material is used to encapsulate different bioactive agents by entrapment in its inner pores, which allows a chemical and physical stabilization between the core and the shell [163]. Waxes become more relevant due to their favourable properties such as hardness, hydrophobicity, scratch resistance and thermal stability. In fact, it is interesting to carry out studies on the microstructure and properties of new waxes to control possible interactions with other components [164,165]. Caseins are a class of milk-derived proteins, similar to whey proteins, containing casein micelles and caseinates as extended forms [166]. Caseins have the facility to form suspensions and, during capsule formulation, have the capacity to emulsify and foam [167]. Table 3 shows the principal coating materials used for encapsulation, as well as their advantages and disadvantages. Although not all these materials have been used yet for PHs encapsulation, they have been used for other molecules and compounds and are good candidates for future applications.

Table 3. Main materials used to form capsule shell.

Materials	Advantages	Disadvantages	Applicable stress type	References
Polysaccharides				
Alginate	• Low toxicity • Bio inert material • Low cost encapsulation process	• Limited changes on mechanical properties • Instability caused by ion-leaching	Biotic	[155,168,169]
Carrageenan	• Not toxic • Biocompatible • Biodegradable	• Potential reaction with bioactive molecules	Abiotic / Biotic	[170–172]
Chitosan	• Not toxic • Enhanced biocompatibility • High stability • Expensive dosing is prevented	• Method of preparation depends on the PHs used	Abiotic / Biotic	[157,173,174]
Gum Arabic	• Abundant availability • Excellent core protection ability	• Limited availability • High cost	Abiotic	[175,176]
Modified starch	• Fully biodegradable • Inexpensive material • Can be easily modified	• Loose structure due to its poor resistance to shearing and stirring • Toxicity of several derivative products	Biotic	[159,177,178]
Maltodextrin	• Low hygroscopicity • Protect bioactive compounds from oxidation	• Poor stability • Low retention	Biotic	[179,180]
Pectin	• Low cost encapsulation process • Possibility to modify its structure	• High swelling degree in unfavourable environments	Biotic	[181–183]
Inorganic				
Amorphous silica	• Biocompatible • High uptake capacity • Controlled drug release system • Low toxicity • Improved loading and releasing properties	• Difficult to predict successful amount of encapsulated drug	Abiotic / Biotic	[162,184]
Synthetic and natural polymers				
Polyvinyl alcohol	• Biodegradable • Not toxic • Biocompatible	• Low stability • Chemical modification	Abiotic	[185,186]
Polyacrylamide	• High stability	• Toxic	Abiotic	[187,188]

Fats and waxes				
Hydrogenated vegetable oils	• Controlled release	• Multiple steps in the preparation process	Biotic	[189,190]
Bees wax	• Highly diverse • Adaptable material to changes in different conditions • Degradable	• Low encapsulation capacity	Abiotic	[191,192]
Paraffin wax	• Structure does not change over time	• Not adjustable • Not adoptable • Toxic	Abiotic	[193,194]
Proteins				
Soft gelatine capsule (SGC)	• High accuracy • Reduces dustiness during manufacturing	• Expensive to produce • Not adaptable	Biotic	[195,196]
Hard gelatine capsule (HGC)	• Rapid drug release	• Problems with cross-linking • Not suitable with hygroscopic compounds	Biotic	[195,197]
Sodium caseinates	• Oxidative stability • Biocompatibility • Increases encapsulation efficiency	• Requires a significant amount of bioactive compound	Abiotic	[166,198,199]

3.3 Release mechanisms of active ingredients

The release of encapsulated bioactive compounds can occur through controlled and uncontrolled mechanisms. The rapid release could be ineffective but, on the contrary, an extremely slow release could decrease their positive effects and cause problems in their entrance through the plant tissue surface. Controlled release requires a trigger stimulation to start. The deployment of this mechanism ensures a long-lasting action of the bioactive molecule with an expected concentration [200]. Furthermore, it is important because its manoeuvrability and predictability characteristics allow the estimation and study of the core release rate [201]. The rate study considers several parameters such as: starting point and duration, kinetics, released quantity, speed and release mechanism [202]. There are five mechanisms of release: i) diffusion, which produces a random movement of the core (usually by a concentration gradient) and in which the release of the active agent depends on the physical-chemical characteristics of the core and the matrix, as well as the ratio between the two [203]; ii) swelling, where differences in solvent concentration cause the whole shell structure to swell with increased pore size, making it difficult to maintain capsule integrity and causing core molecule release [204]; iii) fragmentation, produced by a disruption in the matrix due to physical, chemical or biological stresses and in which the release of the amount of core will depend on the stress magnitude and on the shape and size of the capsule fragments formed [205]; iv) erosion, a process that is caused by several factors such as: temperature, pH, enzymes and mechanical stimulation, among others, and that can occur in two ways: by surface erosion (capsule surface degradation), and by bulk erosion (whole capsule degradation) [206]; and v) dissolution, where the bioactive core is released into an aqueous medium by dissolution or not of the matrix first, starting the dissolution on the surface of the application point or after trespassing it [207]. In the Figure 2B, a schematic procedure of each release mechanism is depicted.

3.4 Encapsulated phytohormones development to enhance plant tolerance to stress

Plants respond to stress in different ways [208] depending on the affected area: nutrient translocation, cell death at the entrance of the affected zone, changes in gene regulation or in the cell wall composition, production of lipids, metabolites and proteins, production of antioxidant compounds, etc. [209,210]. Plant response is influenced by its genotype and stage of development, the duration and intensity of the stress, the combination of different stresses, etc. This response, which is controlled by complex pathway, starts with the stress perception, triggering various molecular events that end with visual, physiological, developmental and metabolic pathways changes [211]. However, in some cases, these response mechanisms are not enough to protect plants because the stress is too radical and produces different internal (cell wall and DNA disruption, lipid peroxidation, protein deformation and mitochondrial cleavage) and external damages (seed germination reduction, biomass reduction, altered root growth and pleiotropic effects) [212]. These problems can be solved using encapsulated

phytohormones that, through their controlled release, allow the correct internalization of the different molecules. Several studies where different plants were treated with encapsulated PHs have shown that encapsulated SA generates pathogenic resistance against *Fusarium verticillioides* and *Sclerotium rolfsii* in maize and rice [213,214], and cold and salt tolerance in sunflower and grape [215,216], respectively. Treatments with encapsulated JA and ABA provide resistance against cold and drought stress in cherry tomato and Arabidopsis [217,218], respectively, and treatments with encapsulated IAA and GAs enhance plant growth and seed germination rates in tomato and bean [219,220]. Once the plant recognizes that it is under stress, signal transduction cascades are triggered which are transduced in PHs, and start a fluctuation between growth and stress response [94]. In the case of encapsulated PHs, the treated plants do not need to activate signal cascades and biosynthesis pathways, on the contrary, they only need to take the released and available phytohormones that could be likely to cause controlled changes. Table 4 compiles the main works where encapsulated PHs are used to promote stress tolerance and growth development.

Although the same phytohormone-loaded nanocapsules could mitigate the harmful effects of different kinds of stress, or even their combination, it should be tested for every different condition. In this context, although the study of two or more combined stress situations has been expanded in the last years, the use of encapsulated PHs for mitigating multifactorial stresses is poorly studied and needs to be explored since at nature, plants are subjected to different negative conditions at the same time [229]. Among the scarce literature in this issue, a recent work has explored the benefits of the application of benzenedicarboxylic acid impregned in calcium nanoparticles to mitigate the combined stress induced by the organic pollutant dichlorodiphenyltrichloroethane and cadmium in Brassica alboglabra plants [230]. This reveals the importance of spreading the use of nanoparticles under stress combination, where encapsulated PHs could bring new strategies in this disturbing scenario.

Table 4. Encapsulated phytohormones used in treatments to improve stress resistance and tissue development in plants.

Encapsulated phytohormone	Encapsulation method	Capsule Material	Plants treated	Agriculture benefit	References
Salicylic acid	• In-situ polymerization	• Alginate	• *Helianthus annuus L.* (Sunflower)	• Tolerance of the tissue to the cold storage	[215]
	• Spray drying	• Amorphous silica and chitosan	• *Arabidopsis thaliana*	• Reduces deleterious effect of SA on treated plants	[18]
	• Ionic gelation	• Chitosan	• Zea mays (maize)	• Control of *Fusarium verticillioides* diseases and act as biostimulant	[213]
	• In-situ polymerization	• Alginate	• *Oryza sativa* (rice)	• Control of *Sclerotium rolfsii* disease	[214]
	• Ionic gelation	• Chitosan	• *Vitis vinifera* (grape)	• Protection against salinity stress	[216]
Jasmonates	• Solvent evaporation	• Gliadin-Casein	• *Solanum lycopersicum* var. *cerasiforme* (cherry tomato)	• Used as coating to enhance cold time storage	[218]
	• Coacervation	• Alginate and chitosan	• *Solanum tuberosum* (potato)	• Tuber postharvest treatment for preserving	[221]
	• Co-extrusion	• PLGA	• *Vitis vinifera* (grape)	• Pest management	[222]
Abscisic acid	• Sol-Gel encapsulation	• Amorphous silica	• *Arabidopsis thaliana*	• Provides resistance against drought stress	[217]
	• In-situ polymerization	• Lignin	• *Oryza sativa* (rice) and *Arabidopsis thaliana*	• Increases drought resistance	[223]
Auxins	• Ionic gelation	• Chitosan	• *Solanum lycopersicum* (tomato)	• Increase germination and seedling growth rate. Acts as biostimulant	[220]
	• Co-extrusion	• Alginate and chitosan	• *Solanum lycopersicum* (tomato)	• Increase morphological characteristics	[224]
	• Ionic gelation	• Chitosan	• *Malus domestica* (apple)	• Promote adventitious rooting	[225]
Gibberellins	• Ionic gelation	• Chitosan	• *Phaseolus vulgaris* (bean)	• Promote germination of seeds and enhances plant fertility	[219]
	• Ionic gelation	• γ-PGA polymer	• *Phaseolus vulgaris* (bean)	• Increase germination rate, and leaf and root development	[226]

	• Interfacial polymerization	• Chitosan and alginate	• *Solanum lycopersicum* (tomato)	• Promote plant development and enhance fruit productivity	[227]
Cytokinins	• Liposome entrapment	• Liposomes	• *Cocos nucifera L. var Makapuno* (Coconut)	• Enhance bioactivity formation of callus in vitro	[228]

4. Conclusions and future perspectives

New forward-thinking solutions to improve crop tolerance to extreme climatic conditions must be obtained. Today, the world demands bioactive compounds that do not affect the environment. The development of biomaterials based on nanotechnology offers new products with applications in agriculture. The encapsulation of PHs could be an affordable solution to fight against environmental stress, reducing its negative effects on plant development and yield, without affecting other characteristics of the crop as its nutritional value and having a minimal impact on the environment.

Recent studies highlight the main role of PHs, such as SA, JA and ABA in plant responses to environmental stress. The exogenous application of PHs activates the response mechanisms that help plants to cope with nutrient deficiency and growth regulation under stress. Studies carried out *in vivo* and *in vitro* have evaluated the bioavailability and controlled release of different products, although the study of the possible interactions between the encapsulated compounds and the matrix within the formulations is still required. In addition, it is important to determine several properties of these nanocarrier systems, such as particle size, charged surface area, surface coating and solubility. These characteristics are essential because they condition the possible toxicological effects. Indeed, toxicology studies based on physical-chemical characteristics, experimental design synthesis and exposure time in the plant would allow the development of new nanocarriers with efficient applications, and those that are not hazardous for the environment and plant health. Encapsulated PHs have become an option for the sustainable development of agriculture due to their desirable characteristics, such as lower production cost, greater availability and eco-friendly profile. In addition, its use can promote the research of new encapsulated bioactive compounds that increase plant tolerance to different stresses.

Further studies are necessary to investigate the synergistic and antagonistic interactions of PHs within plants. This will require the use of genetics, molecular biology, and bioinformatics approaches to identify the metabolites, signals and genes induced during PH treatments. Additionally, studying the interplay between PHs could provide new insights into their role in stress tolerance. Manipulating the endogenous levels of PHs through encapsulation and observing their response in different tissues/organs during various stresses can be an exciting tool for improving plant stress tolerance in modern agriculture. However, it is crucial to consider the interactions between the environment and plant species, as this information can be used to optimize PH behaviour, dosage, and treatment timing. In summary, a better understanding of PH interactions and their effects on plant stress tolerance will require multidisciplinary approaches, and considering the environment-plant species interactions can help us develop effective strategies for using PHs in agriculture.

Funding

This work was supported by MCIN/AEI/10.13039/501100011033 and by the European Union Next Generation (TED2021-129795B-I00) and AGROALNEXT program (funded by MCIN, European Union Next Generation EU -PRTR-C17.I1- and Generalitat Valenciana). Funding was also obtained from Generalitat Valenciana through the programs CIAICO/2021/063 and GRISOLIAP/2020/043.

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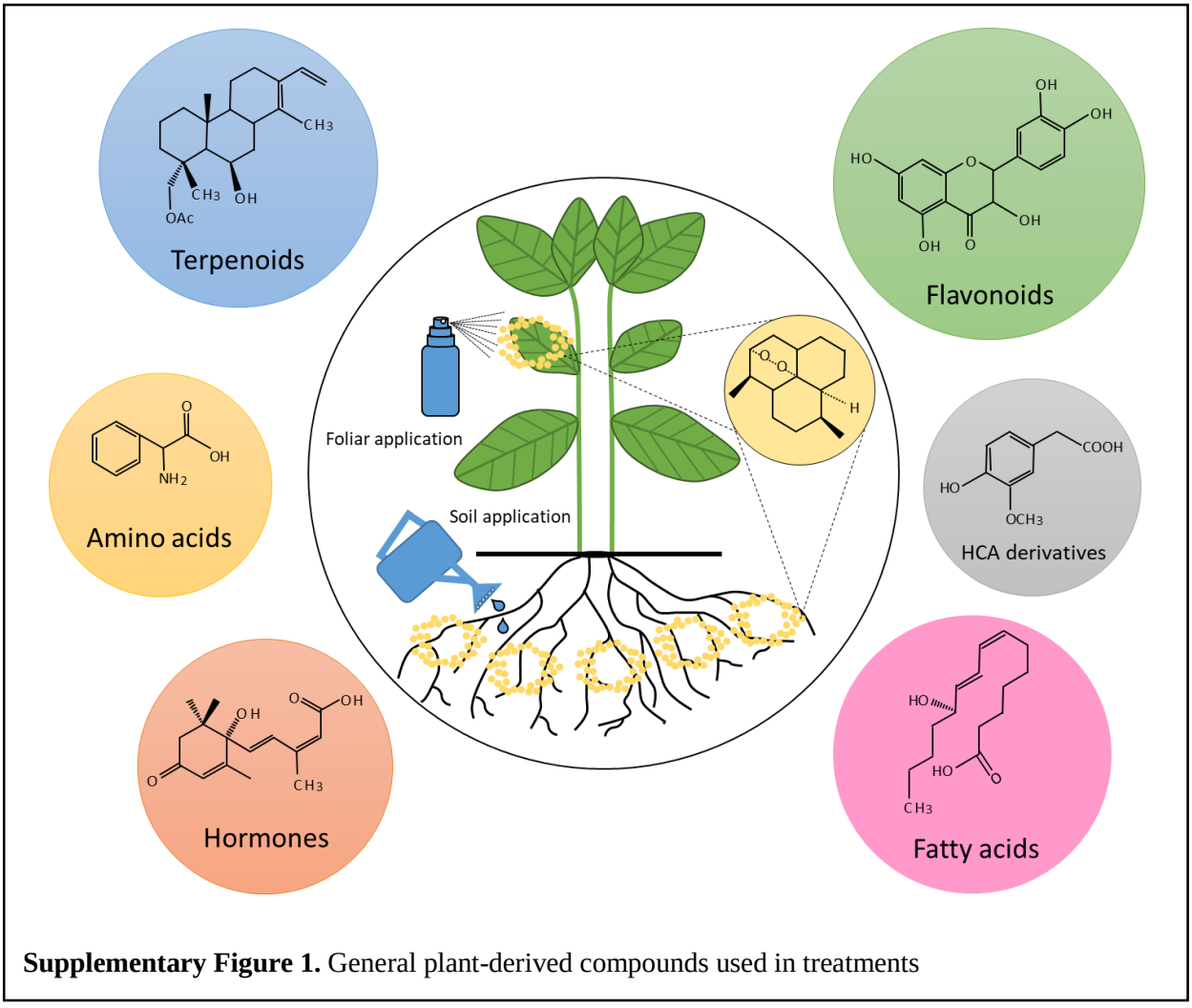
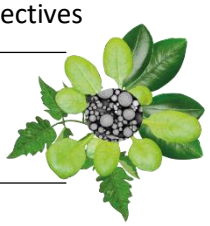
Supplementary material



Supplementary Figure 1. General plant-derived compounds used in treatments

OBJECTIVES



The main objective of this work was to develop of encapsulated salicylic acid particles as an innovative tool for improving plant stress tolerance.

To achieve this aim, sequential objectives were established:

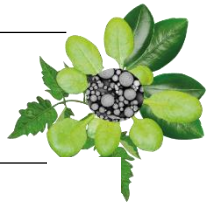
- Formulate encapsulated salicylic acid compounds using silica and chitosan capsules, studying their physical, chemical, and biological properties (Chapter 1).

- Optimize salicylic acid encapsulation through a fractional factorial experimental design, promoting a readily scalable process (Chapter 2).

- Compare the effect of free salicylic acid and encapsulated salicylic acid treatments on biochemical parameters and morphological characteristics of *Arabidopsis thaliana* plants (Chapter 3).

- Determinate the biological effect of encapsulated salicylic acid on promoting stress tolerance in Arabidopsis plants subjected to simple and double stress conditions (Chapter 4).

RESULTS



CHAPTER 1

IMPROVEMENT OF SALICYLIC ACID BIOLOGICAL EFFECT THROUGH ITS ENCAPSULATION WITH SILICA/CHITOSAN

Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., and Clausell-Terol, C. (2022). *International Journal of Biological Macromolecules* **199,** 108-120. https://doi.org/10.1016/j.ijbiomac.2021.12.124

Key words for the tittle: Antifungal activity – Arabidopsis – Root growth

International Journal of Biological Macromolecules 199 (2022) 108–120

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Improvement of salicylic acid biological effect through its encapsulation with silica or chitosan

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ARTICLE INFO

Keywords:
Antifungal activity
Arabidopsis
Root growth

ABSTRACT

Attacks of necrotrophic and biotrophic fungi affect a large number of crops worldwide and are difficult to control with fungicides due to their genetic plasticity. Encapsulation technology is a good alternative for controlling fungal diseases. In this work, encapsulated samples of salicylic acid (SA) with silica (Si:SA) or chitosan (Ch:SA) at three different ratios were prepared by spray drying, and morphological and physicochemical characterised. Therefore, size distribution, specific surface area, thermal stability, encapsulation efficiency, and in-vitro SA release were determined. Biological activity of encapsulated samples were tested against different fungi of agricultural interest at various concentrations (0–1000 μM). Treatments prepared with the lowest ratios for both capsules, were found to have the best antifungal effect in an in vitro system, inhibiting the mycelial growth of Alternaria alternata, Botrytis cinerea, Fusarium oxysporum and Geotrichum candidum. Similarly, treatments with the lowest ratios of both encapsulated samples reduced free SA toxicity on Arabidopsis thaliana seeds. In this system, plants treated with capsules had higher root and rosette development than those treated with free SA. In conclusion, a product with a great potential in agriculture that shows high antifungal capacity and low toxicity for plants have been developed through a controlled and industrially viable process.

1. Introduction

Every year, pathogenic fungi affect leaves, roots, and seeds of important crops causing significant effects and losses in agriculture [1]. The abusive use of chemical fungicides to control fungal diseases in plants has decreased their effectiveness and caused environmental hazards that increase progressively.

Furthermore, the uncontrolled use of these chemical agents have caused the development of fungi resistances [2]. Due to the large number of affected crops, chemical-biological alternatives have recently been applied to control different pathogens and mitigate several environmental stresses. Plants produce natural compounds named phytohormones that control their vegetative growth, floral development, fruit growth and maturation and senescence, among others. Moreover, they act by forming complex phytohormone networks that control and balance plant stress responses, and protect them against several pathogens [3]. Phytohormones can be synthetically produced and then are called Plant Growth Regulators (PGRs) [4]. This group of compounds includes auxins, cytokinins, gibberellins, jasmonic acid, abscisic acid and salicylic acid (SA) [5]. PGRs are widely used in several areas of agriculture to increase the production of crops with better phytosanitary and commercial characteristics [6].

The phenolic ring linked to a hydroxyl group in the SA structure has a vital role on the regulation of crucial processes of plants, such as seed germination, photosynthesis, redox homeostasis, senescence and vegetative growth [7]. SA can be present in the form of a free fraction or in a glycosylated/glucose-ester/methylated conjugate form, and it can be synthesized by two different and compartmentalized routes [8]. In the first one, denominated the phenylalanine route, phenylalanine (Phe) is converted to *trans*-cinnamic acid (t-CA), then t-CA gets oxidized to benzoic acid (BA) and, finally, the aromatic ring of BA is hydroxyled to form SA. On the other route, called the isochorismate route, chorismate is initially transformed in isochorismate (IC) and then in SA [9]. Several studies reveal that SA regulates many tolerance responses to abiotic stress [10,11]; moreover, when seeds are imbibed in SA or it is applied as a foliar treatment through exogenous application, it can control

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https://doi.org/10.1016/j.ijbiomac.2021.12.124
Received 23 September 2021; Received in revised form 9 December 2021; Accepted 19 December 2021
Available online 30 December 2021

Abstract

The present work purpose consisted of developing and characterizing three different ratios of Si:SA and Ch:SA samples. Encapsulated were prepared by spray drying, and characterised for size distribution, thermogravimetry, encapsulation efficiency, specific surface area and in-vitro SA release. Encapsulated were tested against differents fungi at various concentrations (from 0 µM to 1000 µM). The lower ratios Si:SA (1:0.25) and Ch:SA (1:0.5) at 1000 µM, had the best antifungal effect, inhibiting the mycelial growth of *Alternata alternaria*, *Botrytis cinerea*, *Fusarium oxysporum* and *Geotrichum candidum* in 62.5%, 62.0%, 30.2% and 61.6% with Si:SA (1:0.25), respectively, and 80.1%, 80.9%, 22.2% and 29.5% with Ch:SA (1:0.5), respectively. The obtained results demonstrated a potential antifungal product for fungi growth control. At the same way, *Arabidopsis thaliana* seeds were treated with encapsulated at different concentration and the lower ratios Si:SA (1:0.25) and Ch:SA (1:0.5) at 100 µM had a lower toxicity effect in plant development in comparison of higher ratios where roots and rosettes were not able to grow correctly. For the aforementioned, encapsulation reverts the toxic effect of free SA, controlling the amount that plant can absorb. The encapsulated at lower ratios have an incredible potential for agriculture.

1. Introduction

Every year, pathogenic fungi affect leaves, roots, and seeds of important crops causing significant effects and losses in agriculture [1]. The abusive use of chemical fungicides to control fungal diseases in plants has decreased their effectiveness and caused environmental hazards that increase progressively. Furthermore, these chemical agents have caused the development of resistance in fungicides due to their uncontrolled use [2]. Due to the large number of affected crops, chemical-biological alternatives are recently being applied to control different pathogens and mitigate several environmental stresses. Plants produce natural compounds that control their vegetative growth, floral development, fruit growth and maturation, responses to environmental factors, senescence, among others, and these compounds are called phytohormones or plant growth regulators when are produced synthetically [3]. Plant growth regulators (PGRs) are used to modulate plant growth and development, and include auxins, cytokinins, gibberellins, jasmonic acid and salicylic acid (SA) [4]. PGRs are widely used in several areas of agriculture to increase the production of crops with better phytosanitary and commercial characteristics [5]. Phytohormones help plants survive against biotic and abiotic stress conditions by forming a complex phytohormone network that control and balance the stress response, and have also shown protection against several pathogens [6].

The phenolic ring linked to a hydroxyl group in the SA structure has a vital role on the regulation of crucial processes of plants, such as seed germination, photosynthesis, redox homeostasis, senescence and vegetative growth [7]. SA can be present in the form of a free fraction or in a glycosylated/glucose-

ester/methylated conjugate form, and it can be synthesized by two different and compartmentalized routes [8]. In the first one, denominated the phenylalanine route, phenylalanine (Phe) is converted to *trans*-cinnamic acid (t-CA), then t-CA gets oxidized to benzoic acid (BA) and, finally, the aromatic ring of BA is hydroxyled to form SA. On the other route, called the isochorismate route, chorismate is initially transformed in isochorismate (IC) and then in SA [9]. Several studies reveal that SA regulates many tolerance responses to abiotic stress [10,11]; moreover, when seeds are imbibed in SA or it is applied as a foliar treatment through exogenous application, it can control pathogenic diseases and enhance the plant development [12]. The main problem with the exogenous application of SA is to achieve a prolonged and sustained effect, since it can easily degrade when is exposed to light or temperature changes, which can result in a decrease in efficiency and/or loss of activity [13].

Encapsulation is used to coating active agents with protective materials, improving their stability and activity, and even reducing environmental impacts [14,15]. Capsules are generally nanomaterials used as delivery systems for the encapsulation of different active agents, such as enzymes, proteins, genes, metabolites, hormones, among others. The surface of the capsule increases the bioavailability and solubility of the active molecules, besides the small size that they usually present facilitates the encapsulation process and increases the release of the active molecules by an increasing of the specific surface and the contact surface [16]. Additionally, capsules are used for their biocompatibility, controlled and targeted release, and chemical stability [17].

Drug carriers include polysaccharides such as chitosan, which is a natural polymer with useful biocompatibility characteristics, non-toxicity effects and excellent biodegradability. Polysaccharides are an ideal choice as delayed release agents due to their abundance in nature, structural stability, and inexpensiveness [18], which explains the different applications of chitosan carrier systems in crop protection[19]. Like chitosan, silica allows the encapsulation of functional components such as drugs, fluorescent materials and pigments, which are mainly used in drug delivery, imaging and sensing technologies [20]. Silica capsules, with a core–shell hierarchical structure, have recently generated interest as a low-cost, environmental friendly encapsulation technology with short time experimentation requirements [21]. Mesoporous silica has been used for encapsulating abscisic acid (ABA) and tested in *Arabidopsis thaliana*, showing an effective prolonged release of ABA and improving the drought resistance of *Arabidopsis* seedlings [22].

The aim of the present study is to obtain bioactive compounds of SA, encapsulated in chitosan and silica particles (at different capsule:active agent ratios) and to study their biological effect on plant growth and anti-pathogenic activity. In addition to the geometry and size of the encapsulated samples, their physicochemical characteristics and the hormone release mechanism were evaluated. Encapsulated samples were shown to have a greater anti-fungal effect on the growth of different fungi than free SA,

and their effect on *Arabidopsis thaliana* seeds allowed deepening on the regulation of plant growth. The results denote a great potential for applications in agriculture.

2. Materials and methods

2.1 Materials

2.1.1. Raw materials. Chitosan (DG CHI 0.20 g/ml and 85% deacetylated) and pyrogenic amorphous silica (HDK® S13) were purchased from AOXIN (Shanghai, China) and WACKER (Barcelona, Spain), respectively. Salicylic acid (SA), sucrose and tween 80 were purchased from Sigma-Aldrich (St. Louis, USA). Acetone, sodium tripolyphosphate (TPP-Na), potato dextrose agar (PDA), european bacteriological agar and petri dishes were purchased from spanish companies Labkem, Acrilatos SAU, Condalab, Condalab, and Labkem, respectively. Dichloromethane (DCM) was purchased from Fisher Scientific (Lenexa, USA), tween 20 from PANREAC (Barcelona, Spain), and Myo-Inositol and Murashige & Skoog Medium (Basal Salt Mixture) from Duchefa (Haarlem, The Netherlands).

2.1.2. Fungal and plant materials. Five fungus species (*Alternata alternaria*, *Botrytis cinerea*, *Fusarium oxysporum*, *Geotrichum candidum* and *Phytophthora infestans*) were obtained from the Spanish Type Culture Collection (CECT), (Valencia, Spain).

Arabidopsis thaliana wild-type (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Centre. Seeds were surface sterilized with bleach solution (30% v/v sodium hypochlorite and 0.01 % v/v Tween 20) for 10 min incubation, followed by three washes with distilled sterile water.

2.2. Methods

2.2.1. Preparation of silica and chitosan capsules. *Silica encapsulated SA (Si:SA)*: Three different ratios (see Table 1) were formulated: 1:1, 1:0.5 and 1:0.25. The appropriate amount of SA was mixed with 320 ml distilled water by using a planetary mill (Fritsch, Pulverisette®) for 15 min at 120 rpm, using alumina balls as grinding media (□300 g). Amorphous silica was stepwise added and mixed for one hour at 180 rpm.

Chitosan encapsulated SA (Ch:SA): Three different ratios (see Table 1) were prepared (1:1.25, 1:1 and 1:0.5) using the following procedure: 1/ planetary mixing for 5 min at 150 rpm of 138.6 ml of distilled water and 1.4 ml of acetic acid in to acidify the emulsion, 2/ adding 4.2 g of chitosan and planetary mixing for 15 min at 210 rpm, 3/ adding 1.4 ml of tween 80 and planetary mixing for 15 min at 210 rpm, 4/ adding different amounts of salicylic acid (see Table 1) pre-dissolved in dichloromethane (10 min at 500 rpm) and planetary mixing for 15 min at 210 rpm, 5/ adding 2.1 g TPP-Na and 137.9 ml distilled water and planetary mixing for one hour at 210 rpm.

Slurries were maintained in constantly agitation before spray drying. Density was measured in triplicate using 25 ml flasks.

2.2.2. Rheological characterization. The viscosity and rheological behaviour of encapsulated slurries were obtained by conducting tests under steady state conditions using a Bohlin CVO-120 rheometer, controlling the shear stress applied and measuring the shear strain produced. A double gap (DG 40/50) device, composed of two concentric cylinders, was selected to ensure high sensibility with low viscosity suspensions. Initial stirring for 30 s in the rheometer broke up any dispersion inner structure that might have formed, thus eliminating residual history effects. The shear stress was then abruptly reduced to zero. This situation was held for 60 s to enable the dispersion to acquire a controlled, reproducible inner structure. The sequence of the shear test used consisted of an increasing/decreasing logarithmic ramp of shear stress, with twelve pairs of shear rate–shear stress values in each ramp. The samples were thermostated at 25 ºC during testing.

2.2.3. Spray drying. Spray drying was performed with a SD-06 spray drier (Lab Plant, UK), with a standard 0.5 mm nozzle. When the liquid was fed to the nozzle with a peristaltic pump, atomization occurred by the force of the compressed air, disrupting the liquid into small droplets. The droplets, together with hot air, were blown into a chamber where the water in the droplets was evaporated and discharged out through an exhaust tube. The dry product was then collected in a collection bottle and stored in plastic bags at room temperature for further characterization. Figure 1 shows the schematic diagram of the spray drying process.

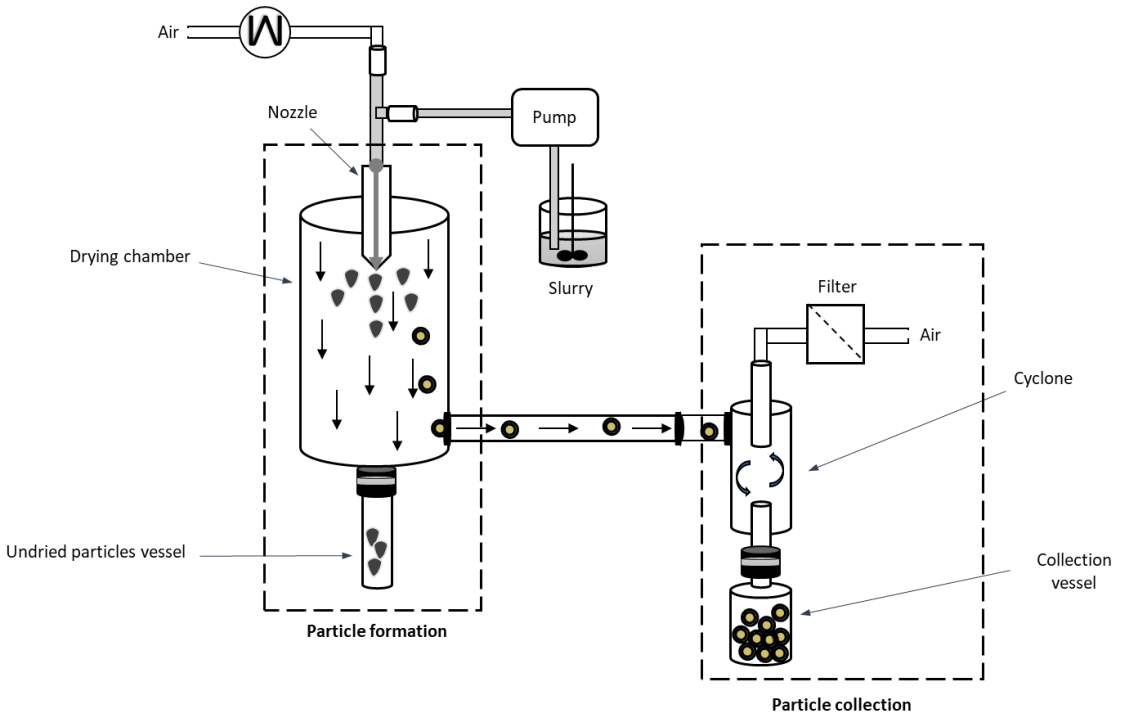

Figure 1. Schematic diagram of the spray dryer. (a) Encapsulated slurry pumped and particles dried, (b) Recollection of dry particles.

In the standard condition, the inlet temperature, spray flow, drying air fan and compressed air pressure were set at 150°C, 10 ml/min, 80% and 1.5 bar, respectively. The drying performance varied from 48 to 79% for the slurries of SA encapsulated in silica (Si:SA) and from 36 to 75% for the slurries of SA encapsulated in chitosan (Ch:SA).

2.2.4. SEM-EDX. The powdered sample was deposited in a brass sample holder using a conducting carbon adhesive tape and, with a view to favouring conductivity, it was coated with platinum. All the prepared samples were observed and photographed with the backscattered electron and secondary electron signal of a field-emission gun environmental scanning electron microscope (FEG-ESEM) Quattro S of Thermo Fisher.

The backscattered electron signal (CBS detector - All mode) provides information on the topography and composition. The higher the average atomic number of the sample, the more intense is the signal, so that the lightest-coloured areas contain the heaviest elements (composition contrast). The secondary electron signal (ETD detector - SE mode) is more superficial, so that it provides information on the morphology of the sample, highlighting surface irregularities such as cracks, pores, and crystal or grain edges.

Samples were also analysed with an energy-dispersive X-ray microanalysis spectrometer (EDS) connected to the microscope. Note that the electron beam interaction volume is of the order of 3µm or higher so that, when analysing very small zones, chemical information is received from the surrounding area. It may furthermore be noted that this analysis system detects elements with an atomic number of 6 or higher (from carbon upwards).

2.2.5. Capsules size distribution. Scanning electron microscopic images were used for determining the capsules size distribution. Image processing and analysing was performed with the image analyser software ImageJ. For the characterization of each sample, four images and more than 800 capsules were measured. The capsule area was determined by ¨Analyse particles function¨, and the diameter was calculated assuming that all encapsulated particles were spherical. Capsules size distributions were obtained by representing the accumulated frequency vs diameter.

2.2.6. Encapsulation efficiency (EE) of encapsulated SA. SA was extracted from both capsules, silica and chitosan. Samples were weighed (5–10 mg) and 1.5 ml of 0.1 M HCl was added to each one. After that, the samples were incubated for 24 h at room temperature. Further, capsules were centrifuged at 12500 rpm and supernatant (containing SA) was measured by ultraviolet–visible (UV–vis) spectrophotometric analysis (Thermo Spectronic) at 297 nm. Experiment was realized with three replications for each ratio of both capsules. EE was calculated by following equation:

$$EE\ (\%) = \frac{(Theoretical\ SA - Determined\ SA)}{Theoretical\ SA} \times 100 \quad (1)$$

where 'Theoretical SA' is the product of initial mass of the sample (mg) and theoretical SA encapsulated (%) and 'Determined SA' is the product of SA supernatant concentration (mg/mL) and supernatant reaction volume (mL). SA supernatant concentration was calculated with a calibration curve of free SA.

2.2.7. Specific surface area. Adsorption/desorption curve, using nitrogen gas as adsorbent, was carried out with a Tristar 3000 equipment from Micromeritics, using the Standard ISO9277:1995. Specific surface area was determined according to the BET method using the adsorption isotherm. This parameter was calculated by means of the multipoint method using the following equation:

$$S_{BET}(m^2 g^{-1}) = n_m \cdot a_{nitrogen} \cdot N_A \quad (2)$$

where n_m is the molar monolayer capacity, $a_{nitrogen}$ is the area of surface occupied by a single adsorbed gas molecule (0.162 nm^2) and N_A is the Avogadro's constant.

The monolayer capacity (nm) was calculated carrying out a linear regression, obtaining the slope and the intercept from the BET equation in which $n_m = (1/(a+b))$, where a is the slope and b is the intercept. The amount of adsorbed nitrogen was measured by means of a static volumetric method.

Before carrying out the test, sample was dried in an oven at 45ºC for 2 hours and, after that; it was outgassed with a nitrogen flux at 80ºC for 3 hours.

2.2.8. Thermal analysis. Thermogravimetric data were recorded on a Mettler-Toledo, TGA/STDA851e model, which allows simultaneous recording of the weight losses (TG), the derivative (DTG), the differential thermal curves (DTA) and the temperature increases (T), in a dynamic nitrogen atmosphere. Analysis conditions used were maximum temperature 1000ºC, heating rate 10 ºC/min and alumina vessel sample holder. The instrument was verified by using different certified reference materials which allow assuring the measurement traceability.

2.2.9. In-vitro SA release. In vitro release study was conducted following the next procedure. 10 mg sample was mixed with 2 ml of distilled sterile water. The experiment was conducted at pH 7 and room temperature under a constant magnetic stirring of 100 rpm. At specific time intervals (0-24h) sample was centrifuged at 12000 rpm for 2 min at 4 °C, and 2 ml supernatant volume was sampled for analysis. Supernatant volume sampled was replaced with an equivalent volume of distilled sterile water in each time tested to keep constant the total volume. The amount of SA released was determined by UV–vis absorption spectroscopy at 297 nm, as described in section 2.2.6. SA release mechanism was evaluated by Korsmeyer-Peppas model [23] using the following equation:

$$\frac{M_t}{M_\infty} = k \cdot t^n \tag{3}$$

where M_t is the amount of SA released at time (t), M_∞ is the amount of SA released at infinite time, k is the kinetic constant, and n is the release exponent. From the value of n it is possible to determine whether the release mechanism is Fickian or non-Fickian (anomalous) release. According to the mathematical model, $n < 0.45$ indicates that the system releases the active agent by diffusion, following Fick's law (case I transport); $n > 0.89$ indicates release by relaxation of the polymeric wall or erosion of the particle, case II transport; and $0.45 < n < 0.89$ indicates release by anomalous transport, with both of the aforementioned mechanisms occurring simultaneously [23].

2.2.10. Antifungal activities. Antifungal activity of the capsules was measured by Poison food technique [9]. Different concentrations (100, 500 and 1000 μM) were tested against five fungus species (see section 2.1.2.). Potato dextrose agar (PDA) medium was mixed with samples and poured in 9 x 15 mm Petri dishes. In the case of fungus species, a 7-days-old mycelial bit (1 x 1 cm) was taken from the peripheral end and placed in the centre of each dish treatment. Following that, dishes were incubated at 25 °C, and the observation of radial mycelial growth was recorded on days 3, 5, 7 and 10. Treatments were performed in quadruple. Inhibition rate was calculated by comparing each dish treatment with control at 10 days and using the following equation:

$$Inhibition\ rate(\%) = \frac{(Mc-Mt)}{Mc} \times 100 \quad (4)$$

where Mc is the mycelial growth in control and Mt is the mycelial growth in the treatment.

2.2.11. Plant growth and treatment conditions. Seeds were sown in 9 x 15 mm dishes containing Murashige and Skoog medium, sucrose, myo-inositol, and vitamins. For each treatment, salicylic acid particles were mixed with medium and poured in Petri dishes. Seeds were distributed individually in the dishes in two rows with 10 seeds per row. Subsequently, seeds were stratified for 24 h at 4°C in the dark to synchronize germination. Dishes were incubated for 0–12 days vertically oriented under long day conditions (16 h of light and 8 h of dark) at 22.5°C and 60% relative humidity. Treatments consisted of four replications for each treatment and twenty plants samples for each replication.

2.2.12. Root development and growth quantification. Petri dishes images were obtained with a scanner (Epson perfection v600 photo) at 600 dpi. The dishes were placed in the scanner with a black surface (included in the scanner) at the top. Different images on days 4, 8 and 12 after sowing were obtained and saved in JPEG format. Size of the roots of each seed were analyzed with MyROOT software [24].

2.2.13. Statistical analysis. Statistical analysis was performed with SPSS software version 21. Significant differences among treatment groups were determined by using the Turkey-Kramer HSD test at $p \leq 0.05$. The experiments replications are detailed in their respective procedure section.

3. Results and Discussion

3.1. Formulation and characterization of Silica (Si:SA) and Chitosan (Ch:SA) encapsulated SA samples

3.1.1. Rheological characterization

Viscosity measurements at different shear rates are needed to evaluate the suitability of a slurry to be atomized. For a pneumatic atomizer, the shear rate in the slurry increases rapidly as it passes through the nozzle, and once the droplets are formed, the shear rate decreases to zero, and the temperature of the slurry increases to the wet-bulb temperature in the drying chamber, which is considerably lower than the programmed inlet temperature (150ºC in our case) [25].

Figure 2 shows the rheological behaviour of the encapsulated slurries prepared with silica (Figure 2a) and chitosan (Figure 2b), for the three capsule:SA studied ratios. The experiments were conducted over the shear rate range of 0.1–1000 s^{-1} from the upward sweep followed by downward sweep. The viscosity of the slurries at the extremes of the range (points A and B in Figure 2) are depicted in Supplementary Table 1, together with the density values of the suspensions.

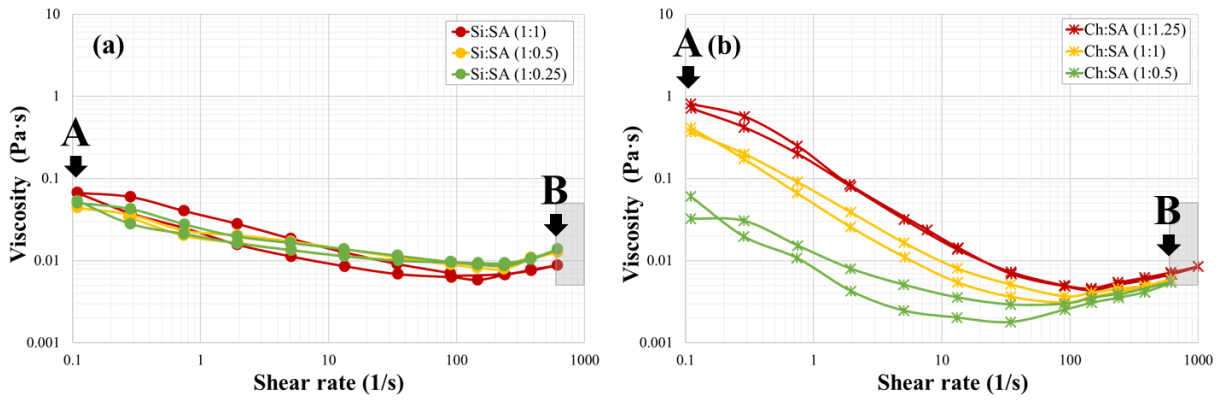

Figure 2. Viscosity flow behaviour for the encapsulated slurries prepared with silica (a) and chitosan (b) with the three capsule:SA studied ratios. A and B points viscosity values are shown in Table 1. The gray area corresponds to the working area in the atomization process.

Silica slurries displayed a low shear thinning behaviour, especially given the nanometric nature of the silica particles, which indicates their good dispersion in the slurry. As shown in Fig. 2a, there is no difference between the three studied ratios, probably because all the samples have the same solid content (See Table 1). Silica capsules present a spherical geometry (Fig. 3a-b-c) which makes them symmetrical. Therefore, discard any option of alignment, which explains their low pseudoplasticity.

Table 1. Characteristic of the formulated Si:SA and Ch:SA samples: Si/Ch:SA ratio, salicylic acid (SA) and solid content (SC) of the prepared slurries and encapsulation efficiency (EE) and BET specific surface area (Se) of the encapsulated materials obtained by spray drying.

SAMPLE	Ratio Si/Ch:SA	SA (%w)	SC (%w)	EE (%)	Se (m^2/g)
Si:SA (1:1)	1 : 1	6.8	13.5	63.6 ± 2.9 ^{ab}	60 ± 3
Si:SA (1:0.5)	1 : 0.5	4.5	13.5	69.4 ± 5.2 ^{ab}	74 ± 4
Si:SA (1:0.25)	1 : 0.25	2.7	13.5	52.6 ± 4.1 ^{a}	83 ± 4
Ch:SA (1:1.25)	1 : 1.25	1.7	3.6	46.6 ± 3.9 ^{ab}	2.3 ± 0.2
Ch:SA (1:1)	1 : 1	1.4	3.4	49.6 ± 1.0 ^{ab}	2.1 ± 0.2
Ch:SA (1:0.5)	1 : 0.5	0.7	2.8	43.9 ± 3.1^{b}	1.8 ± 0.2

Chitosan slurries displayed a mild shear thinning behaviour and a different rheological behaviour between ratios (Figure 2b). The high pseudoplasticity of the chitosan capsules can be explained by their irregular geometry with some laminar particles (Figure 3j-k-l), which can easily align themselves under an external force, decreasing the viscosity of the slurry. The differences observed between ratios tested

in the chitosan samples are mainly due to the different solid content of the slurries (see Table 1). The higher the SA content, the higher the solid content (due to unavoidable needs of the experimental procedure), increasing the inter-particle network and hence the viscosity of the slurries at low shear rates.

The magnitude of shear thinning i.e. reduction in viscosity with respect to increasing shear rate reveals information about inter-particle network formation [26]. High shear thinning behaviour means high inter-particle network, which in turn offers more resistance to flow. Accordingly for the purpose of spray drying, it is preferable to have slurries with weak or no inter-particle network [27], with a low viscosity at high shear rates (grey area in Figure 2a and b) and with a density value lower than 1.4 – 1.6 g/cm^3. Hence, the prepared silica and chitosan encapsulated SA slurries were found to be suitable for spray drying.

Finally, the difference in the viscosities at any given shear rate gives the information about thixotropy which can be defined as the property of the slurry that are thick under normal conditions and flows over time when it is stressed. From the rheographs (Figure. 2a and b), it was evident that the thixotropy was negligible which means that slurries were sufficiently stable and, once again, suitable for the spray drying process.

3.1.2. Size analysis of samples

Image analysis of high resolution SEM micrographs allows to determine their size distribution and shape of capsules [28]. Size analysis of Si:SA samples was performed using SEM micrographs at 5000x magnification, which showed a similar spherical shape in the three studied ratios (see Figure 3a-b-c). SEM micrographs at 10000x magnification (see Figure 3g-h-i) showed the highly porous structure characteristic of silica capsules, where the incorporation of the hormone occurs without altering the structure of the capsule itself.

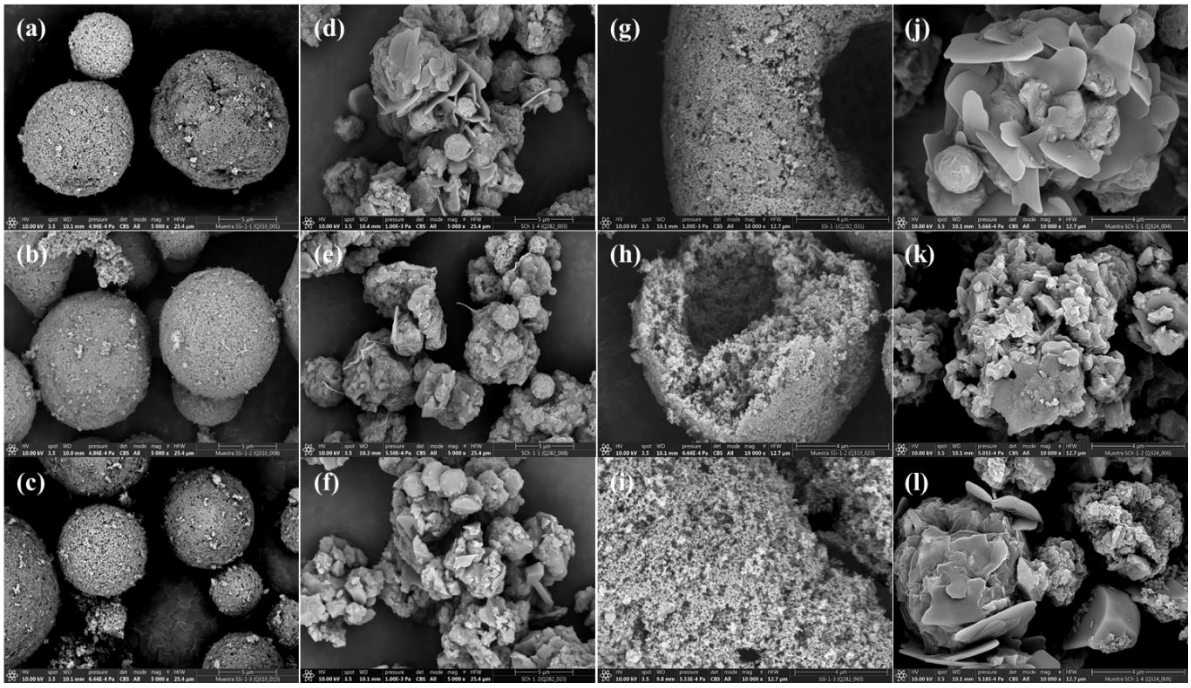

Figure 3. Scanning electron microscopic (SEM) images of the encapsulated samples prepared with silica (a-b-c-g-h-i) and chitosan (d-e-f-j-k-l) for the three capsule:SA studied ratios. Si:SA (1:1) (a-g), Si:SA (1:0.5) (b-h), Si:SA (1:0.25) (c-i), Ch:SA (1:1.25) (d-j), Ch:SA (1:1) (e-k), Ch:SA (1:0.5) (f-l). (a-b-c-d-e-f) 5000x magnification and (g-h-i-j-k-l) 10000x magnification.

However, SEM micrographs at 5000x and 10000x magnification of Ch:SA samples showed an irregular non-spherical shape in the three studied ratios (see Figure 4d-e-f-j-k-l), suggesting that, contrary to Si:SA samples (where SA is embedded into the capsule material [29]), SA should be entrapped within chitosan particles, which chains will be later cross-linked with TPP-Na, that is through an aggregate formation by bonds [30,31].

SEM micrographs analyses were conducted by ImageJ software that calculates the diameter of individual capsules from each sample using the threshold option and estimating the area values, assuming a spherical geometry. Capsule size distribution curves were obtained by representing diameter values vs accumulated area: Figure 4a and b for Si:SA and Ch:SA samples, respectively. D_{90}, D_{50} and D_{10} equivalent diameters were determined from the curves and depicted in Supplementary Table 2. Equivalent diameters designated as D_{10}, D_{50}, and D_{90} indicate that 10%, 50%, and 90% of particles are smaller than such values. As shown in Figure 4 and Supplementary Table 2, the capsule size distributions of all samples were quite similar, with a D_{50} values of 9.6-11.0 μm for silica encapsulated SA samples and of 7.2-8.5 μm for the chitosan ones.

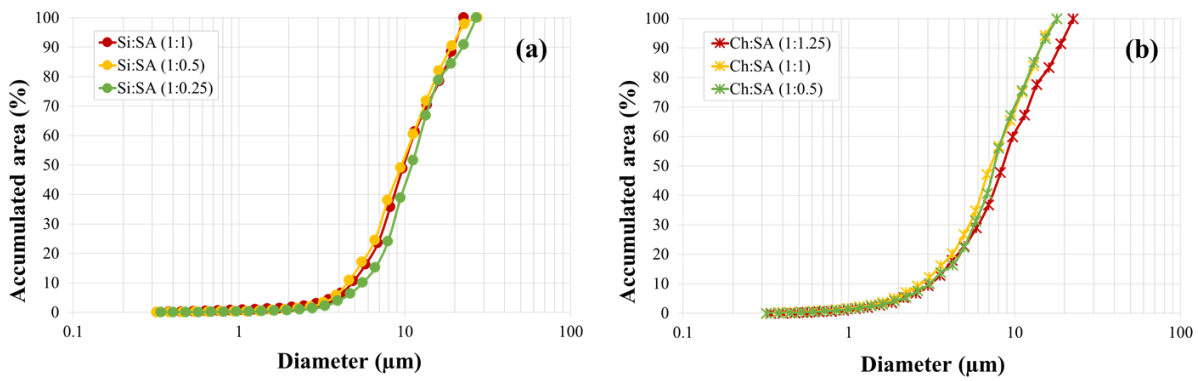

Figure 4. Encapsulate size distributions of the samples prepared with silica (a) and chitosan (b) for the three capsule:SA studied ratios. Characteristic diameters (D_{10}, D_{50} and D_{90}) are shown in Table 3.

An important factor to consider is the toxicity of the samples when applied to living organisms. Certain values of the particle size could increase its toxicity by modifying the characteristics of the surface of the samples and the free energy, enhancing the potential catalytic surface to carry out the chemical reactions between the biological components and the surface of the particles [32]. While the level of toxicity of many materials is well known, it is still unknown how concentration, particle size or particle size distribution can promote new toxicological effects [33]. Therefore, it is important to note that, in our case, all samples have a similar capsule size and capsule size distribution, since, as described above, differences in capsule size can lead to differences in toxicity in the samples [34,35].

3.1.3. Thermal analysis

To assess thermal stability of capsules, thermogravimetric analysis was used. The decomposition temperature (T_d) is the temperature corresponding to the maximum mass loss, which is clearly observed as a peak when the rate of mass loss versus temperature, a so-called DTG thermogram, is plotted [36].

The thermograms of pure and encapsulated materials, for both silica and chitosan encapsulated SA, are presented in Figure 5 (TG-DTG) and in Supplementary Figure 1 (DTA). Pure silica is thermally quite stable, with a maximum mass loss around 5%. However, pure SA undergone complete endothermic decomposition in one stage at 266°C, while pure chitosan experienced two reaction stages of thermal decomposition: a first stage at 90°C (ENDO peak) due to the evaporation of moisture and a second one at 313°C (EXO peak), which is assigned to the dehydration and decomposition of the material. The ENDO DTA peak of pure SA at 168°C correspond to SA fusion.

TG-DTG and DTA thermograms of the encapsulated samples (both with silica and chitosan) show an intermediate behaviour compared to the curves of the pure raw materials, confirming the encapsulation process for the three ratios studied for each capsule material.

The thermal degradation of the encapsulated samples prepared with silica (Figure 5a-b) takes place in one stage, mainly due to the decomposition of the SA, which explains the higher mass loss observed for the higher capsule:SA ratio, that is Si:SA (1:1). Also, SA decomposition rate (observed around 266ºC) decreases for the encapsulated samples, which would result in a slower release of SA.

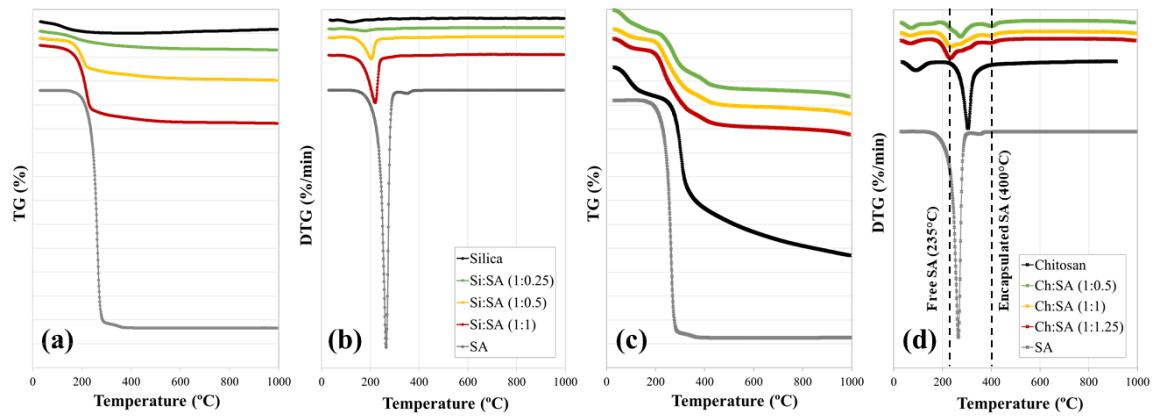

Figure 5. TG (a, c) and DTG (b, d) curves of the encapsulated samples prepared with silica (a-b) and chitosan (c-d) for the three capsule:SA studied ratios and their corresponding raw materials.

The thermal degradation of the encapsulated samples prepared with chitosan are depicted in Figure 5c-d. By comparison with the thermogram of the raw materials, the capsules of Ch:SA manifested two new T_d around 200° and 400°C, which could be ascribed to the loss of free SA and encapsulated SA, respectively. The result, which is in agreement with previous studies from oils of different nature [31,36], reveals the achievement of SA loading into chitosan particles. It should be pointed out that the encapsulated SA decomposed at higher temperature than free SA, reflecting the improved thermal stability of SA by encapsulation. However, the T_d of free SA for the particles prepared by the addition of a smaller initial amount of SA, i.e. Ch:SA (1:0.5), was not as clearly observed (Figure 5d), which should imply that the total amount of SA might be encapsulated into the chitosan particles when low content of SA was added.

3.1.4. SEM-EDX

Semi-quantitative Energy Dispersive X-ray (EDX) microanalysis of SA encapsulated in silica (Si:SA) and chitosan (Ch:SA) are depicted in Figure 6 and 7, respectively, together with the SEM micrographs analyzed in each case. Silica encapsulated SA SEM/EDX results (Figure 6) revealed the presence of three main components: carbon, oxygen and silicon. While oxygen is present in both capsule and encapsulated, carbon can only be found in the SA molecule, and silicon in the silica one, which

facilitates the SEM/EDX results interpretation. In the silica capsules (Figure 6), SA can be easily identified on the surface of the spherical particles, both visually and analytically.

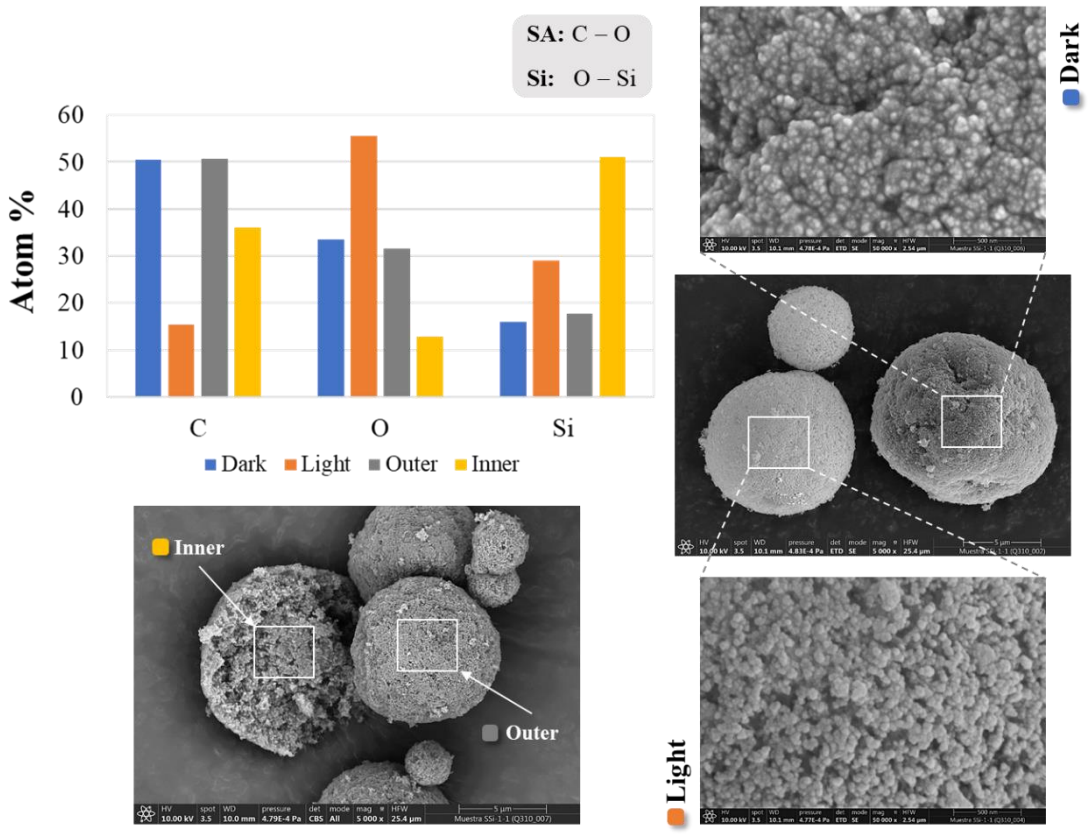

Figure 6. Semi-quantitative Energy Dispersive X-ray (EDX) microanalysis of the encapsulated samples prepared with silica (Si:SA).

Visually by comparing the "Dark" SEM micrograph with the "Light" one and, analytically, by the higher carbon content detected by SEM/EDX analysis on the surface of the loaded silica particles ("Dark" and "Outer" in Figure 6), which can be only associated to the SA molecule. "Inner" SEM/EDX analysis (corresponding to the interior of a loaded silica particle) revealed a decrease in carbon content and an increase in silicon, which means a gradual distribution of SA from the outside to the inside of the spherical silica particles.

Chitosan encapsulated SA SEM/EDX results (Figure 7) revealed the presence of five main components: carbon, oxygen, nitrogen, phosphorous and sodium. In this case, carbon and oxygen are present in both capsule and encapsulated, leaving as differential elements the nitrogen, assigned to chitosan molecule, and phosphorous and sodium, which correspond to the TPP-Na cross-linking material. The lack of a

distinguishing chemical element between capsule and encapsulated makes the interpretation of the SEM/EDX results much more difficult with respect to the previous silica samples. However, from the pictures, an unusual kind of particle with a smoother surface was detected (marked as 1 in Figure 7), since most of the observed particles were similar to those identified as 2 to 5 in Figure 7.

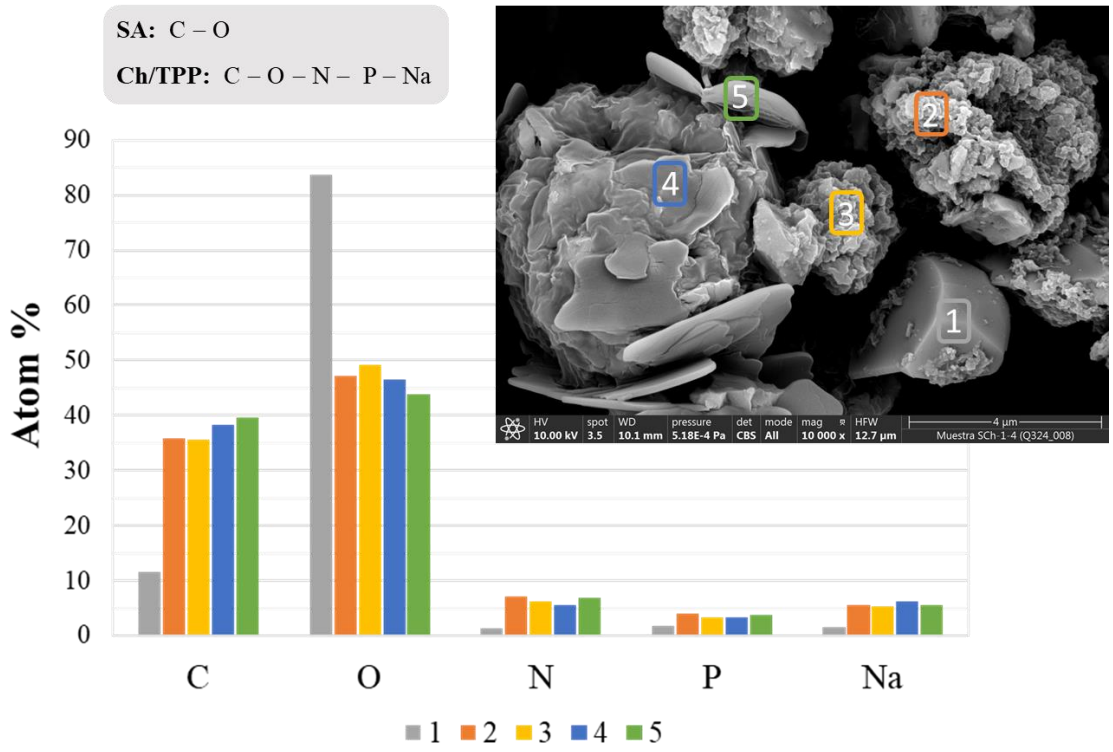

Figure 7. Semi-quantitative Energy Dispersive X-ray (EDX) microanalysis of the encapsulated samples prepared with chitosan (Ch:SA).

This isolate particle has a different chemical composition with lower contents of nitrogen, phosphorus and sodium, which suggest that it could correspond to free SA that have not been adequately encapsulated by chitosan during the process. SEM/EDX analysis of the 2 to 5 marked particles revealed a higher content of nitrogen, phosphorus and sodium, which should correspond to the TPP-Na crosslinked chitosan (capsule material). Indeed, as have been suggested by other authors [31,37], high contents on phosphorus (and sodium in our case) can be related to a worst encapsulation process, since the full SA entrapment within chitosan particles will probably leave no enough space for TPP to be cross-lined with chitosan chains, comparing with unloaded chitosan cross-linked particles. The higher cross-linking density of the unloaded particles would result in a higher phosphorus content (around 50% according to previous findings[31]). Hence the low contents of P and Na identified in our samples

(lower than 10%) were found to be in agreement with data reported in the literature [31], suggesting a good SA entrapment within chitosan particles.

3.1.5. Encapsulation efficiency of SA

Encapsulation efficiency (EE%) results are shown in Table 1. The average EE% of the encapsulated SA with silica and chitosan were 61.9% and 46.7%, respectively, and in both cases the highest EE% value corresponded to the intermediate ratio studied (1:0.5 in the silica samples and 1:1 in the chitosan ones). The differences in EE% found between the two capsules could be explained by the different encapsulation process conducted in each case. During the encapsulation process, SA appears to be embedded in the porous structure of the silica capsule and entrapped within the polymer chains of chitosan and thereafter cross-linked with TPP-Na. Pure silica particles have a high specific surface area (117 m^2/g), allowing a high loading of SA inside, and conducting to a higher EE%.

Contrary to what might be expected (that the highest ratios had the highest EE% values), the results showed that the intermediate ratios had the top EE% for each tested capsule. The reason, as reported in literature, could be attribute to the reduction of the capsule saturation [38]. Higher contents in SA could lead to capsule saturation and an increase of the free SA (unable to bind to saturated chitosan or to penetrate saturated silica), reducing the EE%.

3.2. In vitro kinetics of SA release

Encapsulated systems were evaluated to determine their kinetics and their release mechanism. In vitro cumulative release was performed on the three studied ratios of Si:SA and Ch:SA samples (Figure 8a and b, respectively). The constant movement applied by the magnetic stirrer was kept at the lowest speed allowed by the equipment so no physical damage was induced to the capsule by the mechanical movement and SA could detached from the capsule following a natural extraction process [39]. Results showed that SA was released in the first 6 hours in one stage, for both capsules and for the three tested ratios, where chitosan capsule results are in agreement with precedent studies conducted [40].

Korsmeyer-Peppas model, which has already been used to evaluate the release kinetics of another encapsulated phytohormones, i.e. gibberellic acid [35], was used to determine the release mechanism and kinetics of SA from the two capsules studied. The value of n in Eq. 3 determines the kind of release mechanism, that is Fickian or non-Fickian (anomalous) diffusion, while the k value in the same equation determines the speed of release [41].

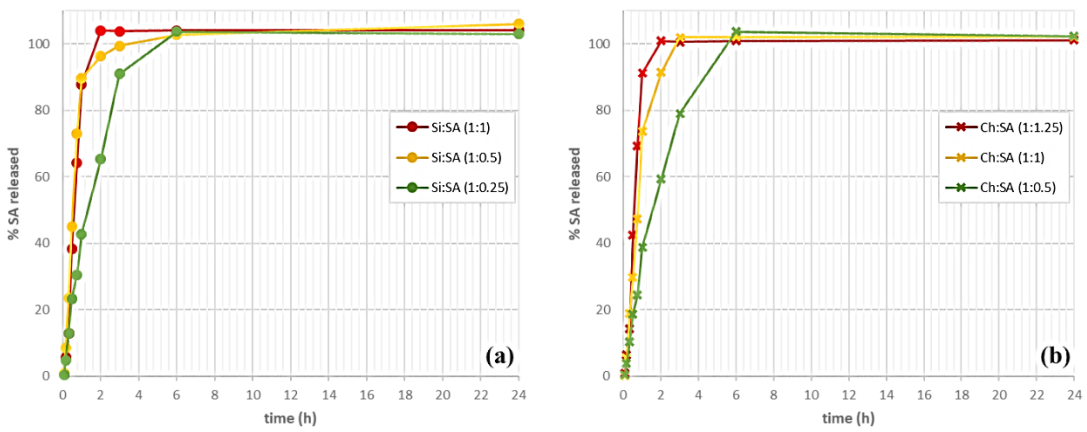

Figure 8. SA release from encapsulated samples prepared with silica (a) and chitosan (b) for the three capsule:SA studied ratios.

The linear plot of $ln(M_t/M_\infty)$ versus $ln(t)$ yielded the diffusion exponent (n), the regression values (r^2) and the diffusion constant (k), depicted in Table 2. The results showed that the release of SA from silica and chitosan capsules followed a non-Fickian (case II transport) release mechanism for both capsules and for all the studied ratios, where active substance is released by dissolution or relaxation of polymer chains inside of the capsule [42,43]. Diffusion exponent (n) ranged from 1.16-1.66 for silica encapsulated SA samples and from 1.22-1.57 for chitosan encapsulated ones. In both capsules the lowest n value corresponded to the lowest ratios, that is 1:0.25 and 1:0.5, respectively.

Table 2. Mathematical values obtained from Korsmeyer-Peppas model. K, n and r^2 represents kinetic constant, release exponent and Pearson coefficient, respectively, of Si:SA and Ch:SA samples.

SAMPLE	k (h^{-n})	n	r^2
Si:SA (1:1)	0.70	1.66	0.92
Si:SA (1:0.5)	0.59	1.26	0.83
Si:SA (1:0.25)	0.28	1.16	0.86
Ch:SA (1:1.25)	0.66	1.57	0.91
Ch:SA (1:1)	0.48	1.52	0.86
Ch:SA (1:0.5)	0.31	1.22	0.88

The value of k (Table 2) is positively correlated with the release rate kinetics [44]. The highest values of k were observed with the highest ratios tested, which indeed corresponded to the highest EE%. The increase of SA in the polymer matrix, which resulted in an increase of Ch-TPP nanogel hydrophilic

character, seems to favour the release of the encapsulated hormone, as previously found in the literature [45]. During the swelling and relaxation process, the water embedded in the chitosan matrix untangled and loosened the polymer chains, which provided more mobility for the hormone to diffuse from the polymer matrix to the surrounding medium [46].

3.3. Antifungal activity of Si:SA and Ch:SA samples

It is known that SA has antifungal effects as a decoupling agent of organelle membranes [47] and that SA affects directly the fungal development in *Eutypa lata* by a displacement of the hydroxyl group on the aromatic ring of SA structure [48], which reduced the fungal growth. To test if the hormone has also toxic effects on differents fungi of agronomical importance, a preliminary toxicity curve of SA with different concentrations was conducted on *A. alternata*, *F. Oxysporum*, *G. candidum*, *F. infestans* and *B. cinerea* (see Supplementary Table 3 and Supplementary Figure 2 and 3). *A. alternata* and *B. cinerea* inhibition rates were found to be 45.72 % and 42.11 %, respectively; both recorded at maximum SA concentration of 1000 µM. Inhibition rates for *F. oxysporum* and *G. candidum* were 19.83% and 21.27%, respectively, while *P. infestans* did not show mycelial inhibition at any concentration of SA. *A. alternata* and *B. cinerea* showed mycelial inhibition between 100 to 1000 µM SA concentration, and *F. oxysporum* and *G. candidum* in the 700-1000 µM range. In view of these results, 100, 500 and 1000 µM SA concentrations were used to test the Si:SA and Ch:SA samples.

Supplementary Table 4 and Supplementary Figure 4 and 5 depict the results obtained for 100 µM treatments. First, it is shown that empty capsules did not have any antifungal effect on their own. Mycelial growth of *A. alternata* and *B. cinerea* was reduced by 100 µM SA, with inhibition rates of 16.5% and 11.6%, respectively, while Si:SA (1:0.25) and Ch:SA (1:0.5) applied at 100 µM caused inhibition rates of 34.1% and 43.1% for *A. alternata* and 17.3% and 38.8% for *B. cinerea*, respectively. These values exceed the *A. alternata* and *B. cinerea* inhibition rates caused by the free hormone, which means that encapsulation increased antifungal activity and the capsules are acting only as carriers as they have no effect on fungi by themselves. The others studied ratios at 100 µM did not affect the mycelial growth of *A. alternata* and *B. cinerea* (see Supplementary Table 4 and Supplementary Figure 5). In the case of *F. Oxysporum*, *G. candidum* and *F. infestans*, the three capsule:SA studied ratios at 100 µM did not affect their mycelial growth (see Supplementary Table 4 and Supplementary Figure 5).

In the same way, Supplementary Table 4 and Supplementary Figure 6 and 7 depict the results obtained for 500 µM treatments. The radial growth of *A. alternata* and *B. cinerea* was highly inhibited in 30.8% and 20.4%, respectively. Treatments with Si:SA (1:0.25) and Ch:SA (1:0.5) at 500 µM caused higher inhibition rates of 52.8% and 55.9% for *A. alternata* and of 31.9% and 57.1% for *B. cinerea*, respectively. Inhibition rate for *F. oxysporum* was 16.4 % at this SA concentration and 22.9% with the Si:SA (1:0.25) treatment. The others studied ratios at 500 µM did not affect the mycelial growth of *A.*

alternata, *B. cinerea* and *F. oxysporum* (see Supplementary Table 4 and Supplementary Figure 7). In the case of *G. candidum* and *F. infestans*, the three capsule:SA studied ratios at 500 µM did not affect their mycelial growth (see Supplementary Table 4 and Supplementary Figure 7).

In the same way, Supplementary Table 4 and Supplementary Figure 6 and 7 depict the results obtained for 500 µM treatments. The radial growth of *A. alternata* and *B. cinerea* was highly inhibited in 30.8% and 20.4%, respectively. Treatments with Si:SA (1:0.25) and Ch:SA (1:0.5) at 500 µM caused higher inhibition rates of 52.8% and 55.9% for *A. alternata* and of 31.9% and 57.1% for *B. cinerea*, respectively. Inhibition rate for *F. oxysporum* was 16.4 % at this SA concentration and 22.9% with the Si:SA (1:0.25) treatment. The others studied ratios at 500 µM did not affect the mycelial growth of *A. alternata*, *B. cinerea* and *F. oxysporum* (see Supplementary Table 4 and Supplementary Figure 7). In the case of *G. candidum* and *F. infestans*, the three capsule:SA studied ratios at 500 µM did not affect their mycelial growth (see Supplementary Table 4 and Supplementary Figure 7).

At 1000 µM (see Supplementary Table 4 and Figure 9 and 10), Si:SA (1:0.25) and Ch:SA (1:0.5) treatments displayed a strong inhibition rate for *A. alternata* (62.5% and 80.1%, respectively) and *B. cinerea* (62.0% and 80.9%, respectively). The inhibition rates values for *F. oxysporum* were 30.2% with the Si:SA (1:0.25) treatment and 22.2% with the Ch:SA (1:0.5) one. Mycelial growth inhibition rate for *G. candidum* was 61.6% and 29.5% with Si:SA (1:0.25) and Ch:SA (1:0.5), respectively. It may be notice that these values were higher than the SA 1000 µM values of 45.8%, 37,3%, 24,7% and 20,4% which correspond to *A. alternata*, *B. cinerea*, *F. oxysporum* and *G. candidum*, respectively.

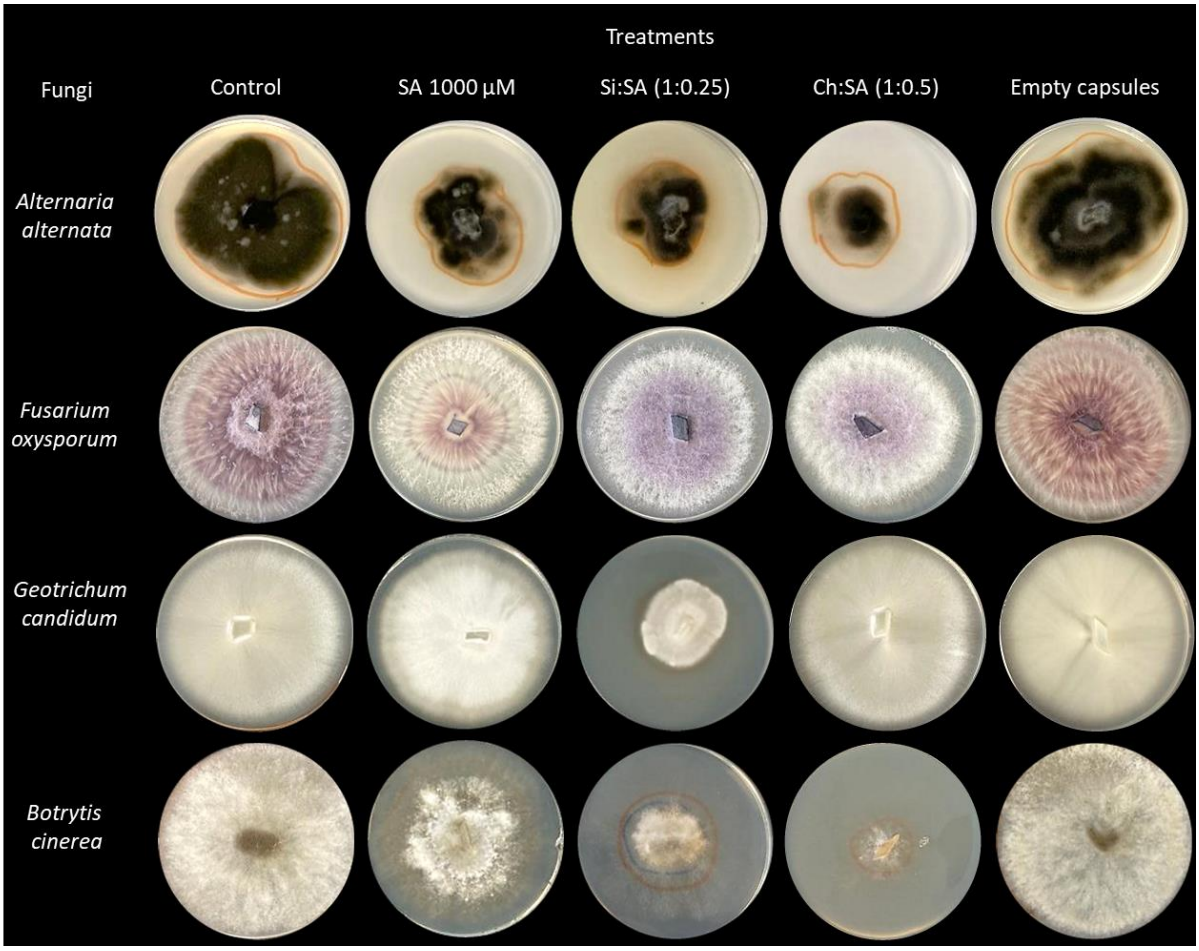

Figure 9. Antifungal effects of Si:SA (1:0.25) and Ch:SA (1:0.5) capsules at 1000 µM. Last column represents capsules without salicylic acid. The others studied ratios are not in the figure because they did not have representative mycelial inhibition effect. *Phytophthora infestans* is not in the figure because three capsule:SA studied ratios at 1000 µM had not a representative growth inhibition effect on it.

The radial growth of fungi was reduced by the smallest ratios of Si:SA and Ch:SA samples in a ratios dependent way, which might be explained by the maximum content of SA inside each capsule. Silica capsule is a porous material and SA could become oversaturated its internal surface, producing an agglutinate and preventing the correct release of SA in Si:SA higher ratios [49]. On the other hand, chitosan higher ratios could avoid the correct formation of links between Ch-TPP and SA. Therefore, form structures that are not entirely stable with inefficient SA release. In addition, as observed in the kinetics analysis, samples with lower ratios of SA release it in a more controlled way, affecting fungus growth for a longer time than the free SA. In summary, the encapsulation process with both capsules (silica or chitosan) increases the antifungal activity of SA, being the samples with lowest ratios of SA those that provide the best results.

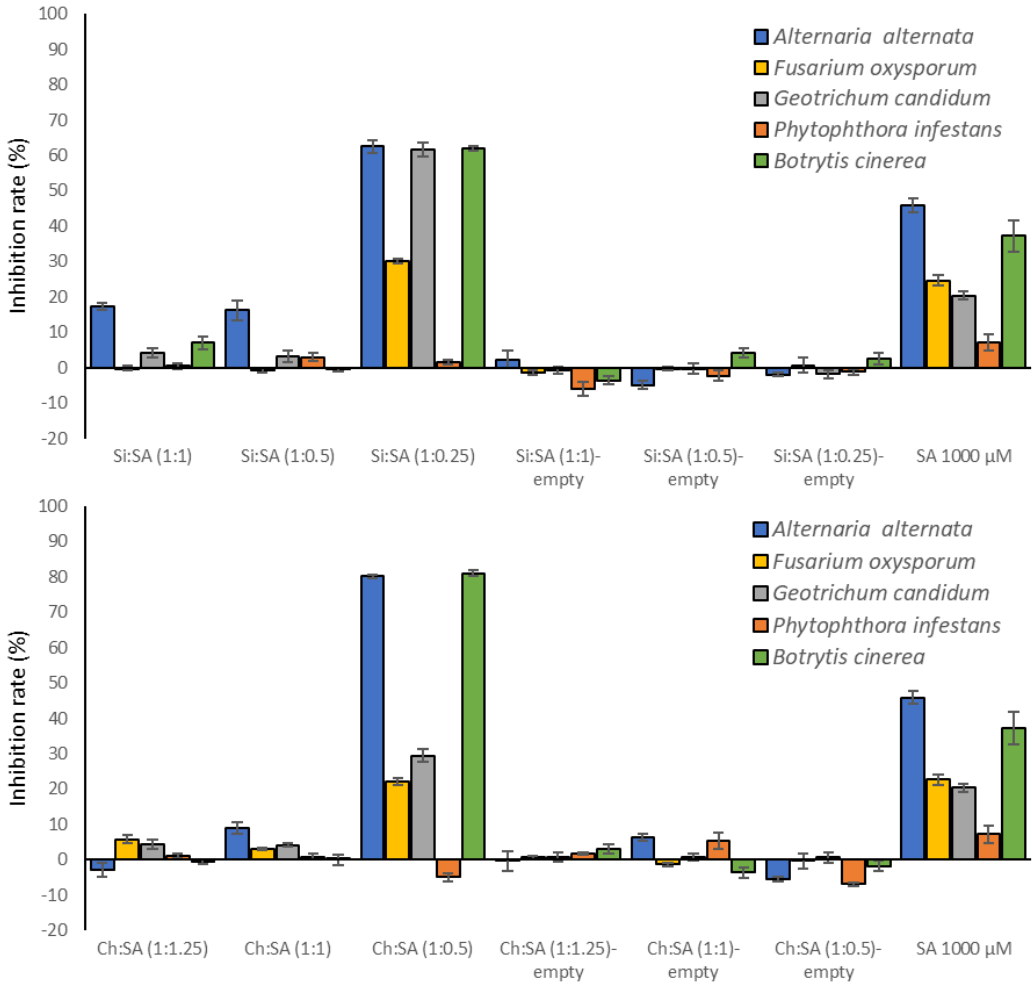

Figure 10. Fungal inhibition rates of capsules at 1000 µM. "Empty" samples correspond to capsules without salicylic acid. Fungi species *Alternaria alternata*, *Fusarium oxysporum*, *Geotrichum candidum*, *Phytophthora infestans* and *Botrytis cinerea* are representing with blue, yellow, silver, orange and green, respectively.

3.4. Effect of Si:SA and Ch:SA samples on Arabidopsis roots growth

The effect of Si:SA and Ch:SA treatments on plant development was evaluated with three doses (100, 500 and 1000 µM) of SA to determine the maximum SA concentration that Arabidopsis plants can tolerate without conditioning their growth. The results (see Supplementary Figure 8) showed an intense inhibition growth at concentrations of 500 and 1000 µM. Our results showed a totally inhibited-germination of Arabidopsis seeds at SA 1000 µM, probably because SA is playing a negative regulator

role, inducing oxidative stress [50,51]. Data also show a plant growth inhibition with roots and aerial parts less developed than controls in the case of SA 100 µM treatment.

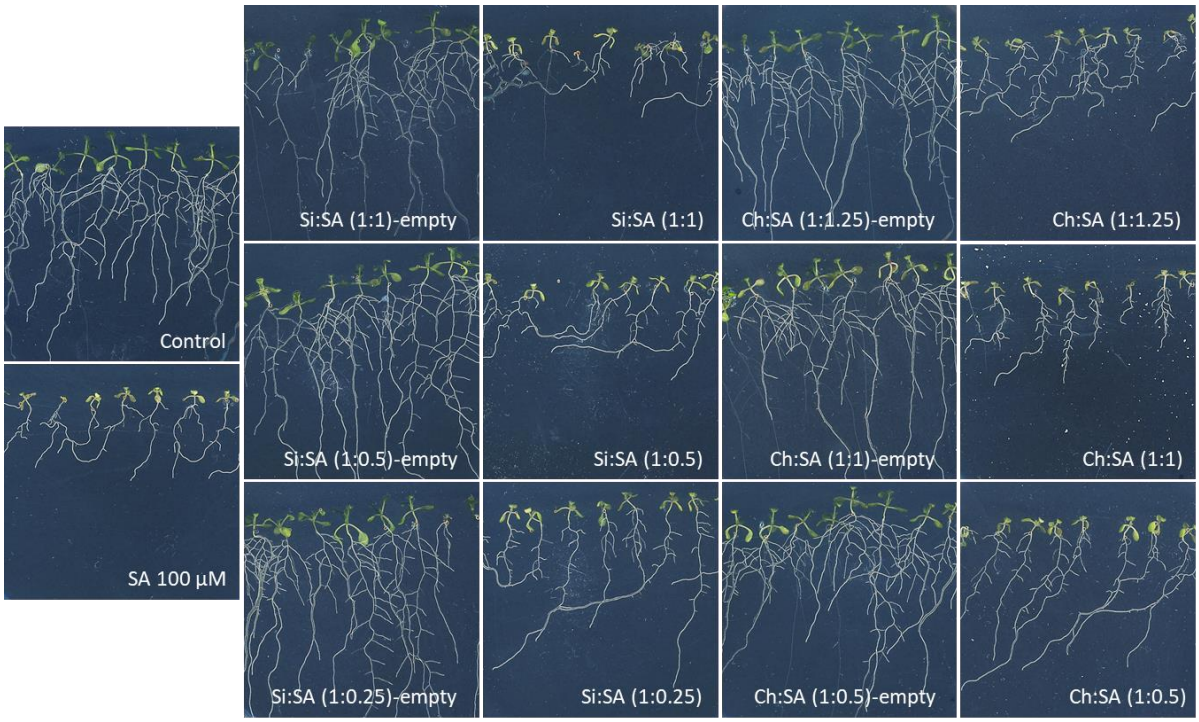

Figure 11. Effect of different capsules at 100 µM on growth and development of *Arabidopsis thaliana* plants, 12 days after seeds sowed. "Empty" samples correspond to capsules without salicylic acid.

From preliminary results, 100 µM was the dose chosen for further experiments. Figure 11 and 12 show that treatments with lowest ratios of SA (1:0.25 for Si:SA and 1:0.5 for Ch:SA) were more effective in increasing Arabidopsis roots in comparison with those treated with free SA (see Figure 12a-c). The day 12 after sowing, roots treated with Si:SA (1:0.25) and Ch:SA (1:0.5) at 100 µM had a growth of 27.1 mm and 29.2 mm, respectively. In contrast, treatment with free SA leads to a root growth of 17.3 mm (see Figure 12b-d). Therefore, data show that encapsulation reverts the toxic effect of free SA.

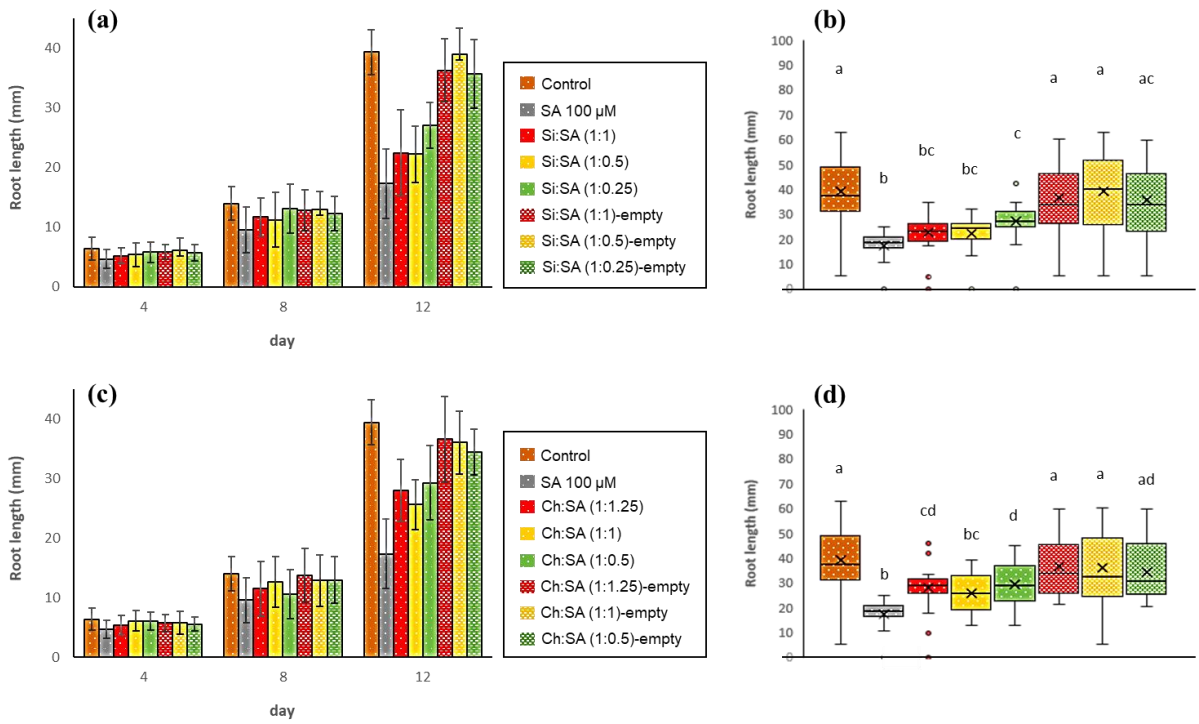

Figure 12. Root length quantification of different capsules at 100 µM. *Arabidopsis thaliana* plants at 4, 8 and 12 days after seeds sowed for the encapsulated samples prepared with silica (a) and chitosan (c). Box plot at 12 days after seeds sowed for the encapsulated samples prepared with silica (b) and chitosan (d). "Empty" samples correspond to capsules without salicylic acid.

In Arabidopsis, SA has a central role in the regulation of several plant functions and it induces antioxidant defences in abiotic or biotic stress [52]. Nevertheless, over accumulation of SA induces a programmed cell death. During stress, there is a trade-off between resistance and growth and SA endogenous accumulation triggers the immune responses that allow survival but penalize growth [53]. Treatments with exogenous SA reduce in a dose-dependent manner the Arabidopsis root elongation, inhibiting cell proliferation. Moreover, high-dosage of SA inhibits cell cycle progression and induces the auxin accumulation that inhibits lateral root development [54].

Encapsulation of SA to lower ratios is highly effective due to allow a controlled SA release. As aforementioned, this could be explained for differences in kinetics release since lower values of the kinetic constant (k) allows a slower release of SA, reducing the amount of SA available in the medium susceptible to be absorbed by the plant and decreasing its over accumulation and its toxicity. Higher ratios samples (1:0.5 and 1:1 for Si:SA and 1:1 and 1:1.25 for Ch:SA) and free SA gave worse results since large amounts of SA increase toxicity in plant cells because encapsulation at higher ratios is inefficient and probably the excess of SA remains on the surface of silica and with the quitosan do not

correctly form closed capsules, leaving a considerable amount of SA free [55]. It should be also noticed that the capsules per se did not show any significant effect on Arabidopsis roots growth.

4. Conclusions

In summary, this work describes characteristics and biological effects of encapsulated systems composed of silica or quitosan as carriers for the SA phytohormone. The capsule:SA lower ratios provided a controlled release than the others studied ratios because of lowest amount of SA was able to correctly encapsulate and did not saturate the capsule. In vitro assays against *A. alternata, B. cinerea, F. oxysporum,* and *G. candidum* of Si:SA (1:0.25) and Ch:SA (1:0.5) treatments, had a stronger inhibition effect in mycelial growth than free SA where a slow SA release affect effectively for longer times. Necrotrophic and biotrophic fungi attack around of 200 crops at worldwide causing water-soaking of tissues following of appearance of grey masses on leaves, stems and fruits [56], and these are difficult to control with fungicides due to its genetic plasticity [57]. For this reason, the encapsulated samples are a great alternative for anti-fungal activity control and can be tested *in vitro* and *in vivo*, in others pathogenic fungi. Moreover, the efficacy of the treatments allows to carry out new studies to formulate new active fungicide for crop protection. Similarly, in biological assays of Arabidopsis seeds, the lowest ratios samples reverse the toxic effect in the development of plants growth by reducing the over accumulation of SA free in the medium which is absorbed during the development of the plant. In the case of the higher ratios samples and free SA, the effect of these in the plants was considerable worse, avoiding the correct formation and growing of roots and rosettes. These results can be extrapolated to biotic and abiotic stress assays in plants where a determinated phytohormone will be a controlled and enhanced effect, giving to the plant the capacity to resist and mitigate a specific stress application. Finally, the use of capsule systems for any phytohormone can enhance its biological activity and efficiency when its used in the field, resulting in a great agricultural product with a higher quality and important economic value.

Acknowledgement

This work was supported by the Spanish Ministerio de Ciencia e Innovación (PID2019-104062RB-I00), Universitat Jaume I (UJI-B2019-11, UJIB2020-13) and Generalitat Valenciana - GRISOLIAP/2020/043.

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Supplementary material

Supplementary Table 1. Density and viscosity (point A and B in Figure 1) of the prepared slurries ($g/cm3 \pm 0.002$ and $mPa{\cdot}s \pm 1$).

SAMPLE	$\boldsymbol{\rho}$ $(\boldsymbol{g/cm^3})$	$\boldsymbol{\eta_A}$ $(\boldsymbol{mPa \cdot s})$	$\boldsymbol{\eta_B}$ $(\boldsymbol{mPa \cdot s})$
Si:SA (1:1)	1.048	67	9
Si:SA (1:0.5)	1.058	44	13
Si:SA (1:0.25)	1.066	50	14
Ch:SA (1:1.25)	1.061	717	7
Ch:SA (1:1)	1.052	369	6
Ch:SA (1:0.5)	1.041	32	5

Supplementary Table 2. Characteristic diameters of the capsules size distributions shown in Figure 3 (mm ± 0.2).

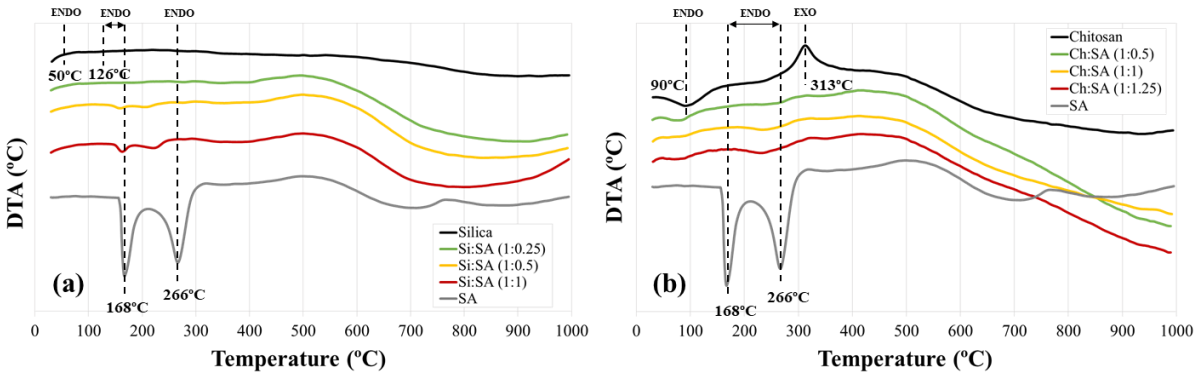
SAMPLE	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)
Si:SA (1:1)	4.8	9.9	16.9
Si:SA (1:0.5)	4.5	9.6	19.1
Si:SA (1:0.25)	5.6	11.0	22.2
Ch:SA (1:1.25)	3.1	8.5	18. 5
Ch:SA (1:1)	2.7	7.2	14.3
Ch:SA (1:0.5)	3.1	7.6	14.4

Supplementary Table 3. Effect of different salicylic acid concentrations against fungi species.

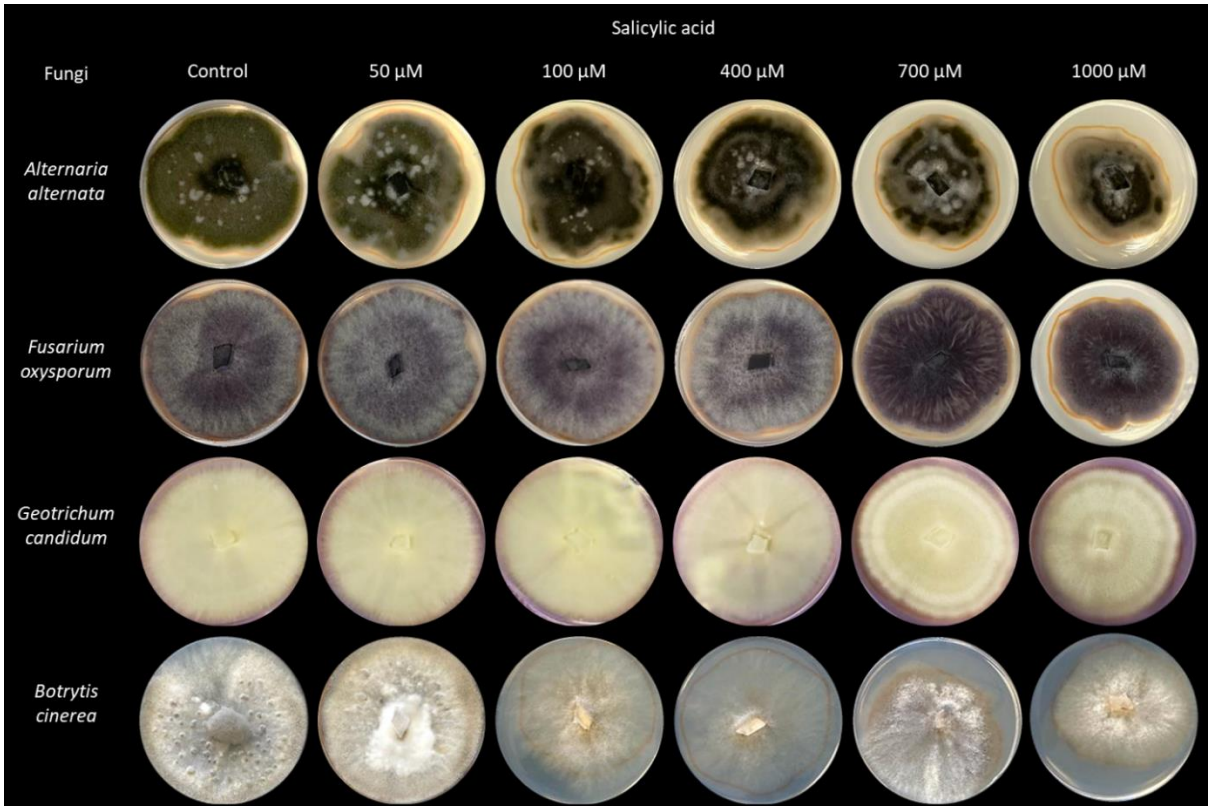
	% Inhibition rate				
SA (μM)	*Alternaria alternata*	*Fusarium oxysporum*	*Geotrichum candidum*	*Phytophthora infestans*	*Botrytis cinerea*
Control	0.000 ± 0.0 ^{a}	0.000 ± 0.0 ^{a}	0.000 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.000 ± 0.0 ^{a}
50	8.51 ± 1.4 ^{ab}	0.193 ± 0.4 ^{a}	0.941 ± 0.4 ^{a}	-1.1 ± 1.3 ^{a}	0.213 ± 0.3 ^{a}
100	15.92 ± 0.7 ^{b}	1.346 ± 0.6 ^{a}	1.97 ± 0.6 ^{ab}	0.77 ± 0.9 ^{a}	10.69 ± 1.0 ^{b}
400	21.9 ± 0.2 ^{bc}	5.25 ± 0.6 ^{ab}	2.27 ± 0.5 ^{ab}	4.65 ± 1.0 ^{a}	22.4 ± 0.9 ^{bc}
700	32.2 ± 1.2 ^{cd}	11.7 ± 0.9 ^{bc}	10.30 ± 1.2 ^{b}	5.49 ± 1.2 ^{a}	30.32 ± 0.2 ^{c}
1000	45.72 ± 0.9 ^{d}	19.83 ± 2.1 ^{c}	21.27 ± 2.0 ^{c}	8.61 ± 0.4 ^{a}	42.11 ± 3.5 ^{d}

Supplementary Table 4. Resume of different samples against fungi species. "Empty" samples correspond to capsules without salicylic acid**.**

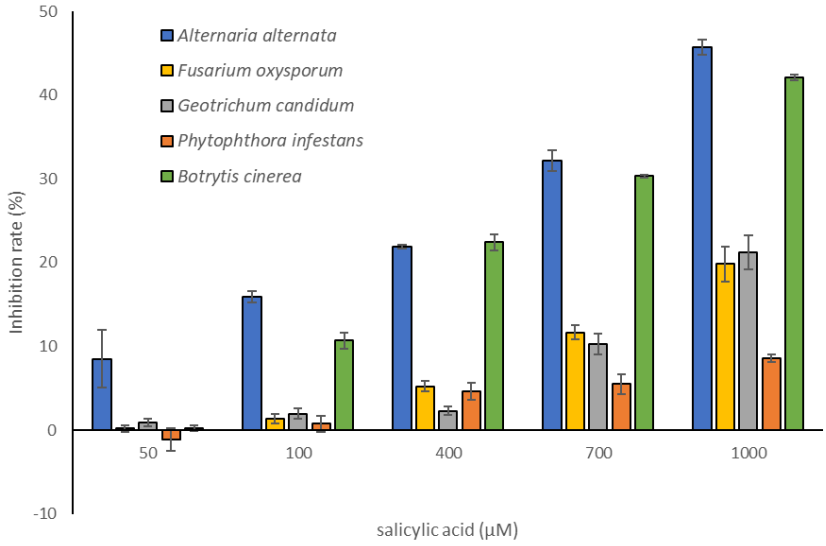
Treatments	% Inhibition rate														
	Alternaria alternata			*Phytophthora infestans*			*Fusarium oxysporum*			*Geotrichum candidum*			*Botrytis cinerea*		
	100 µM	500 µM	1000 µM	100 µM	500 µM	1000 µM	100 µM	500 µM	1000 µM	100 µM	500 µM	1000 µM	100 µM	500 µM	1000 µM
Control	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}
SA	16.5 ± 1.2 ^{b}	30.8 ± 1.5 ^{b}	45.8 ± 1.9 ^{b}	1.68 ± 1.0 ^{a}	-4.5 ± 0.7 ^{a}	7.19 ± 2.4 ^{a}	1.30 ± 0.8 ^{a}	16.4 ± 1.4 ^{b}	24.7 ± 1.6 ^{b}	1.51 ± 0.4 ^{a}	6.59 ± 0.6 ^{a}	20.4 ± 1.2 ^{b}	11.6 ± 0.6 ^{b}	20.4 ± 1.1 ^{b}	37.3 ± 2.7 ^{b}
Si:SA (1:1)	6.41 ± 0.9 ^{a}	3.20 ± 1.6 ^{a}	17.4 ± 0.9 ^{a}	1.43 ± 0.8 ^{a}	-1.0 ± 1.1 ^{a}	0.43 ± 0.9 ^{a}	1.14 ± 1.0 ^{a}	5.65 ± 1.4 ^{a}	-0.2 ± 0.7 ^{a}	4.84 ± 0.5 ^{a}	4.20 ± 1.3 ^{a}	4.20 ± 1.3 ^{a}	2.34 ± 2.3 ^{a}	1.18 ± 1.8 ^{c}	7.03 ± 1.7 ^{a}
Si:SA (1:0.5)	-5.6 ± 0.5 ^{a}	2.97 ± 1.2 ^{a}	16.3 ± 2.8 ^{a}	3.00 ± 1.1 ^{a}	2.92 ± 1.1 ^{a}	2.92 ± 1.1 ^{a}	0.27 ± 1.2 ^{a}	7.23 ± 2.2 ^{a}	-0.7 ± 0.6 ^{a}	2.05 ± 0.4 ^{a}	10.9 ± 1.0 ^{a}	3.51 ± 1.6 ^{a}	-1.5 ± 1.0 ^{a}	2.54 ± 0.4 ^{a}	-0.5 ± 0.5 ^{a}
Si:SA (1:0.25)	34.1 ± 2 ^{cd}	52.8 ± 1.1 ^{c}	62.5 ± 1.7 ^{cd}	-1.1 ± 0.9 ^{a}	-5.8 ± 0.7 ^{a}	1.67 ± 0.6 ^{a}	-2.1 ± 0.7 ^{a}	22.9 ± 0.7 ^{c}	30.2 ± 0.6 ^{b}	2.06 ± 0.2 ^{a}	1.08 ± 1.2 ^{a}	61.6 ± 1.9 ^{c}	17.3 ± 3.2 ^{b}	31.9 ± 0.6 ^{c}	62.0 ± 1.5 ^{c}
Si:SA (1:1)-empty	5.40 ± 2.4 ^{a}	-1.8 ± 0.3 a	2.27 ± 2.4 ^{a}	-10 ± 0.3 ^{a}	-5.9 ± 2.0 ^{a}	-5.9 ± 1.9 ^{a}	-1.0 ± 0.3 ^{a}	-4.5 ± 2.1 ^{a}	-1.3 ± 0.6 ^{a}	-0.2 ± 0.4 ^{a}	-0.8 ± 1.1 ^{a}	-0.78 ± 1.1 ^{a}	1.45 ± 1.7 ^{a}	-3.6 ± 1.2 ^{a}	-3.6 ± 1.2 ^{a}
Si:SA (1:0.5)-empty	0.50 ± 1.5 ^{a}	4.99 ± 0.3 ^{a}	-4.98 ± 1.1 ^{a}	-1.5 ± 1.9 ^{a}	1.46 ± 0.6 ^{a}	-2.3 ± 1.5 ^{a}	-2.1 ± 1.9 ^{a}	2.97 ± 1.2 ^{a}	-0.3 ± 0.4 ^{a}	0.62 ± 0.6 ^{a}	-0.3 ± 1.5 ^{a}	-0.33 ± 1.5 ^{a}	0.47 ± 1.8 ^{a}	-1.8 ± 1.4 ^{a}	4.32 ± 1.3 ^{a}
Si:SA (1:0.25)-empty	-3.6 ± 0.5 ^{a}	- 1.1 ± 0.5 ^{a}	-1.92 ± 0.4 ^{a}	0.30 ± 1.5 ^{a}	1.23 ± 0.3 ^{a}	-1.1 ± 0.9 ^{a}	0.30 ± 1.5 ^{a}	-2.2 ± 1.3 ^{a}	0.63 ± 2.1 ^{a}	-1.3 ± 1.0 ^{a}	-1.8 ± 1.2 ^{a}	-1.84 ± 1.2 ^{a}	0.57 ± 1.2 ^{a}	-2.9 ± 0.9 ^{a}	2.53 ± 3.4 ^{a}
Ch:SA (1:1.25)	0.83 ± 2.4 ^{a}	3.57 ± 2.4 ^{a}	-2.8 ± 0.5 ^{a}	6.60 ± 0.9 ^{a}	-3.6 ± 1.4 ^{a}	1.17 ± 1.1 ^{a}	2.60 ± 0.9 ^{a}	-2.6 ± 0.9 ^{a}	5.86 ± 0.9 ^{a}	0.30 ± 0.9 ^{a}	-4.9 ± 0.3 ^{a}	4.24 ± 1.7 ^{a}	-2.1 ± 1.0 ^{a}	4.36 ± 1.7 ^{a}	-0.4 ± 0.8 ^{a}
Ch:SA (1:1)	4.85 ± 0.7 ^{a}	6.03 ± 0.9 ^{a}	8.95 ± 1.9 ^{a}	-5.0 ± 1.2 ^{a}	0.91 ± 0.7 ^{a}	0.91 ± 0.7 ^{a}	-2.0 ± 1.2 ^{a}	-3.3 ± 1.7 ^{a}	3.08 ± 1.3 ^{a}	-0.2 ± 0.6 ^{a}	0.82 ± 1.4 ^{a}	4.20 ± 1.3 ^{a}	5.36 ± 1.0 ^{a}	2.46 ± 0.6 ^{a}	0.09 ± 0.6 ^{a}
Ch:SA (1:0.5)	43.1 ± 1.2 ^{d}	55.9 ± 0.8 ^{c}	80.1 ± 1.6 ^{d}	6.12 ± 0.7 ^{a}	-4.9 ± 0.8 ^{a}	-4.9 ± 0.8 ^{a}	1.22 ± 0.7 ^{a}	13.4 ± 1.9 ^{bc}	22.2 ± 0.4 ^{b}	0.97 ± 0.2 ^{a}	8.53 ± 0.5 ^{a}	29.5 ± 0.6 ^{d}	38.8 ± 2.5 ^{c}	57.1 ± 0.7 ^{d}	80.9 ± 0.8 ^{d}
Ch:SA (1:1.25)-empty	-5.2 ± 0.6 ^{a}	7.92 ± 1.7 ^{a}	-0.3 ± 0.7 ^{a}	-1.7 ± 1.4 ^{a}	2.18 ± 0.4 ^{a}	1.69 ± 0.6 ^{a}	1.7 ± 1.4 ^{a}	0.05 ± 0.9 ^{a}	0.63 ± 2.1 ^{a}	-3.2 ± 2.2 ^{a}	0.18 ± 1.0 ^{a}	0.81 ± 1.5 ^{a}	3.57 ± 2.1 ^{a}	-2.6 ± 1.1 ^{a}	3.08 ± 1.5 ^{a}
Ch:SA (1:1)-empty	0.63 ± 2.5 ^{a}	6.51 ± 1.4 ^{a}	6.32 ± 2.7 ^{a}	-1.6 ± 1.4 ^{a}	3.48 ± 0.2 ^{a}	5.40 ± 0.3 ^{a}	-1.0 ± 1.4 ^{a}	1.97 ± 1.5 ^{a}	-1.3 ± 0.6 ^{a}	-2.6 ± 1.6 ^{a}	-3.5 ± 0.9 ^{a}	0.88 ± 1.4 ^{a}	1.52 ± 1.1 ^{a}	4.27 ± 2.0 ^{a}	-3.6 ± 1.2 ^{a}
Ch:SA (1:0.5)-empty	-4.1 ± 0.8 ^{a}	5.97 ± 2.0 ^{a}	-5.40 ± 1.0 ^{a}	-8.2 ± 3.6 ^{a}	-1.4 ± 1.4 ^{a}	-6.9 ± 2.3 ^{a}	-3.2 ± 0.6 ^{a}	2.97 ± 1.2 ^{a}	-0.3 ± 0.4 ^{a}	2.7 ± 1.5 ^{a}	1.01 ± 0.8 ^{a}	0.68 ± 0.9 ^{a}	1.77 ± 0.6 ^{a}	-2.38 ± 1.3 ^{a}	-1.8 ± 1.4 ^{a}



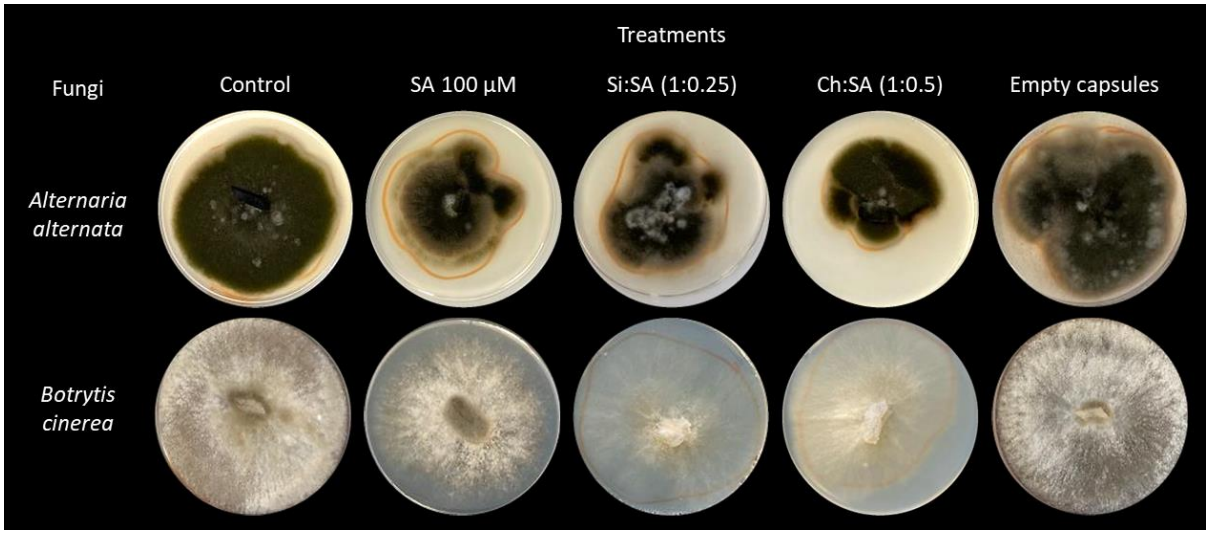
Supplementary Figure 1. DTA thermograms of the encapsulated samples prepared with silica (a) and chitosan (b) for the three capsule:SA studied ratios and their corresponding raw materials.



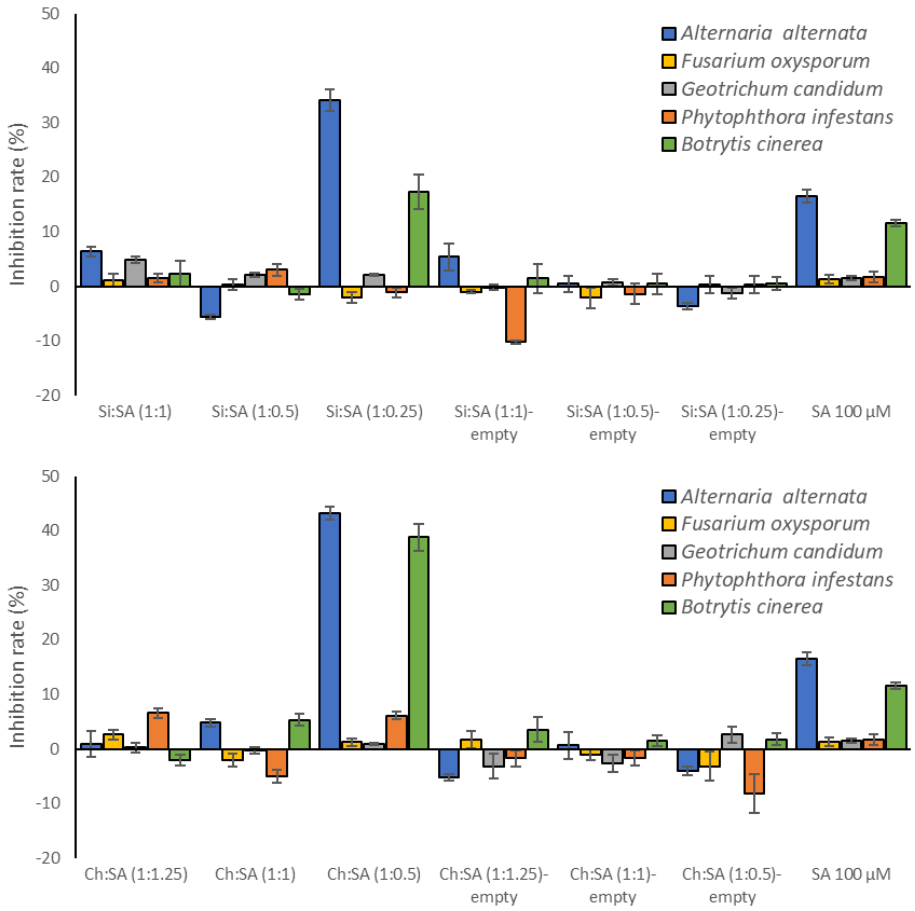
Supplementary Figure 2. Antifungal effects of different SA concentrations. *Phytophthora infestans* is not in the figure because different SA concentrations had not a representative growth inhibition effect on it.



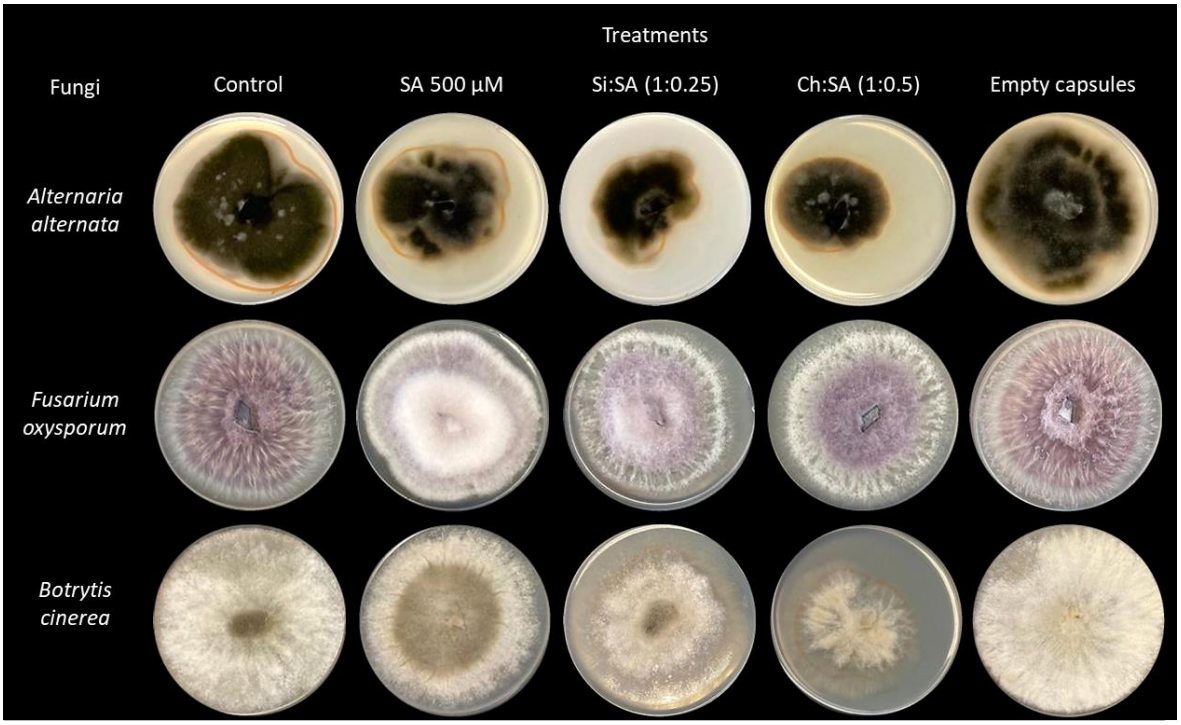
Supplementary Figure 3. Fungal inhibition rates of different SA concentrations. Fungi species *Alternaria alternata*, *Fusarium oxysporum*, *Geotrichum candidum*, *Phytophthora infestans* and *Botrytis cinerea* are representing with blue, yellow, silver, orange and green, respectively.



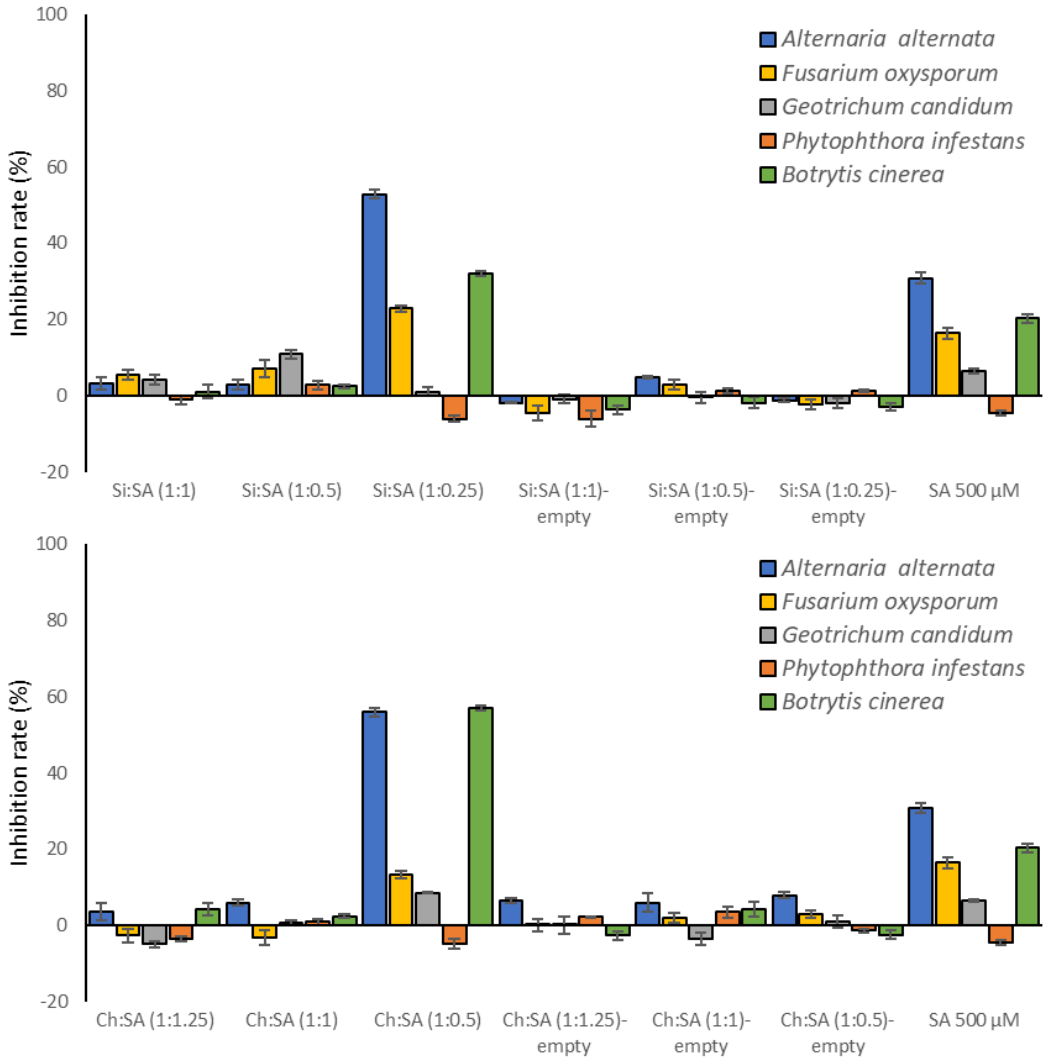
Supplementary Figure 4. Antifungal effects of Si:SA (1:0.25) and Ch:SA (1:0.5) at 100 µM. Last column represents capsules without salicylic acid. The others studied ratios are not in the figure because they did not have representative mycelial inhibition effect. *Phytophthora infestans*, *Fusarium oxysporum* and *Geotrichum candidum* are not in the figure because three capsule:SA studied ratios at 100 µM had not a representative growth inhibition effect on them.



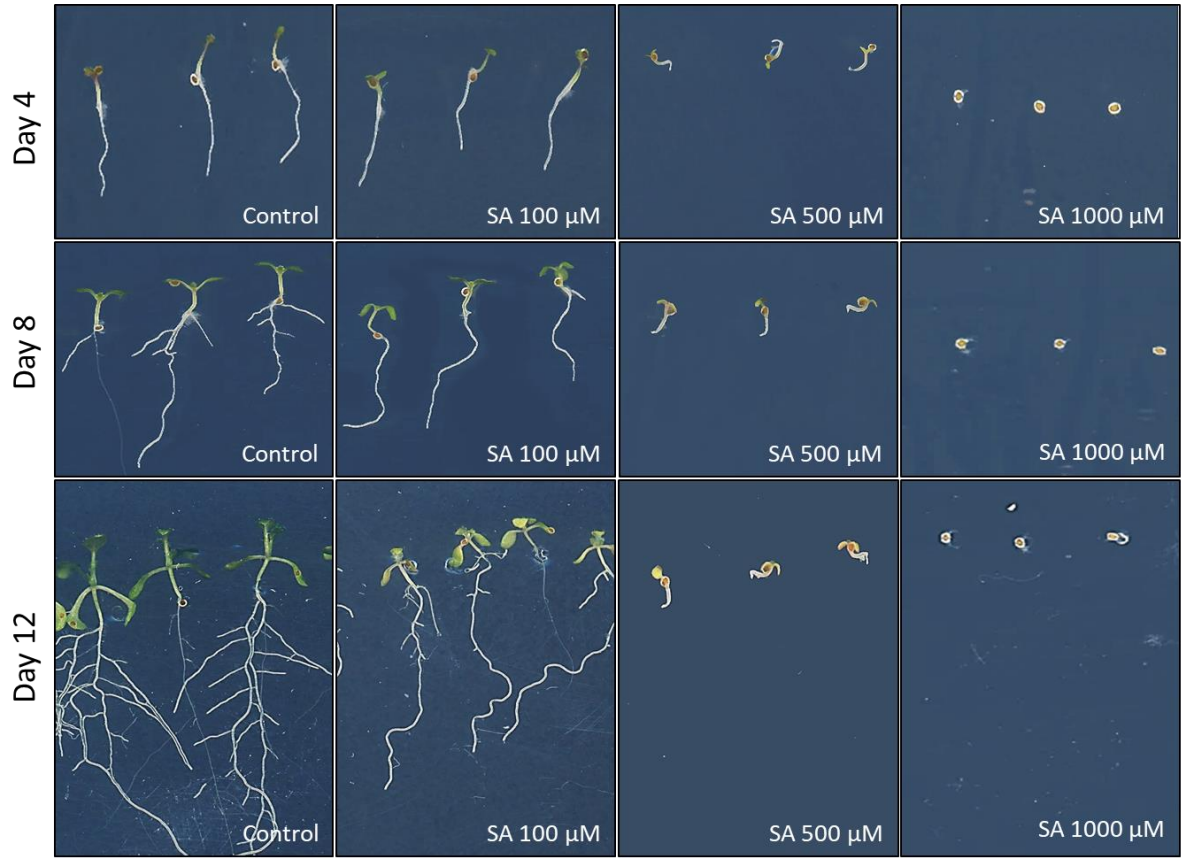
Supplementary Figure 5. Fungal inhibition rates of different capsules at 100 µM. Sample ratio-empty represent capsule without salicylic acid. Fungi species *Alternaria alternata*, *Fusarium oxysporum*, *Geotrichum candidum*, *Phytophthora infestans* and *Botrytis cinerea* are representing with blue, yellow, silver, orange and green, respectively.



Supplementary Figure 6. Antifungal effects of Si:SA (1:0.25) and Ch:SA (1:0.5) at 500 µM. Last column represents capsules without salicylic acid. The others studied ratios are not in the figure because they did not have representative mycelial inhibition effect. *Phytophthora infestans* and *Geotrichum candidum* are not in the figure because three capsule:SA studied ratios at 500 µM had not a representative growth inhibition effect on them.

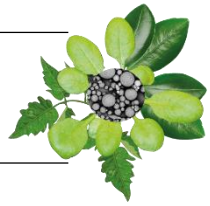


Supplementary Figure 7. Fungal inhibition rates of different capsules at 500 µM. Sample ratio-empty represent capsule without salicylic acid. Fungi species *Alternaria alternata*, *Fusarium oxysporum*, *Geotrichum candidum*, *Phytophthora infestans* and *Botrytis cinerea* are representing with blue, yellow, silver, orange and green, respectively.



Supplementary Figure 8. Effect of different SA concentrations in growth and development of *Arabidopsis thaliana* plants at 4, 8 and 12 days after seeds sowed.

CHAPTER 2



OPTIMIZATION OF PROCESS VARIABLES FOR INDUSTRIALLY SCALABLE ENCAPSULATION OF SALICYLIC ACID IN AN ENVIRONMENTALLY FRIENDLY SETTING

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Key words for the tittle: Amorphous silica – Chitosan – Factorial design – Fungi – Spray-drying – Phytohormone

Unpublished

Abstract

Encapsulated phytohormones are gaining attention as a novel palliative treatment for plants to cope with environmental stress. Exogenous treatments using encapsulated salicylic acid (SA) promote plant stress tolerance while enabling normal growth and development. Several methods exist to produce encapsulated active molecules, and recently, spray drying has emerged as a particularly appealing process for formulating these compounds. However, phytohormone encapsulation has not been properly established yet. In a previous study, silica/quitosan SA encapsulated samples were formulated at different ratios, and their physical, chemical, and kinetic characteristics were analyzed, resulting in a promising antifungal product [1]. However, it is unknown whether the encapsulated SA is affected in its structure and, thus, in its properties due to the spray temperature. Therefore, to decrease the spray temperature, silica/chitosan SA samples were formulated using a water-acetone mixture, and their characteristics studied and compared with the samples previously formulated in water.

This study reveals the dispensability of using an organic solvent to reduce the spray-drying temperature during atomization, as the antifungal potential of the silica/quitosan-encapsulated SA samples does not improve. Acetone- and water-based encapsulates effectively inhibited the mycelial growth of two necrotrophic fungi (*Alternaria alternata* and *Penicillium digitatum*) by approximately 50%. However, avoiding the use of organic solvents in the formulation mitigate associated issues such as environmental impact, safety, health and toxicity concerns, cost, regulatory compliance, material compatibility, and handling.

Furthermore, the water-based encapsulation process was optimized through a fractional randomized experimental design. Six process variables at two levels were selected: i) solid content, ii) milling speed, iii) milling time, iv) spray temperature, v) feed rate, and vi) airflow, resulting in 16 randomized experiments that allowed the establishment of optimal conditions for the encapsulation of SA. This optimization enables the reduction of raw material loss and production costs, fostering environmental sustainability.

1. Introduction

Climate change leads to extreme weather alterations, including cold spells, droughts, heat waves, heavy rainfall, and floods. These unfavourable events can disturb plants, altering their typical growth rate and development [2]. Plants have the ability to respond to environmental challenges by activating several signal transduction pathways that trigger molecular and physiological changes [3]. The perception of external stimuli is modulated by phytohormones, which in turn regulate growth and defense responses. These phytohormones exhibit dynamic interactions and adaptations in the face of adverse conditions.

Among the pivotal phytohormones are salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and auxins (IAA), each assuming crucial roles.

A growing trend involves the application of exogenous treatments using these phytohormones to augment plant resilience, as evidenced by an escalating trajectory [4]. Exemplifying this approach is the exogenous application of SA, which triggers plant defense mechanisms and reduces stress injuries. To optimize the efficacy of SA treatment, innovative methods such as controlled release technologies are being explored [5]. These methodologies encompass the encapsulation of bioactive compounds within protective matrices, enabling gradual and precisely regulated release. As a result, this approach provides enduring protection to treated plants exposed to challenging environmental conditions.

Numerous studies have utilized SA as the core material, encased in amorphous silica and chitosan shells. This encapsulation strategy effectively prevents SA degradation, preserving its integrity for efficient agricultural applications [6]. However, careful consideration of the application method is crucial, whether it involves tissue spraying, automatic irrigation, or in vitro systems. This consideration ensures the precise delivery of SA to specific plant areas, ultimately optimizing its efficacy while minimizing associated cost [7–9]. Additionally, it is important to acknowledge that the encapsulation process and application method may vary based on factors such as shell material, core molecule, plant species, and testing conditions.

Spray-drying has garnered considerable attention as an encapsulation method, largely due to its compatibility with biodegradable and biocompatible materials, cost-effectiveness, reproducibility, and scalability [10]. The process involves atomizing a liquid feed solution into a hot air stream, resulting in rapid drying within mere seconds [11]. This is achieved through the highly efficient heat and mass transfer capabilities of the equipment [12]. The spray drying process is conducted in two main steps: i) formulating a liquid feed using planetary milling to mix the shell and core components through rotational force, and ii) achieving a dry powder of the encapsulated sample through atomization [13]. The spray-drying process is governed by multiple variables that collectively influence the final particle properties [14]. For instance, increasing atomization pressure and reducing feed rate result in the formation of smaller droplets, leading to a finer dry powder [15]. Conversely, a higher viscosity of the feed suspension yields larger droplet size [16]. Particle shape is determined by the inlet temperature, while the airspeed rate impacts the drying and particle separation processes [17]. These parameters collectively afford control over particle properties, including surface morphology, owing to the dryer's versatility. The selection of atomization parameters depends on the bioactive working molecule, as a significant alteration could induce degradation and undesired interactions, ultimately reducing the collection of dry particles [18]. Therefore, adjusting these parameter values is a crucial step that can significantly influence the entire process and its final outcome.

Organic solvent spray-drying can serve as a viable alternative to aqueous methods when preparing encapsulated active molecules. This method utilized a range of compounds, including acetone, dichloromethane (DCM), ethanol, methylene chloride, and tetrahydrofuran [19]. It proves particularly advantageous when the necessity arises to lower the process temperature to safeguard the compound activity [20]. Nevertheless, it is important to take into account the drawbacks associated with the use of organic solvents, which include: i) increasing air pollution, ii) leading to air quality problems, iii) posing health and safety risks due to their toxicity, iv) producing hazardous waste, v) contributing to resource depletion, vi) incurring high operational costs, and vii) hindering the implementation of environmentally friendly alternatives [21,22].

This study addresses two primary objectives. First, it seeks to formulate encapsulated samples of SA using an organic solvent to reduce the operational temperature during the atomization stage. The study assesses any potential loss or deterioration of SA during the encapsulation process and investigates the necessity of employing this type of liquid medium for preparing suspensions. To achieve this, a comparison will be made among physical, chemical, and kinetic characteristics, as well as the biological activity, of the samples encapsulated using an organic solvent with those formulated in water in a previous study [1]. The optimal grinding speed to prevent alumina contamination resulting from grinding wear will be also determined, as this contamination can potentially reduce the encapsulation efficiency of SA and affect the biological properties of the encapsulated materials.

Secondly, the study focuses on optimizing the entire encapsulation process, which includes both planetary milling and spray drying procedures. A randomized experimental design will be employed to analyze the impact of the key process variables: i) solid content, ii) milling speed, iii) milling time, iv) spray temperature, v) feed rate, and vi) airflow volume percentage. The overarching goal is to determine the most effective combination of parameters to enhance the encapsulation process. This optimization serves a dual purpose: reducing energy consumption and associated costs while minimizing the environmental footprint of the industrial-scale process.

2. Materials and Methods

2.1 Materials

2.1.1. Raw materials. The reagents and materials used in this work are as follows: Pyrogenic amorphous silica (Si) (HDK® S13), Chitosan (Ch) (DG CHI 0.20 g/mL and 85% deacetylated), and Salicylic Acid (SA) were purchased from WACKER (Barcelona, Spain), AOXIN (Shanghai, China), and Sigma-Aldrich (St. Louis, USA), respectively. Acetone, potato dextrose agar (PDA), sodium tripolyphosphate (TPP-Na), tween 80, and petri dishes (9 x 15 cm) were purchased from Spanish

companies Labkem, Condalab, Acrilatos SAU, PANREAC, and Labkem, respectively. Acetic acid (glacial) and Dichloromethane (DCM) were purchased from Fisher Scientific (Lenexa, USA).

2.1.2. Fungi. The three fungal species (*Alternata alternaria*, *Fusarium oxysporum*, and *Penicillium digitatum*) used in this study were obtained from the Spanish Type Culture Collection (CECT) in Valencia, Spain.

2.2. Methods

2.2.1. Formulation and optimization of encapsulated SA samples in acetone

Grinding wear study

This study was conducted using chitosan-encapsulated SA samples due to their lower solid content, which promotes grinding wear. The findings from this study were then extrapolated to silica-encapsulated SA samples.

The general procedure for formulating chitosan encapsulated SA samples is carried out using a planetary mill (Fritsch, Pulverisette®) and alumina balls as grinding media (approximately 300 g). To prepare a Ch:SA suspension with a weight percentage of 2.5-3.6 w/v%. Ch was dissolved in a 1 v/v% acetic acid solution, and Tween 80 v/v% was used as a dispersant. Then, a 1 w/v% TPP-Na solution was added to crosslink the chitosan polymer chains. The process operated at a speed of 210 rpm, with 15-minute breaks between the steps. The final homogenization lasted for 60 minutes.

To control grinding wear in suspension formulations, various solvent compositions (acetone/water) and milling rates (rpm) were investigated using a 1:1 capsule-to-SA w/w ratio [Ch:SA (1:1)]. Initially, three different solvent mixtures were tested: 100% acetone, acetone/water (75/25 v/v%), and acetone/water (50/50 v/v%) to determine the optimal solvent ratio. Once the optimal acetone/water ratio was identified, the milling rates for both the stepwise addition of raw materials (Vo) and the final homogenization stage (Vf) were evaluated. Vo was adjusted within the range of 75-150 rpm, and Vf within the range of 100-210 rpm.

Organic solvent study

For the organic solvent study, chitosan-encapsulated SA samples were prepared in three capsule-to-SA ratios (1:1.25, 1:1, and 1:0.5) using the selected solvent composition (acetone/water) and the update milling rate, following the same protocol as in the previous section. Silica-encapsulated SA samples were also prepared in three capsule-to-SA ratios (1:1, 1:0.5, and 1:0.25) using the same planetary mill and grinding media. Si:SA slurries were prepared by mixing the respective amount of SA with distilled

water for 15 min at 120 rpm, and adding the amorphous silica stepwise, homogenizing the mixture for 1 h at 180 rpm.

To simplify reading and working, the encapsulated samples have been named with the prefix "ace" (for acetone), followed by the capsule type (Si or Ch) and its respective ratio.

2.2.1.1 Spray drying. Spray drying was carried out on silica and chitosan-encapsulated SA samples, labelled as 'ace Si:SA' and 'ace Ch:SA', respectively. This process was performed using an SD-06 spray dryer (Lab Plant, UK) equipped with a standard 0.5 mm nozzle. The suspensions were atomized by the force of compressed air, resulting in the formation of small droplets. These atomized droplets were then dried by hot air, causing the evaporation of water. The resulting dry powder was gathered in a collection bottle and subsequently stored in plastic bags at room temperature for further characterization. The spray drying process was conducted under the following conditions: i) inlet temperature of 150°C; ii) spray flow rate of 10 mL/min; iii) drying air fan of 80%; and; iv) compressed air pressure of 1.5 bar.

2.2.2. Optimization of milling and spray drying process by experimental design. The most important variables from both milling and spray drying processes were selected, and a multivariate optimization was applied. To determine the optimal conditions for both processes, a fractional factorial design was employed with 6 variables (A, B, C, D, E, F) and 2 levels (-1, +1), resulting in a 2^{6-2} combination, as described in Table 1a. The principal objective of this method was to reduce the extensive number of factorial experiments by eliminating repetitive combinations. To minimize unexpected variability in the observed responses, a total of 16 accurately randomized experiments were conducted, as shown in Table 1b. The selection of variables and levels was based on previous studies conducted by our group ([1,9]). The resulting responses included: i) performance; ii) grinding wear; iii) density; iv) thermogravimetric (mass loss); v) specific surface (Se); vi) moisture; vii) viscosity; viii) efficiency of encapsulation (EE); ix) particle diameter (D_{50}); x) antifungal effect (inhibition rate); and; xi) release rate (kinetic). The procedures for calculating these responses are elucidated in the following sections.

Table 1. Variables studied in the 2^{6-2} factorial fractional design. a) Tested variables with their respective levels (+1, -1). b) Experiments design from 1 to 16 with randomized level combinations for each tested variable**.**

a

	Variables	Level (+1)	Level (-1)
A	Solid content	6.4%	4.0%
B	Milling speed	220 rpm	160 rpm
C	Milling time	80 min	40 min
D	Spray temperature	160°C	130°C
E	Feed rate	12 mL/min	5 mL/min
F	Airflow	90%	70%

b

Experiments	A	B	C	D	E	F
1	-1	-1	-1	-1	-1	-1
2	1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	1
4	1	1	-1	-1	-1	-1
5	-1	-1	1	-1	1	-1
6	1	-1	1	-1	-1	1
7	-1	1	1	-1	-1	1
8	1	1	1	-1	1	-1
9	-1	-1	-1	1	-1	1
10	1	-1	-1	1	1	-1
11	-1	1	-1	1	1	-1
12	1	1	-1	1	-1	1
13	-1	-1	1	1	1	1
14	1	-1	1	1	-1	-1
15	-1	1	1	1	-1	-1
16	1	1	1	1	1	1

2.2.3. Characterization of physical, chemical, and kinetic parameters of encapsulated samples

2.2.3.1. Density. Density was determined using the pycnometer method, with triplicate measurements of the suspension in 25 mL flasks, and the results were reported in g/mL.

2.2.3.2. Viscosity. The rheological behaviour of the slurries was determined by subjecting them to shear stress and measuring shear strain using a Bohlin CVO-120 rheometer equipped with a double gap (DG

40/50) device consisting of two concentric cylinders. The testing procedure commenced with an initial 30-second stirring period to ensure the proper dispersion and homogeneity of the slurries. Subsequently, a logarithmic ramp of shear stress was applied, with twelve pairs of shear rate-shear stress values measured in both the increasing and decreasing directions of the ramp. All measurements were conducted at a constant temperature of 25°C.

2.2.3.3. Performance. The performance parameter refers to the amount of dry powder (in grams) obtained after the spraying process. To calculate the performance metric, the mass of the collected powder is divided by the theoretical solid content of the formulated slurry (in grams), expressed as a percentage.

2.2.3.4. Grinding wear. The grinding wear parameter refers to the wear caused by the alumina balls and the bowl during the grinding process. The method for its calculation involves weighing both the bowl and the alumina balls at the beginning and at the end of the grinding process. Grinding wear was calculated using the following equation:

$$Grinding\ wear = \frac{(initial\ bowl\ weight + initial\ balls\ weight)}{(final\ bowl\ weight + final\ balls\ grinding)} \times 100 \quad (1)$$

2.2.3.5. Moisture. The moisture content of the atomized powder was determined by weight difference. Between 50 to 100 mg of the atomized powder was placed in a drying oven (J.P. SELECTA®) at 100ºC for 24 hours. After drying, the atomized powder was weighed, and the moisture content was calculated using the following equation:

$$Moisture = \frac{(initial\ weight - final\ weight\ )}{final\ weight} \times 100 \quad (2)$$

2.2.3.6. SEM-EDS. Samples were subjected to analysis using an energy-dispersive X-ray microanalysis spectrometer (EDS). Samples were examined and photographed using the backscattered electron and secondary electron signals of a Quattro S field-emission gun environmental scanning electron microscope (FEG-ESEM) (Thermo Fisher). The electron beam interaction volume is on the order of 3 µm or greater, which means that chemical information is also obtained from the surrounding area when examining very small regions. This analysis system can identify elements with an atomic number of 6 or higher (starting from carbon and above).

2.2.3.7. Thermal analysis. Thermogravimetric assays were performed using a Mettler-Toledo TGA/STDA851e model. The equipment recorded simultaneous temperature increases (T), differential thermal curves (DTA), weight losses (TG), and derivative weight loss (DTG), under a dynamic nitrogen

atmosphere. The analysis conditions included a maximum temperature of 1000ºC, a heating rate of 10ºC/min, and an alumina vessel sample holder.

2.2.3.8. Specific surface area. Specific surface area was determined by BET method, using a Tristar 3000 equipment from Micromeritics with the Standard ISO9277:1995. Adsorption isotherm was calculated using the following equation:

$$S_{BET}(m^2 g^{-1}) = n_m \cdot a_{nitrogen} \cdot N_A \quad (3)$$

where n_m represents the molar monolayer capacity, $a_{nitrogen}$ represents the surface occupied area by a single adsorbed gas molecule (0.162 nm^2) and N_A represents the Avogadro's constant.

To determine the monolayer capacity (nm), a linear regression analysis was performed. The slope (a) and intercept (b) values were obtained from the BET equation: $n_m = (1/(a+b))$. The measurement of the adsorbed nitrogen was conducted using a static volumetric method. Before starting experimental procedure, the sample was subjected to a drying process in an oven at 45ºC for 2 hours. Following the drying step, the sample was outgassed with a nitrogen flux at 80ºC for 3 hours.

2.2.3.9. Capsules size distribution. Scanning electron microscopy (SEM) images were utilized to determine the size distribution of the capsules. Image processing and analysis were conducted using the ImageJ software. Four captured images were used to measure approximately 800 particles. The area of each capsule was determined using the "Analyze Particles" function, and spherical shape assumptions were made to calculate their diameter. The size distributions of the capsules were obtained by plotting the accumulated frequency against the corresponding diameter values.

2.2.3.10. Encapsulation efficiency (EE) of encapsulated SA. SA encapsulated samples were weighed (between 5-10 mg), and the inner SA was released by adding 1.5 mL of 0.1 M HCl. The samples were then incubated at room temperature overnight. After the incubation period, the samples were centrifuged at 12500 rpm, and the supernatant was collected for measuring the presence of SA using ultraviolet-visible (UV-vis) spectroscopy (Thermo Spectronic) at 297 nm. The experiment was conducted with three replicates for each sample, and the encapsulation efficiency (EE) was calculated using the following equation:

$$EE\ (\%) = \frac{(Theoretical\ SA\ -\ Determined\ SA\ )}{Theoretical\ SA} \times 100 \quad (4)$$

'Theoretical SA' represents the initial weight of the sample (measured in mg) divided by the theoretical encapsulated SA (%). On the other hand, 'Determined SA' refers to the concentration of SA in the

supernatant (measured in mg/mL) multiplied by the volume of the supernatant (in mL). The SA supernatant concentration was determined by employing a calibration curve for free SA.

2.2.3.11. In-vitro SA release. Each sample weighing 10 mg was mixed with 2 mL of sterile distilled water in a 2 mL Eppendorf tube. The tubes were placed on a magnetic stirrer set at a constant speed of 100 rpm at room temperature. At different time intervals ranging from 0 to 24 hours, 2 mL of supernatant was collected and centrifuged at 12000 rpm for 2 minutes at 4°C for analysis. The supernatant was replaced with an equivalent volume (2 mL of sterile distilled water at each time point, ensuring that the initial volume remained constant. The amount of SA released in the supernatant was measured using UV-visible absorption spectroscopy at 297 nm, following the procedure described in section 2.2.3.10. The mechanism of SA release was evaluated using the Korsmeyer-Peppas model, which is described by the following equation:

$$\frac{M_t}{M_\infty} = k \cdot t^n \tag{5}$$

where M_t represents the amount of SA released at time (t), M_∞ represents the amount of SA released at infinite time, k is the kinetic constant, and n is the release exponent. The n value can be used to determine the release mechanism (Fickian or non-Fickian) of the SA. Based on the mathematical model, different ranges of n value, imply different release mechanisms: i) If n is less than 0.45, it suggests that the system releases the SA through diffusion, following Fick's law (referred to as case I transport); ii) if n is greater than 0.89, it signifies that the release occurs due to relaxation or erosion of the shell (known as case II transport); and; iii) if the value of n is between 0.45 and 0.89, it indicates an anomalous release, where diffusion and relaxation/erosion mechanisms are involved simultaneously.

2.2.4. Determination of antifungal activities. The antifungal activity of the encapsulated samples was determined using the Poison food technique [23]. The experiment was conducted in 9 x 15 cm petri dishes by mixing different concentrations of encapsulated samples (100, 500, and 1000 μM) with Potato dextrose agar (PDA) medium. For each treatment, a 7-day-old mycelial piece (1 x 1 cm) of the respective fungus (see section 2.1.2) was placed in the centre of each dish. The dishes were incubated at 25°C for 10 days, and the radial mycelial growth was recorded on the 3rd, 5th, 7th, and 10th day. Each treatment was performed in quadruplicate. The inhibition rate was calculated by comparing the growth in each treatment dish with the control dish at the 10th day, using the following equation:

$$Inhibition\ rate(\%) = \frac{(Mc - Mt)}{Mc} \times 100 \tag{6}$$

where Mc refers to the mycelial growth in control and Mt refers to the mycelial growth in the treatment.

2.2.5. Software and statistical analysis. The fractional factorial design was conducted using package FrF2 version 2.3-1 of R. The statistical analysis was conducted using SPSS version 21. Turkey-Kramer HSD test was employed with a significance of $p \leq 0.05$, to identify significant differences among treatments. Individual – PCA graphics was constructed using R package factoextra [24].

3. Results and Discussion

3.1. Organic solvent approach

To achieve the initial objective of this study, which involves encapsulating SA using an organic solvent to reduce atomization temperature and assess the potential loss or degradation of SA in the process, a mixture of water and acetone was chosen. In a previous stage, the appropriate grinding speed was also determined to prevent alumina contamination from grinding wear, which can reduce the encapsulation efficiency of SA and/or diminish the biological effect of the encapsulated materials.

3.1.1. Decreasing the rotation speed helps to control alumina contamination

Encapsulation of active molecules by the spray drying technique consists of two stages: first, the formulation and homogenization of the raw materials in the planetary mill to achieve the needed rheological characteristics of the suspension, followed by the atomization in the spray dryer to obtain a dry powder [25]. The milling process affects the rheological characteristics of the slurry, not only due to the degree of homogenization achieved but also because the grinding wear produced during the process. Grinding wear, in our case, refers to the detachment of alumina particles due to the tapping between the alumina balls and the bowl during the homogenization of the slurry [26].

The formulation of Ch:SA samples involves multiple steps, compared to silica-encapsulated SA. These additional steps can complicate the encapsulation process, potentially leading to increased alumina wear and subsequently affecting the spray drying process. Furthermore, the introduction of additional steps can result in increased energy consumption, leading to process inefficiency. For these reasons, chitosan-encapsulated SA was selected for slurry formulation optimization.

As an initial stage, various ratios of acetone to water were tested to formulate a slurry without solid waste (solid residues resulting from poor homogenization at the bottom of the bowl). The testing conditions are outlined in Table 2a, with the best results achieved at a 1:1 ratio of acetone to water, resulting in zero solid waste and a density of 0.968 g/mL.

Various rotational speeds (rpms) were tested for the Ch:SA (1:1) formulation (see section 2.2.1), and three parameters were analyzed to assess the characteristics of the formulations: i) density, ii) solid waste, and iii) grinding wear (Table 2b). Density values remained consistent, averaging around 0.9 g/mL for most testing conditions. However, the combination of the lowest rates (75/100 rpm) exhibited

a lower density of 0.791 g/mL, likely due to poor dispersion of the raw materials in the solvent. A low density suggests that the rotation speed fell below the critical speed, directly impacting the suspension homogeneity [27].

Table 2. Acetone optimization results. a) The best solvent ratio and speed milling conditions for SA encapsulation are highlighted in green. b) The best speed milling conditions for decreasing grinding wear are highlighted in blue.

a

Samples w/w ratio	v/v% solvent ratio (acetone/water)	Density (g/mL)	Solid waste (g)	Milling conditions	
Ch:SA (1:1)	100	1.739	5.4	Vo: 150 rpm	Vf: 210 rpm
Ch:SA (1:1)	75/25	0.890	3.4	Vo: 150 rpm	Vf: 210 rpm
Ch:SA (1:1)	50/50	0.968	0	Vo: 150 rpm	Vf: 210 rpm

b

Samples w/w ratio	v/v% solvent ratio (acetone/water)	Vo	Vf	Density (g/mL)	Solid waste (g)	Grinding wear (%)
Ch:SA (1:1)	50/50	75 rpm	100 rpm	0.791	2.74	20.10
Ch:SA (1:1)	50/50	100 rpm	150 rpm	0.953	0.89	28.13
Ch:SA (1:1)	50/50	100 rpm	130 rpm	0.897	1.78	26.21
Ch:SA (1:1)	50/50	120 rpm	125 rpm	0.833	1.34	25.11
Ch:SA (1:1)	50/50	120 rpm	160 rpm	0.963	0	30.37
Ch:SA (1:1)	50/50	120 rpm	180 rpm	0.983	0	42.59
Ch:SA (1:1)	50/50	150 rpm	210 rpm	1.053	0	58.27

In the slurry production process, the rotation speed produces a large centrifugal force at the highest rates. Consequently, high speed maintains a favourable grinding rate, controlling particle dispersion and promoting adequate homogenization, but it also increases energy consumption [28]. The rotation speed must be high enough to support the homogenization process while preventing the formation of solid waste. However, it should be low enough to avoid excessive wear of the grinding media [29] and to minimize any unnecessary increase in energy consumption.

The results showed that speeds ≤150 rpm were insufficient to homogenize the raw materials adequately, leading to the presence of sedimented material at the end of the grinding process. Optimal conditions were determined based on the percentage of residual alumina. Values exceeding 35% were considered unacceptable because a high alumina content could compete with SA for space within the capsules,

potentially affecting the encapsulation process. Ultimately, the best formulation was achieved with milling speed combinations of Vo/Vf at 120/160 rpm. This formulation exhibited a residual alumina content of around 30%, a density of 0.963 g/mL, and no solid waste (Table 2b).

3.1.2. Acetone as a versatile solvent for spray-drying optimization

After establishing the optimal milling conditions for Ch:SA samples, Si:SA and Ch:SA samples were prepared using acetone, as described in section 2.2.1, to lower the atomization temperature and explore potential SA degradation during spray drying at temperatures above 100°C. These slurries were then subjected to spray drying, utilizing the process variable values shown in Table 3, which yielded the parameter results presented in the same table.

Acetone lower boiling point allows for a reduced spraying temperature (100°C) [30]. This temperature reduction not only decreases energy consumption but also mitigates the risk of overheating and material degradation. The solvent evaporation rate plays a vital role in the spray drying process, impacting both process variables and the final product characteristics [31]. It is important to consider that atomizing slurries previously formulated with acetone carries the risk of fire, explosions, and health problems. This issue can be effectively controlled by using an acetone/water mixture, which reduces the evaporation rate and improves work security during the atomization process.

Generally, silica-encapsulated SA samples prepared with acetone exhibit better performance values (ranging from 41.6% to 59.8%) when compared to chitosan-encapsulated SA samples (ranging from 17.8% to 24.4%) due to the higher solid content of the slurries. This trend is similar to that observed in silica/chitosan-encapsulated SA samples previously prepared in water (refer to Table 3). However, there is a performance disparity between the two solvents, with a more pronounced effect observed in chitosan, which is attributed to the higher speeds employed in the water-based solvent samples. In the case of chitosan samples, potential partial solubility during the grinding process should also be considered.

The performance of these samples is influenced by the rheological behavior of the slurries [32]. However, when comparing the viscosity of samples prepared in water and acetone at different ratios (as shown in Table 3), no significant differences are observed, with values ranging from 0.006 to 0.014 Pa*s. Additionally, the density values fall within the range of 0.96 to 1.07 g/mL. For ace-Si:SA samples, the performance percentage remains consistent within the customary production range when water is used as the solvent, displaying minimal variation and modest loss of raw materials. The introduction of an organic solvent in the formulation of the slurries did not alter their rheology, making them suitable for the spray-drying process and very similar to the previous water-based formulations [1].

Table 3. Comparison of SA spraying variables and encapsulated SA parameters using water or acetone as solvent**.**

Variables	Si:SA			ace-Si:SA			Ch:SA			ace-Ch:SA		
	1:1	1:0.5	1:0.25	1:1	1:0.5	1:0.25	1:1.25	1:1	1:0.5	1:1.25	1:1	1:0.5
Spray Drying												
Aspirator flow (%)	80	80	80	80	80	80	80	80	80	80	80	80
Spray gas flow pressure (bar)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Slurry flow rate (%)	10	10	10	10	10	10	10	10	10	10	10	10
Inlet temperature (°C)	150	150	150	100	100	100	150	150	150	100	100	100
Outlet temperature (°C)	82	82	83	54	54	54	83	82	80	56	57	53
Encapsulated SA simples parameters												
Density (g/mL)	1.05	1.06	1.07	0.99	0.99	0.99	1.08	1.07	1.07	0.98	0.97	0.96
Viscosity (Pa*s)	0.009	0.013	0.014	0.007	0.010	0.013	0.007	0.006	0.005	0.008	0.007	0.006
Performance (%)	47.6	63.0	79.4	41.6	49.1	59.8	35.7	70.1	74.7	17.8	20.6	24.4
Moisture (%)	53.9	7.7	12.5	65.2	61.1	69.4	15.9	5.5	6.5	15.9	15.9	7.1
S_{BET} (m^2/g)	60	74	83	47	66	82	2.3	2.1	1.8	3.0	3.3	3.7
D_{50} (µm)	9.90	9.57	11.2	11.1	13.7	15.3	8.55	7.30	7.53	11.2	12.4	11.3
EE (%)	63.6	69.4	52.7	47.1	45.2	52.1	46.6	49.6	43.9	54.2	44.7	45.2
Kinetic (h^{-n})	0.70	0.59	0.28	0.97	0.89	0.40	0.66	0.48	0.31	0.79	0.41	0.26
Release exponent (n)	1.66	1.26	1.16	1,16	0.94	0.98	1.57	1.52	1.22	1.48	1.38	1.21

In contrast, the performance of ace-Ch:SA samples is affected by the low solid content (ranging from 2.8% to 3.6%) compared to the 13.6% solid content in ace-Si:SA samples. By decreasing grinding wear and, in conjunction with spraying temperatures below 140°C, two phenomena occur: i) a decrease in the evaporation capacity, resulting in the atomized encapsulates retaining significant humidity [33]; and ii) an increase of moisture in the chamber base, leading to the adherence of wet particles to the chamber walls [34]. The higher performance values in Ch:SA samples can also be attributed to the higher grinding speed used compared to ace-Ch:SA samples, with rates of 57-50% versus 28-32%, respectively. This suggests that by reducing the grinding speed, the performance aligns more with the real scenario where the interference of grinding waste is minimized.

In the comparison between ratios, the performance of silica/chitosan-encapsulated SA samples prepared using acetone exhibit a trend in which performance improves as the encapsulation ratio decreases. When compared to encapsulated samples prepared in water, the performance behavior is similar (as shown in Table 3). This suggests that a smaller amount of SA can be homogenized more effectively with the capsules, becoming trapped inside without saturating them, as previous indicated in our findings [1].

The high moisture values (between 61.1-69.4%) of the ace-Si:SA samples (refer to Table 3) can be attributed to high S_{BET} values, which generate create a larger surface area capable of retaining more water [35]. The ace-Si:SA samples showed S_{BET} values from 47 to 88 m^2/g, while ace-Ch:SA samples showed lower moisture values (between 7.1-15.9%) due to low S_{BET} values (from 3.0 to 3.7 m^2/g). In general, the acetone/water combination increases the dissolvent evaporation rate. This combined with a large surface area where the solvent can be trapped, results in powders with high humidity [36].

In terms of the size and morphology of the encapsulates, silica-encapsulated SA samples, prepared in both water and acetone, exhibit a similar spherical shape (see Supplementary Figure 1) with D_{50} values ranging from 9.90 µm to 15.3 µm (refer to Table 3). In contrast, chitosan-encapsulated SA samples exhibit an irregular geometry, much more pronounced in the case of samples prepared in water (Supplementary Figure 2) and with a D_{50} values ranging from 7.5 µm to 11.3 µm (Table 3). The morphological difference between Ch:SA and ace-Ch:SA samples could be attributed to the potential dissolution in the acetone/water solution and its subsequent crystallization during atomization [36].

The SEM/EDS results for the silica-encapsulated samples (Figure 1) revealed the presence of three main components in both samples, water-based (Si:SA) and organic solvent (ace-Si:SA): carbon, oxygen, and silicon. While oxygen is present in both the capsule and the encapsulated material, carbon is unique to the SA molecule and silicon is associated with the silica component. SA can be readily identified on the surface of the spherical particles, both visually and analytically. Visual identification can be achieved by comparing the ‘Dark’ SEM micrograph with the ‘Light’ one, corresponding to loaded and unloaded capsules, respectively. Analytically, the higher carbon content detected by EDS analysis on the surface of the loaded silica particles (‘Dark’ in Figure 1) can be attributed solely to the SA molecule. SEM results demonstrated that the use of an organic solvent did not lead to any differences in chemical composition, but it did result in a slightly porous surface morphology, possibly related to the higher evaporation rate.

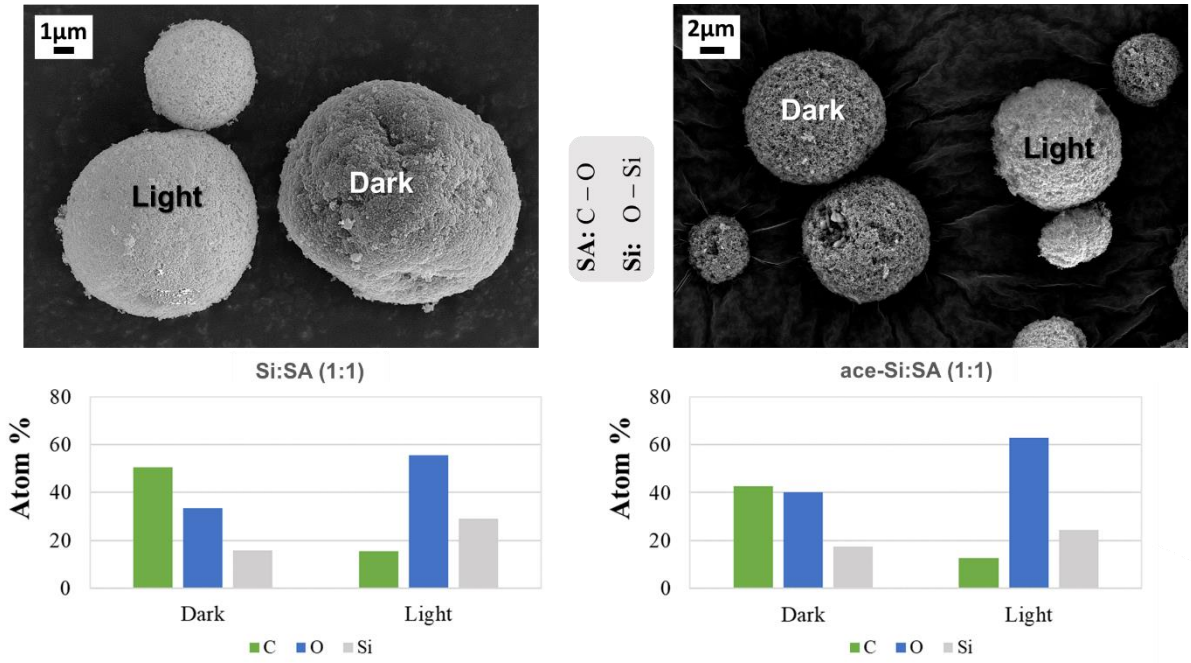

Figure 1. Semi-quantitative Energy Dispersive X-ray (EDS) microanalysis of the silica-encapsulated SA samples.

The SEM/EDS analysis of chitosan-encapsulated SA samples, both with (ace-Ch:SA) and without the addition of acetone (Ch:SA), revealed the presence of five main components: carbon, oxygen, nitrogen, phosphorus, and sodium (Figure 2). While carbon and oxygen are found in both the capsule and the encapsulated material, nitrogen, phosphorus and sodium are exclusively present in the capsule, either in the chitosan or in the TPP-Na cross-linking material. This lack of a distinctive chemical element between the capsule and the encapsulated material made interpretation more challenging than with previous silica-encapsulated SA samples. The analysis identified an unusual particle with a smoother surface (marked as 1 in both SEM micrographs in Figure 2), which differed from particles labeled as 2 to 4. As revealed by EDS, this isolated particle had a different chemical composition with lower levels of nitrogen, phosphorus, and sodium, suggesting that it might correspond to free SA that was not encapsulated during the process [37].

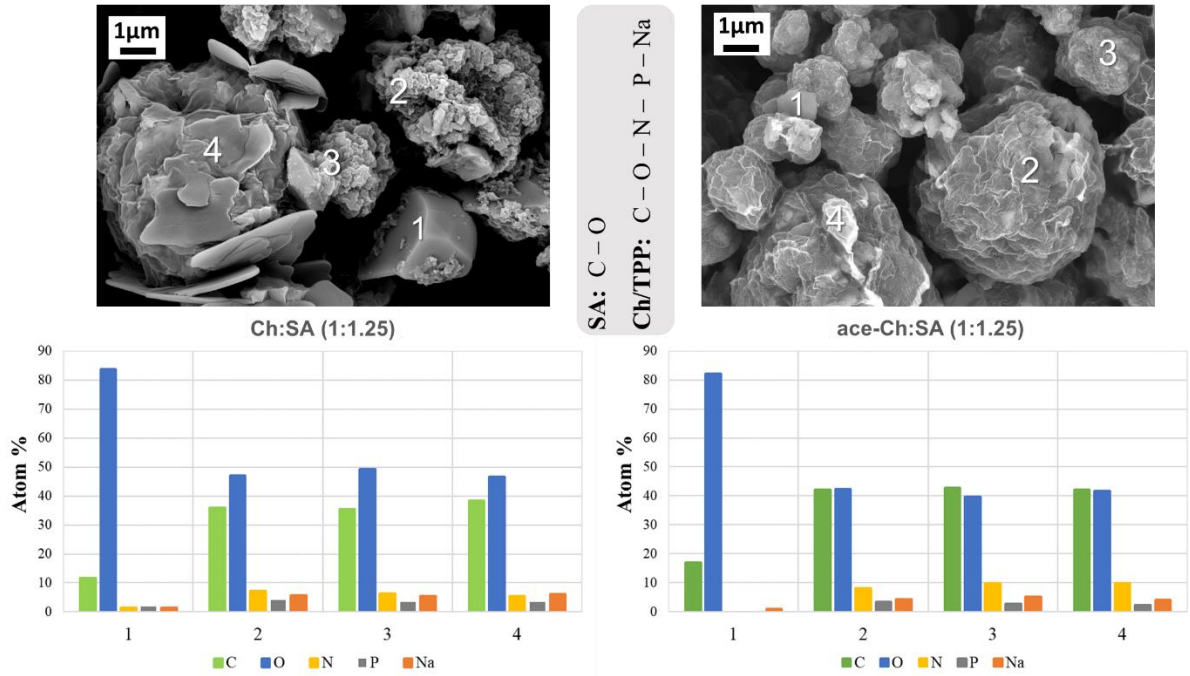

Figure 2. Semi-quantitative Energy Dispersive X-ray (EDS) microanalysis of the chitosan-encapsulated SA samples.

Further EDS analysis of particles marked 2 to 4, regardless of the introduction of acetone, revealed higher content of nitrogen, phosphorus, and sodium, consistent with the TPP-Na crosslinked chitosan. High phosphorus and sodium content may indicate a less effective encapsulation process. Complete entrapment of SA within chitosan particles might limit space for TPP cross-linking with chitosan chains compared to unloaded chitosan cross-linked particles. Higher cross-linking density in unloaded particles leads to higher phosphorus content (approximately 50% based on previous findings [38]). Our samples, with low P and Na contents (below 10%), align with literature data [38], suggesting successful SA entrapment within chitosan particles. The results also demonstrate that salicylic acid (SA) is consistently encapsulated, irrespective of the solvent used, effectively entrapped within the chitosan capsule during its formation. Similar to the silica-encapsulated samples, the surface morphology undergoes changes upon the introduction of the organic solvent, likely due to different evaporation rates and partial solubilization/crystallization of the chitosan.

Pure silica exhibits high thermal stability, with a maximum mass loss of approximately 5%. Similarly, pure salicylic acid (SA) experiences complete endothermic decomposition around 400°C. In the case of pure chitosan, two distinct thermal decomposition stages are evident: the first at 90°C, attributed to moisture evaporation, and the second at 313°C, associated with dehydration and material decomposition [1].

Encapsulated samples, whether prepared with silica (ace Si:SA) or chitosan (ace Ch:SA), exhibit noteworthy thermal behavior (see Supplementary Figure 3). Silica-encapsulated SA samples undergo thermal degradation in a single stage, primarily due to SA decomposition, resulting in higher mass loss for samples with the highest capsule:SA ratio (ace Si:SA (1:1)). Notably, the complete decomposition of silica-encapsulated SA samples occurs at higher temperatures and a slower rate compared to free SA, indicating enhanced thermal stability through encapsulation.

Chitosan-encapsulated samples exhibit two distinctive peaks around 200°C and 400°C, corresponding to the loss of free SA and encapsulated SA, respectively. This observation aligns with our previous studies [1,37,39], indicating successful SA loading into chitosan particles. Significantly, encapsulated SA undergoes decomposition at higher temperatures than free SA, highlighting the improved thermal stability achieved through encapsulation.

Upon comparing samples formulated in both water and acetone, for both silica and chitosan, no significant differences in mass loss are observed. This consistent trend suggests that encapsulation is equally effective in both water and acetone. When comparing the samples formulated in both water and acetone, for both silica and chitosan, it is observed that there are no significant differences in mass loss. This consistent trend indicates that encapsulation occurs equally effectively in both water and acetone.

3.1.3. Acetone does not alter the SA kinetic and antifungal properties

Analysis of kinetic rates revealed a reduction in the release rate as the encapsulation ratio decreases (see Table 3), with values of 0.40 and 0.26 h^{-1} for ace-Si:SA (1:0.25) and ace-Ch:SA (1:0.5), respectively. However, the same release mechanism (case II transport), associated with the relaxation or erosion of the shell, was observed for all ratios ($n>0.89$). These findings are in accordance with studies on water-produced SA-encapsulated samples [1] and other literature [40], where SA exhibited a slow release at the lowest ratio for both capsules [1,40].

Previous studies have demonstrated the antifungal effects of SA, and its encapsulation in both silica and chitosan further enhances this effect at the lowest ratios of 1:0.25 and 1:0.5, respectively [1]. Figure 3 illustrates the inhibition rates at the 10th day for *A. alternata*, *F. oxysporum*, and *P. digitatum* when treated with free-SA and SA-encapsulated samples, formulated in both water and acetone at 1000 µM. Notably, *A. alternata* and *P. digitatum* exhibited the highest inhibition rates among these fungi (refer to Supplementary Figure 4, and Supplementary Figure 5, respectively).

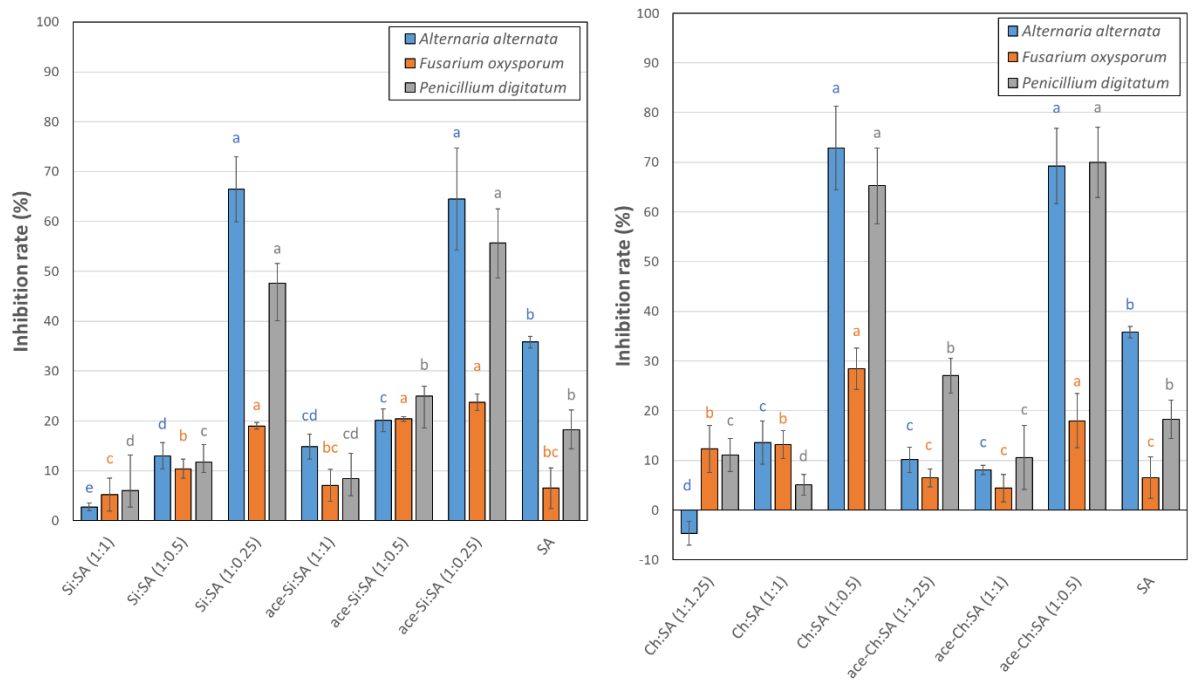

Figure 3. Fungal inhibition rates of the silica/quitosan-encapsulated SA samples at 1000 µM. Statistics analysis was performed by comparing all samples for each fungus.

Si:SA and ace-Si:SA samples at the lowest ratio exhibited similar inhibition rates, approximately 65% for *A. alternata* and 50% for *P. digitatum*. Similarly, Ch:SA and ace Ch:SA samples at the lowest ratio produced similar inhibition rates, around 70% for *A. alternata* and 65% for *P. digitatum*. In contrast, for *F. oxysporum*, all SA-encapsulated samples at the lowest ratios displayed a limited antifungal effect, with an average value of approximately 20% (see Figure 3, and Supplementary Figure 6). This difference could be attributed to the fact that SA tends to impact necrotrophic fungi growth more efficiently than hemibiotrophic fungi [41].

The absence of differences observed between treatments with encapsulated samples performed in water vs acetone on fungal growth can be attributed to similar characteristics during slurry formulation, including homogenization, dispersion and encapsulation behaviours. Notably, the addition of acetone results in a consistent and homogenous solution, with no changes in the desired SA characteristics [42]. Once again, significant antifungal properties were observed at the lowest ratios, suggesting the successful encapsulation of the optimal amount of SA without saturating the capsules.

SA significantly influences fungal growth. Both silica and chitosan SA-encapsulated samples, formulated in both acetone and water, displayed a robust antifungal capacity, particularly effective

against necrotrophic fungi, representing the most significant challenges in agriculture [43]. No discernible effects on mycelial growth were observed in the three fungi treated with the empty Si and Ch capsules (Supplementary Figure 7).

In conclusion, upon careful comparison with previous experiments, there appears to be no justification for incorporating acetone in the formulation of encapsulated SA. Acetone neither modifies the characteristics of the SA encapsulation process nor enhances its biological activity. On the contrary, the use of acetone could pose numerous disadvantages from environmental, economic, and industrial safety perspectives.

3.2. Optimization of the encapsulation process

The comparison between the encapsulated samples formulated in water vs acetone showed no differences, suggesting that the atomization temperature does not affect the structure of the SA and, therefore, does not alter its properties. Once it was proven that it is not necessary to use another solvent, the spray drying conditions were optimized by changing the values of the most influential variables of the whole process.

3.2.1. Fractional factorial design as a potent tool for optimization of the SA encapsulation

Fractional factorial design is a method employed to analyse the effects of multiple factors and their combinations, limiting the experiments to only a fraction of all possible ones [44]. This statistical method is useful when the experimental conditions are extremely large, reducing the required resources and saving time and costs [45]. Fractional factorial designs at 2-levels or 3-levels are frequently employed in agricultural experiments [46] and industrial settings [47] to determine the effects of different factors in a process. The fractional factorial design at 2-levels is particularly advantageous during the initial phases of experimental research [48,49], especially when there are no more than 6 process parameters or design factors.

To optimize SA encapsulation, a randomized fractional factorial design with 2-levels was selected as the preferred approach. This decision was driven by the numerous critical factors involved in the preparation of encapsulated SA samples. Process variables for the design were selected from both the milling and the spray drying processes, acknowledging their significant impact on the ultimate quality characteristics of the encapsulated products.

During the milling process, one of the most important considerations is the friction generated by ball impacts. This friction is directly related to the milling speed and milling time of grinding operation. Prolonged high-speed rates can lead to increase wear on the balls and bowls within the planetary mill, while low-speed rates may result in balls sliding without achieving proper slurry homogenization [50].

Furthermore, extended impacts can disrupt correct homogenization of the core and shell materials, potentially causing agglomeration and impacting the subsequent spray drying process [51]. In the context of the spray drying process, both inlet temperature and slurry flow rate are critical factors that determine proper powder drying and exert significant influence over particle size and morphology [52]. Also, optimal drying conditions and control over particle size are guaranteed by accurately setting the aspiration flow [53]. An overarching factor that affects both processes is the solid content, wherein any increase or decrease has a direct impact on feed formulation and collected powder.

In our research, the FrF2 package provided us with randomly selected conditions (Table 1b), and the trials were conducted in a completely random order. The six principal process variables previously described in point 2.2.2 were evaluated, and the response results are shown in Table 4.

Table 4. Parameters obtained for SA encapsulation optimization through the fractional factorial design.

Experiment (nº)	Performance (%)	Grinding_Wear (%)	Density (g/mL)	Mass_Loss (%)	S_{BET} (m^2/g)	Moisture (%)	Viscosity (Pa*s)		EE (%)	D_{50} (µm)	Inhibition_Rate (%)	Kinetic (h^{-n})
							A	B				
1	29.39	35.18	1.01	16.81	56	33.33	0.0541	0.0049	25.5	19.9	20.1	0.58
2	22.34	19.69	1.02	18.71	68	59.09	0.0402	0.0055	32.2	18.4	17.6	0.48
3	36.44	57.73	1.01	17.32	45	22.22	0.0185	0.0051	31.6	17.5	10.1	0.52
4	33.31	38.01	1.02	21.26	54	19.88	0.0099	0.0055	35.2	17.2	23.5	0.93
5	41.42	57.56	1.01	16.17	45	23.63	0.0582	0.0048	31.7	17.1	16.9	0.54
6	37.39	37.99	1.02	16.78	60	50.00	0.0346	0.0055	28.0	26.7	8.7	0.55
7	67.72	94.00	1.03	15.74	32	13.33	0.1405	0.0052	25.3	18.5	13.0	0.93
8	33.46	64.79	1.03	25.10	37	27.88	0.0113	0.0057	53.6	18.9	58.4	0.30
9	32.82	39.86	1.01	10.43	59	41.66	0.0502	0.0049	20.6	18.6	18.9	0.60
10	25.16	17.05	1.02	21.61	65	57.50	0.0886	0.0055	53.5	19.5	13.9	0.88
11	45.13	62.98	1.02	15.02	43	9.99	0.1126	0.0042	30.9	18.7	19.4	0.60
12	36.49	39.85	1.02	10.72	61	9.99	0.0542	0.0056	29.2	19.9	15.6	0.50
13	42.33	56.63	1.01	12.93	46	62.50	0.0386	0.0049	29.9	21.3	10.9	1.00
14	33.67	31.19	1.02	16.03	62	33.03	0.0648	0.0057	37.3	19.6	14.0	0.53
15	59.99	96.71	1.02	15.18	30	19.84	0.0254	0.0052	28.6	19.2	14.3	0.39
16	42.79	61.84	1.03	14.79	44	30.00	0.0592	0.0064	33.7	19.5	12.3	0.85

3.2.2. The parameters of the SA encapsulated samples are interrelated

The assessed variables play a crucial role in determining the parameters of encapsulated salicylic acid (SA). The results in Table 4 evidence that the density of the 16 encapsulated samples did not exhibit any significant differences. The viscosity at point B showed no discernible variations, while differences were observed at point A (refer to Supplementary Figure 8). The discrepancy in viscosity for the resting suspensions can be attributed to the solid content and milling speed used for the slurry formulation. Suspensions with high solid content showed elevated viscosity values at point A (Experiments 7 and 11), suggesting the presence of an inter-particle network [54,55]. The remaining slurries exhibited viscosity values at point A that were relatively similar (Table 4).

To ensure the optimal execution of the spray drying process, it is crucial to assess the rheological behaviour of the formulations, as this will influence the atomization process involving the passage of the feed solution through the needles of the sprayer. A solution with high viscosity tends to create an encapsulated sample with thicker walls, affecting the spraying process and the thermal properties of the obtained powder [56]. However, the viscosity values obtained at point A did not influence the process after the fractional analysis, being this consistent with the easy atomization of all slurries.

The particle diameter is directly influenced by the viscosity of the formulation. Higher viscosity can hinder the proper dispersion of particles in the suspension, leading to agglomeration or uneven distribution [50]. Additionally, these formulations demonstrated effective particle dispersion and maintained stable slurries, even when the solid content percentages were at their lowest, rendering them suitable for the spray drying process [1]. SEM images were acquired to analyze the morphology and particle size distribution of the encapsulated samples—factors that can profoundly influence the outcomes of experimental biological assays and particle toxicity [57].

In terms of morphology, a consistent doughnut shape was observed in all cases. This characteristic is primarily influenced by flow rate, temperature process conditions, and the potential addition of surfactants [53,54], (see Figure 4). The equivalent diameter of the average particle size (D_{50}) was calculated based on the granulometric curves (Supplementary Figure 9), revealing values within the range of 17 to 26 μm, with no significant differences noted. This correlation aligns with the viscosity results obtained under spray conditions (point B), as similar slurry viscosities lead to comparable droplet sizes when the feed passes through the nozzle during spraying, resulting in similar particle sizes [30]. These findings underscore that the atomization process consistently yields particles that exhibit homogeneity in both size and shape.

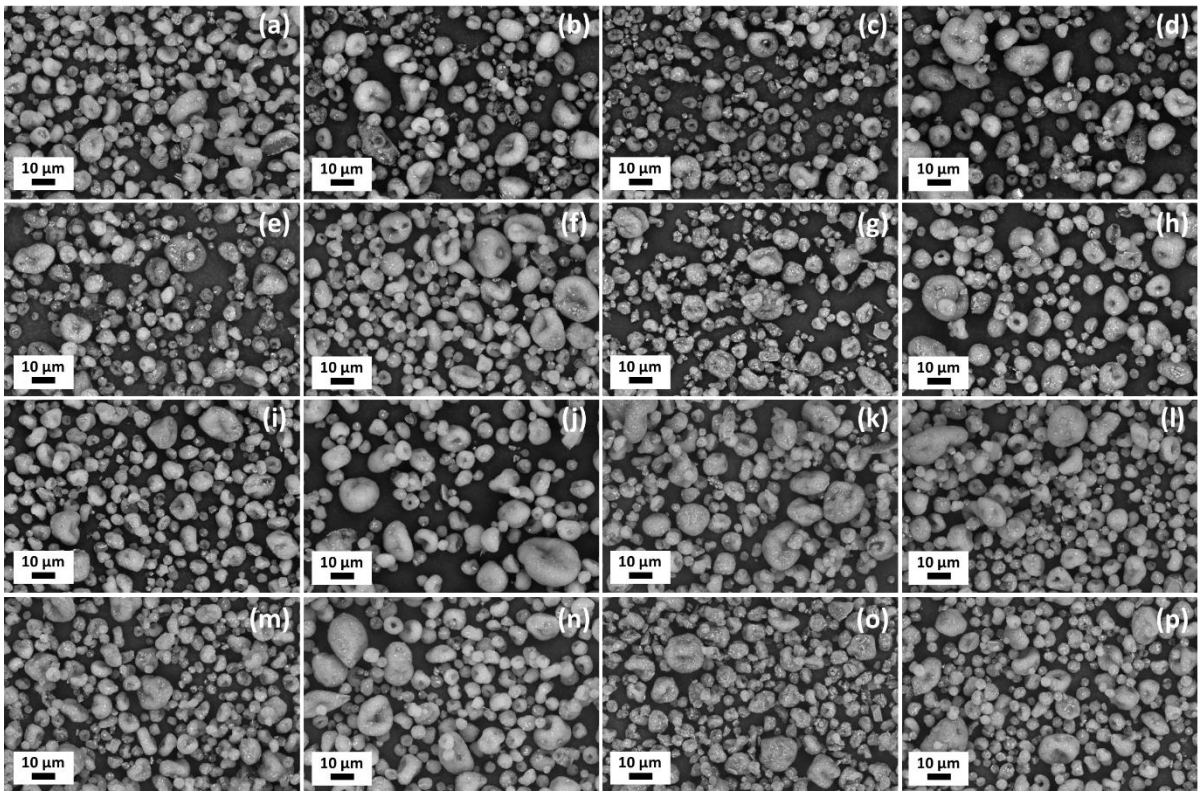

Figure 4. Scanning electron microscopy (SEM) images of the 16 silica-encapsulated SA samples prepared in water at (1:1) ratio. Images were taken at 1000x magnification. Figures a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, correspond to experiments from 1 to 16, respectively.

Performance and grinding wear exhibit a close correlation, suggesting that enhanced performance may be associated with substantial wear. The wear material appears to adhere to the walls of the encapsulates or becomes trapped within the capsules along with the SA, potentially contributing to improved performance. This non-formulated raw material might also result in a diminished mass loss when the samples are exposed to heat, owing to the well-established high thermal properties of alumina [58]. Furthermore, variations in specific surface area (S_{BET}) can be linked to the alumina content. Increased alumina contamination is observed to reduce S_{BET}, subsequently leading to a decrease in moisture percentage, as evidenced in Experiments 7 and 15 (Table 4). This reduction in moisture content may be attributed to the alumina encapsulated in the silica, which diminishes the surface area, thereby providing less space for water retention [59].

In terms of production yield and economic considerations, the paramount response is performance, as it directly correlates with the quantity of powder collected after the drying process [34]. Powder loss typically occurs when the drying process is incomplete, leading to powder adherence to the walls of the drying chamber. The significance of these performance values lies in their ability to provide insights

into the efficiency of silica-alumina (SA) encapsulation. Our findings reveal a spectrum of performance values ranging from 20% to 68%. A high-performance value is often associated with a heightened alumina content produced through grinding wear. Conversely, elevated alumina values correspond to a lower encapsulation percentage, suggesting that alumina may be encapsulating instead of the intended SA. Understanding these performance metrics is crucial for optimizing production yield and aligning with economic considerations.

Encapsulation efficiency (EE) stands as a pivotal output, serving as a crucial indicator of the accurate encapsulation of bioactive compounds. In Table 4, Experiments 8 and 10 stand out with highlighted values of 53.6% and 53.5%, respectively, marking them as the most successful among the 16 conducted experiments. Achieving a commendable EE necessitates elevating the inlet temperature to facilitate the rapid drying of the encapsulated molecule, thereby preventing its adhesion to the chamber [56]. Moreover, EE is influenced by both the amount of SA and alumina contamination. The alumina content impacts SA encapsulation by occupying the inner space of the silica. A noteworthy distinction in kinetic parameters is evident when comparing Experiments 8 and 10, with Experiment 8 exhibiting the best kinetic parameter value (0.30 h^{-1}) compared to Experiment 10 and other experiments (see Supplementary Figure 10). Although both experiments yield similar EE values, the variance in kinetic release can be attributed to a higher proportion of alumina encapsulation rather than SA, resulting in a rapid SA release (0.88 h^{-1}). This, in turn, leads to a rapid release of SA, diminishing its antifungal effectiveness.

In the assessment of antifungal activity, once again, Experiment 8 demonstrated superior results, yielding an inhibition rate of 58.4% against *Penicillium digitatum*. The heightened efficacy observed in these results appears to be intricately linked to the controlled release facilitated by encapsulation. This controlled release mechanism enhances the inhibition of mycelial growth, ensuring a sustained and prolonged impact on the fungi over time [60]. Collectively, these findings unequivocally position Experiment 8 as the optimal choice for a genuine biological application, as illustrated in Figure 5.

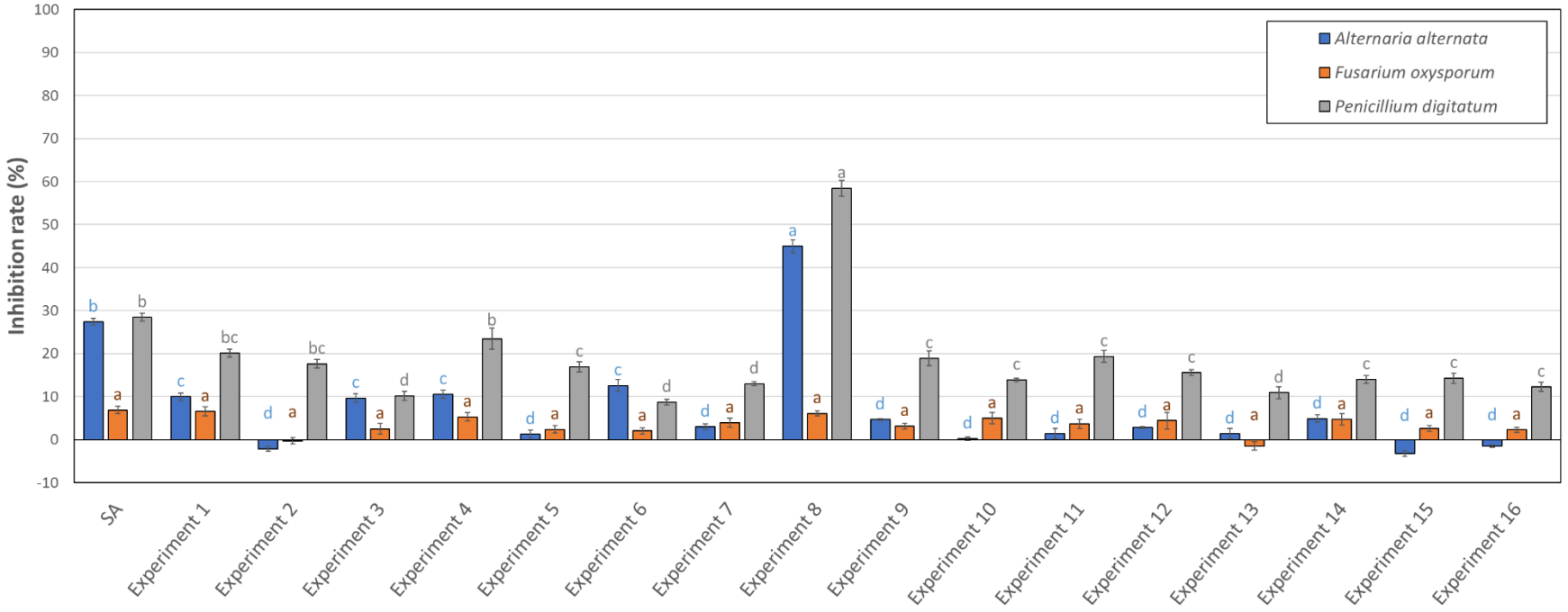

Figure 5. Fungal inhibition rates of the 16 experiments. Statistics analysis was performed by comparing all samples for each fungus.

3.2.3. Optimization of the process variables enhances the final encapsulated SA properties

Once the interrelations between the responses were elucidated, the next step involved identifying the process variables with the most significant impact on the overall encapsulation process for optimization. Employing the FrF2 package, main effect plots (as the ones shown in Figure 6) and normal plots (Supplementary Figure 11) for each response were generated. Normal plot or normal probability plot allows to assess magnitude, direction, and significance of the effects. In this plot, effects that deviate significantly from 0 are considered statistically significant [61]. The main effect plot aids in comprehending the parameters that exert the most influence on each response at every tested level [62]. Figure 6 illustrates the impact of each process variable on the studied responses, with solid content standing out among 7 out of the 12 responses: performance, grinding wear, mass loss, viscosity, density, average capsule size (D_{50}), S_{BET}, and EE.

Upon individual analysis of each graph, it becomes evident that certain studied variables under study, such as inlet temperature and slurry flow rate, exhibit minimal to no impact on moisture, viscosity, density, D_{50}, S_{BET}, kinetic rate, and inhibition rate (Supplementary Figure 11). Notably, in the case of viscosity (Figure 6g) and moisture (Figure 6f.), only two variables had a discernible effect: milling speed and solid content, respectively.

The influence of solid content on rheological behaviour is particularly noteworthy. It operates by reducing flow resistance, attributed to the diminished internal friction between particles, thereby enabling free movement [63].

The milling speed influences grinding wear, leading to significant alumina contamination, particularly at high speeds. The escalation of solid content, driven by alumina, directly impacts S_{BET} (Figure 6e). This influence stems from its interference with SA encapsulation, resulting in a subsequent reduction in the available surface area for contact [35]. SA may be absorbed into the alumina [58], fostering a interaction between the carboxylic group of SA and the hydroxyl group of alumina [64]. This phenomenon finds support in Experiments 7 and 15, wherein elevated grinding wear values of 94.00% and 96.71%, respectively, substantially diminish the S_{BET} to 32 and 30 (m^2/g) (Table 4).

The reduction in S_{BET} subsequently contributes to a decrease in powder moisture. A study on water absorption ability reported that up to 70% of water can be adsorbed on the alumina surface [59]. Consequently, with a diminished free surface, less water becomes trapped, facilitating a more efficient drying process. Once more, Experiments 7 and 15 substantiate this correlation, demonstrating that low values of S_{BET} – 32 and 30 (m^2/g), respectively – are associated with reduced moisture levels of 13.33% and 19.84% (Table 4).

Performance (Figure 6a), grinding wear (Figure 6b), and density (Figure 6c) were mainly affected by three variables: i) solid content, ii) milling speed, and iii) milling time. A lower solid content during

high-speed and prolonged milling intensifies the internal impact between the grinding media and the bowls walls, leading to increased alumina content through abrasive wear. Mass loss (Figure 6d) is influenced by solid content and aspiration flow. The decrease in solid content reduces slurry viscosity, subsequently lowering the required aspiration flow rate to break down the feed into small droplets [65].

The inlet temperature is intricately linked to mass loss, particularly at higher spray temperatures, where inadequate encapsulation renders the bioactive molecule more susceptible to degradation. As depicted in Supplementary Figure 12, Experiments 9 and 12 exhibited the lowest mass loss values at 400°C, and consistently maintained the lowest values even at 1000°C, outperforming the other experiments. Conversely, Experiments 8 and 10 displayed the highest mass loss values at 400°C, with degradation persisting until 1000°C (Supplementary Figure 13). The progressive and continuous degradation observed beyond 400ºC can be attributed to superior encapsulation and enhanced thermal stability, as the protective shell shields the SA when exposed to elevated temperatures [37]. Conversely, lower mass loss after 400ºC may be indicative of poor encapsulation, leading to the decomposition of most SA at 400°C.

Considering that silica experiences almost no mass loss and SA completely decomposes at 400°C, several insights can be drawn from Supplementary Figure 13. i. The observed misalignment between the blue and grey bars across all experiments suggests that mass loss persists at temperatures beyond 400°C, providing clear evidence of the SA encapsulation process. ii. Given that all samples are prepared with equal SA content, those exhibiting the highest total loss (grey bar) are indicative of the least SA loss during atomization, signifying a more efficient encapsulation process. This heightened efficiency may be attributed to factors such as reduced alumina content from grinding (Experiment 10), which could interfere with the process, or a lower drying temperature (Experiments 4 and 8). iii. The greater the disparity between the grey and blue bars, the more efficient the encapsulation process. In this regard, Experiment 8 not only allowed for the encapsulation of the highest amount of SA but also did so in the most efficient manner.

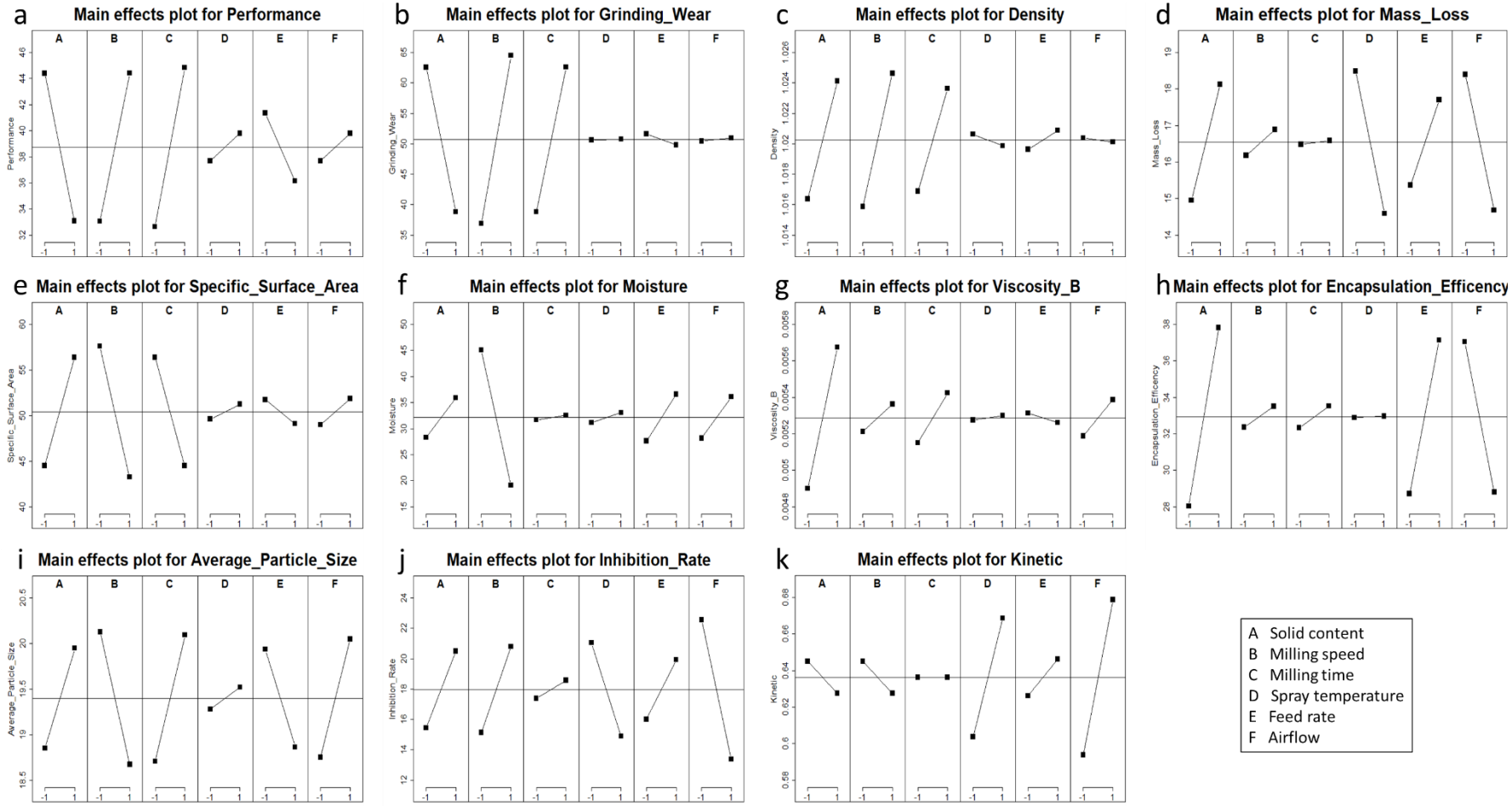

Figure 6. Main effect plots for experimental responses.

As shown in section 3.2.2, the D_{50} (Figure 6i) exhibited no differences among samples, and the tested variables showed no influence on its distribution. This consistency is attributed to the effective dispersion and homogenization achieved under all conditions used, ensuring a uniform and consistent capsule size distribution. EE (Figure 6h), kinetic (Figure 6k), and inhibition rate (Figure 6j) are properties contingent upon the physical and chemical characteristics of the encapsulates. Furthermore, both kinetic and inhibition rate are subject to external variables such as pH [66], physical/chemical force stimulation [67], fungal species [68], environmental temperature [69] or tested concentration [70]. In this contest, Experiment 8 uniquely exhibits a correlation between all three parameters, where a high EE allows for a controlled and gradual release (kinetic rate), consequently exerting a substantial impact on fungal growth.

3.2.4. SA potential is conditioned to spray drying variables

Following a thorough examination of the variables influence on the responses, an assessment of the correlation between the levels of the tested parameters and the resulting responses was conducted through PCA analysis. The utility of PCA in discerning potential correlations among the tested conditions has been demonstrated [71]. The individual PCA plot, representing the experimental conditions, revealed that the two main components collectively contribute to approximately 55.6% of the total variance, with 32.7% attributed to Dim1 and 22.9% to Dim2. Figure 7 displays three distinct relationships, grouping Experiments 2 – 10 and Experiments 7 - 15 while underscoring the significance of Experiment 8.

Experiments 2 and 10 shared similar tested conditions, including solid content (+1), milling speed (-1), milling time (-1), and feed rate (+1), with variations only in spray temperature (-1 and +1) and aspiration (+1 and -1), as shown in Table 1b. Although these experiments exhibited modest performance values, and demonstrated the lowest grinding wear values, the decreased alumina content resulted in increased EE values. However, the lowest mass loss values between 400°C and 1000°C were observed, suggesting poorer encapsulation of SA. Despite achieving good EE values, the anticipated controlled SA release and substantial antifungal effect were not realized, highlighting a deficiency in SA encapsulation.

Conversely, Experiments 7 and 15 shared similar tested conditions, including solid content (-1), milling speed (+1), milling time (+1), and feed rate (-1), with variations in spray temperature (-1 and +1) and airflow (+1 and -1), as outlined in Table 1b. These experiments demonstrated the highest performance values, attributed to considerable grinding wear values. The low feed rate employed, prolonged the formulation's chamber residence time, leading to improved drying and reduced powder moisture. However, this extended drying time, coupled with the low feed rate, resulted in poorer EE values as a substantial amount of SA was not fully encapsulated. Moreover, high mass loss values suggest good SA encapsulation, but due to the high amount of grinding wear, it is not fully realized. This is further

corroborated by the fact that neither of these experiments exhibited satisfactory release or antifungal effect.

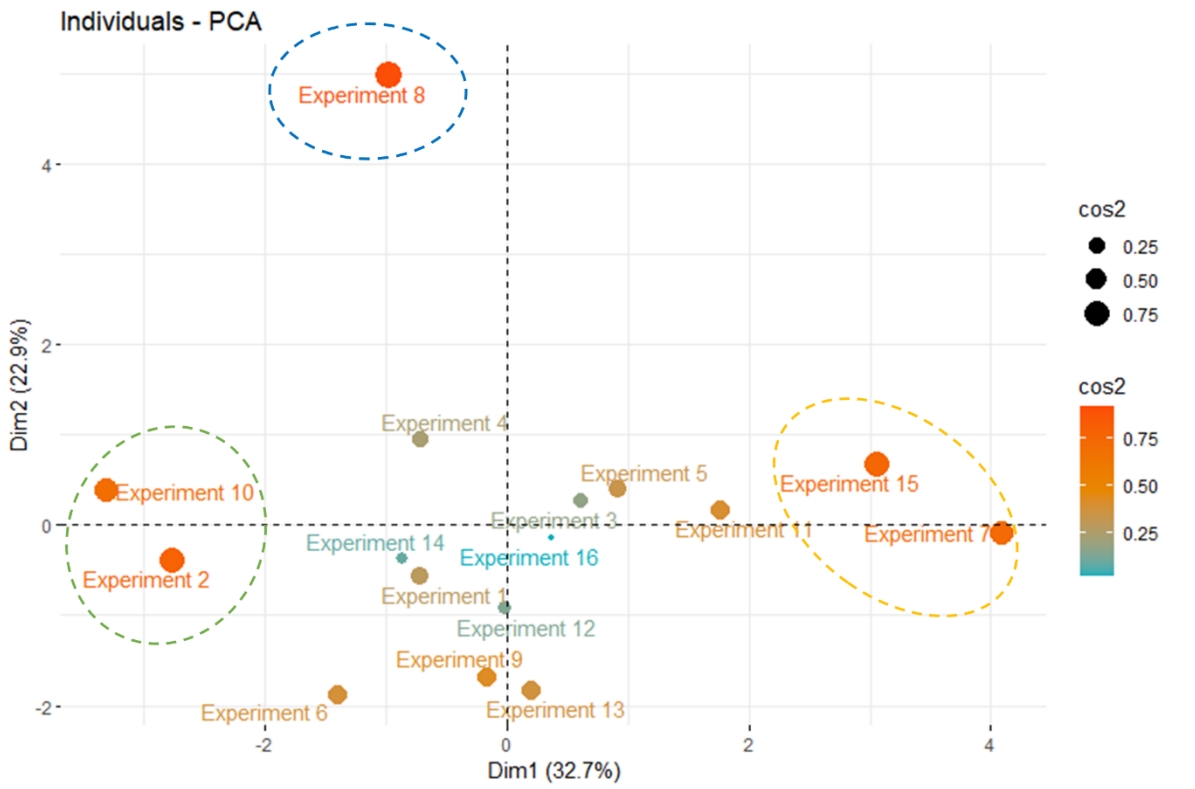

Figure 7. Principal component analysis (PCA) of the 16 experiments.

Finally, Experiment 8 yielded the best results in terms of EE, kinetic rate and inhibition rate. Its tested conditions included solid content (+1), milling speed (+1), milling time (+1), spray temperature (-1), feed rate (+1) and airflow (-1). Both performance and grinding wear displayed average values. Additionally, an average moisture level was observed, likely resulting from an equilibrium between the quantity of SA and grinding wear within the formulation. The relatively good performance and controlled presence of grinding wear contributed to an improved EE. This heightened EE value facilitated a desired controlled release, subsequently enhancing the antifungal effect (refer to Table 4). Similarly, lower aspiration maintained more controlled airflow, preventing powder loss. It is worth noting that the low tested temperature condition proved effective in adequately drying the slurry without adversely affecting the encapsulated SA properties.

4. Conclusions

The optimization of the SA encapsulation process was successfully achieved through a systematic study of the key variables associated with the two unit operations involved: wet-milling and spray-drying. The study of acetone-based encapsulated SA samples revealed that SA does not undergo degradation during spray drying at a temperature of 150°C. Consequently, there is no need to use organic solvents to formulate SA encapsulated samples. This not only yields significant cost savings but also mitigates environmental impact.

Despite some differences were found between encapsulated samples prepared in water versus acetone in terms of performance and moisture, critical parameters such as encapsulation efficiency and kinetic rate remained consistent. This supports the notion that both treatments were equally effective when applied as antifungal control.

The optimization of the water-based encapsulation process through a fractional factorial design revealed that the most influential variables in the whole atomization process are solid content, milling speed, and milling time in comparison with spray temperature, feed rate and airflow. These three variables play a crucial role in material homogenization and determine the final amount of grinding wear. Lower solid content, combined with higher speed and extended time, can overestimate performance values or encapsulation efficiency, directly impacting moisture and specific surface area of the encapsulates as alumina wear binds to the capsules, hindering proper SA encapsulation.

However, feed rate, airflow and spray temperature are critical for the proper drying of the slurries. A fast slurry feed rate coupled with low airflow fails to provide sufficient time for proper powder drying, leading to adherence to the walls. Besides, a powder not properly dried suggests problems during spray drying, and subsequently, low SA encapsulation efficiency values, thus decreasing its antifungal effect.

The fractional factorial design facilitated the establishment of optimal values for process variables, thereby improving the encapsulation process and the final properties of the encapsulated SA samples. These findings could serve as a valuable starting point for formulating other plant-derived molecules that play a crucial role in stress tolerance.

Acknowledgment

This work has been cofounded by Project PID2022-137825OB-I00 (MCIN /AEI /10.13039/501100011033 / FEDER, UE) and by Project AGROALNEXT/2022/010 (GVA, PRTR, MRR, NextGenerationEU). JS was supported by program Santiago Grisolia from GVA.

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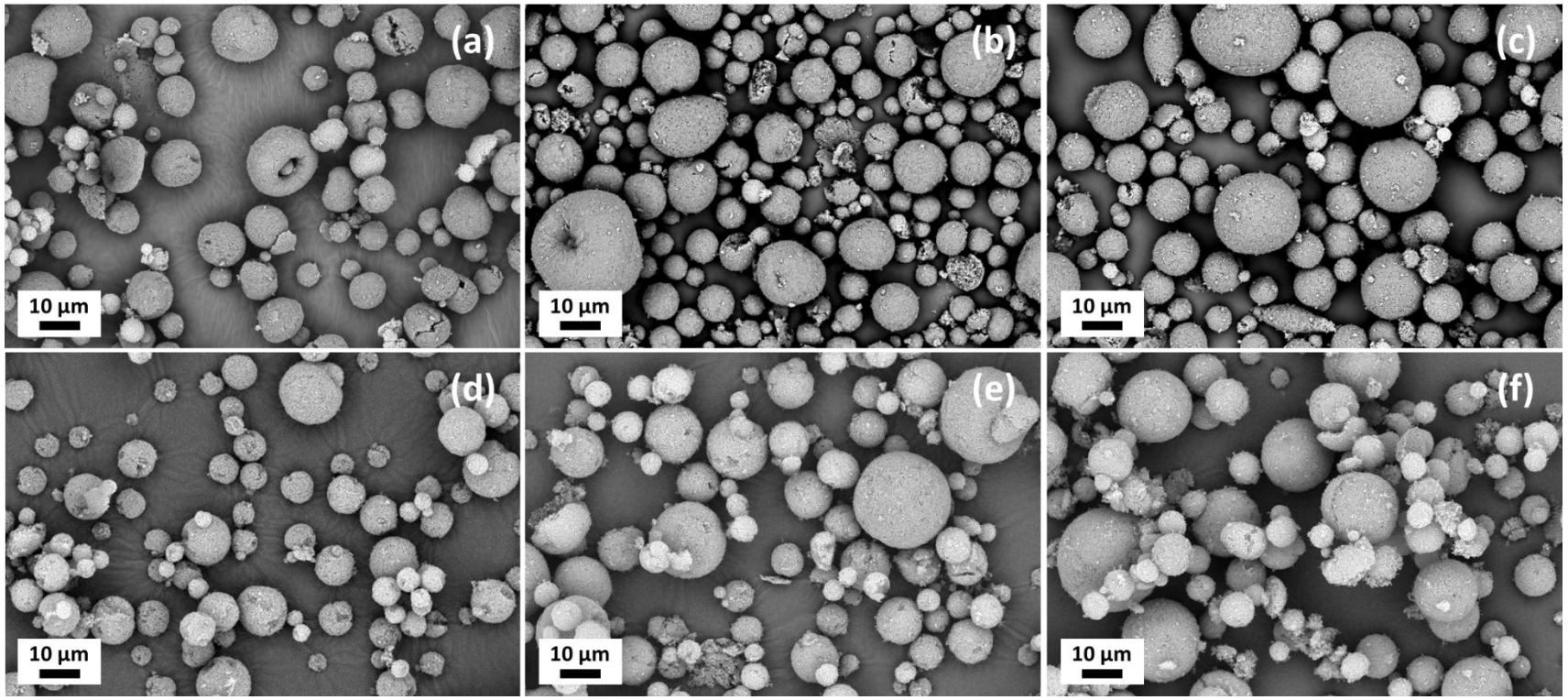
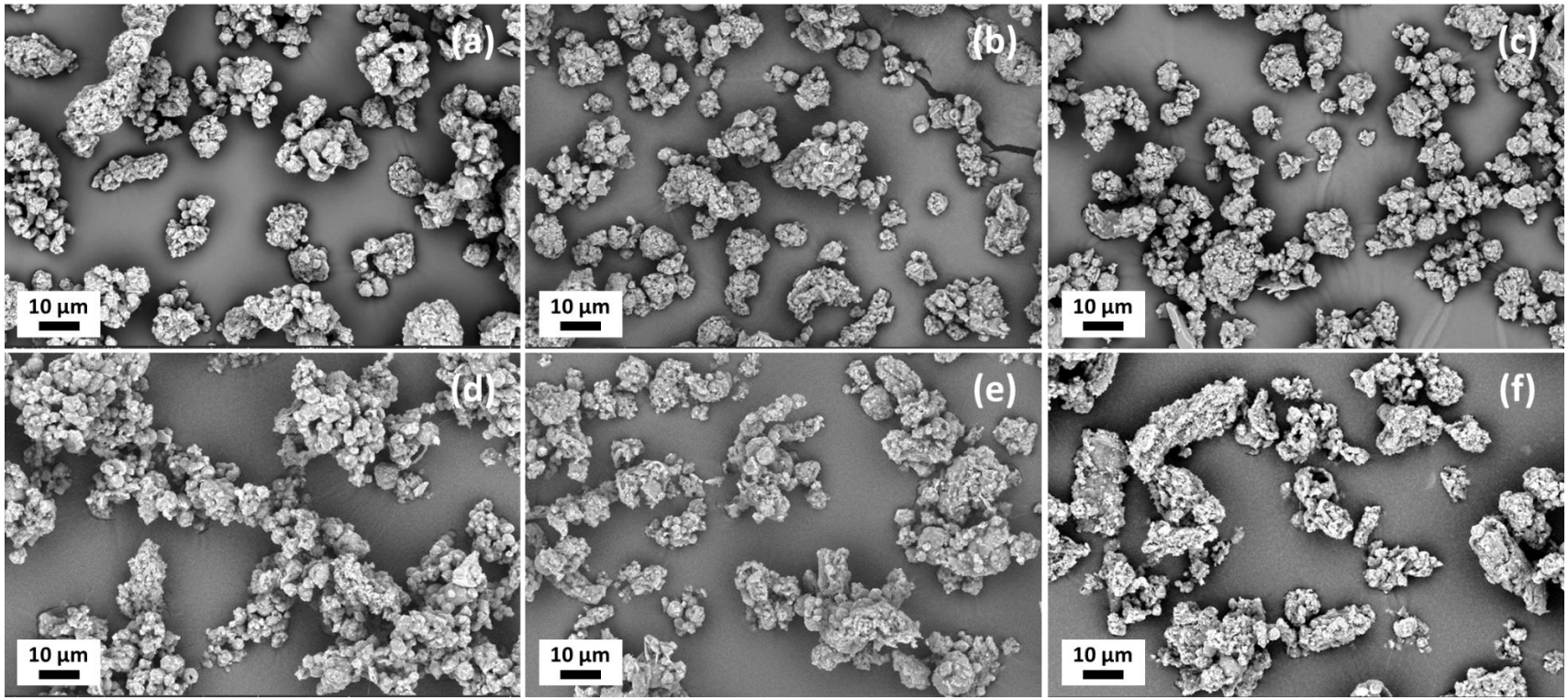
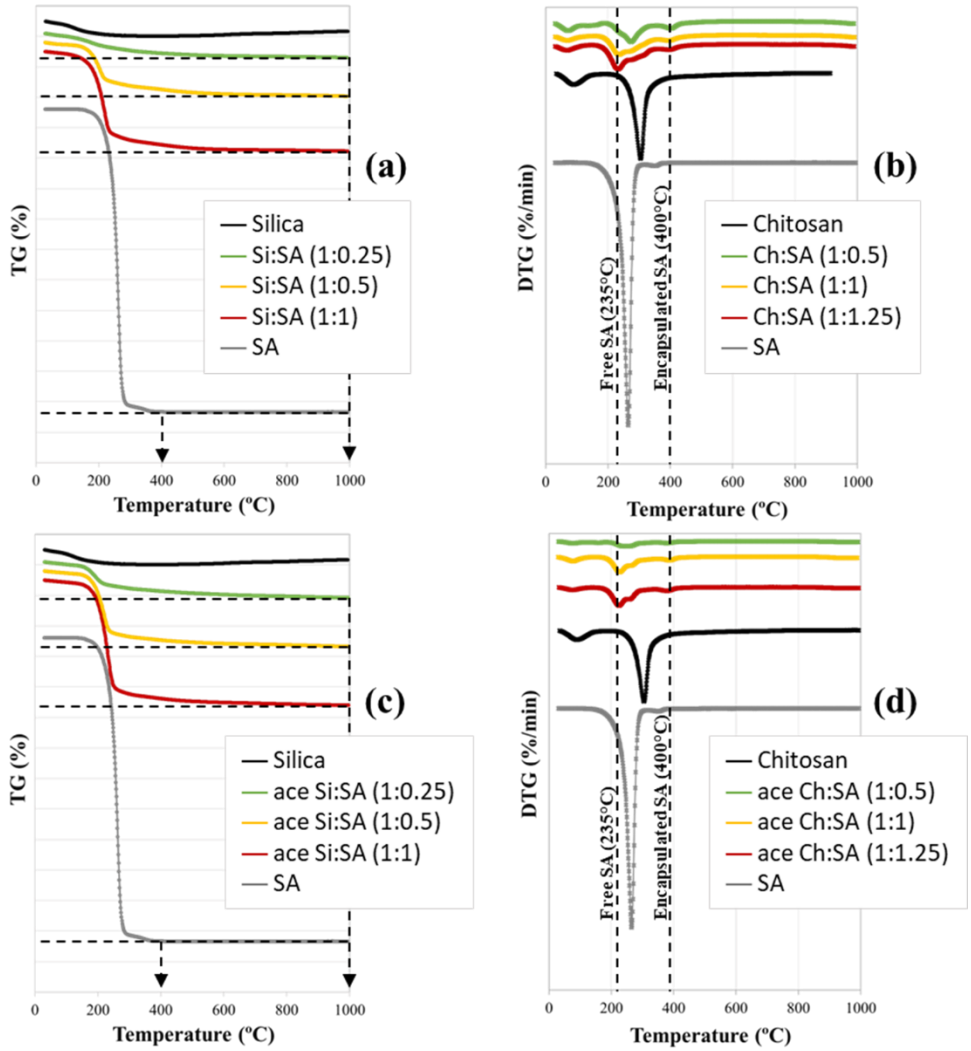
Supplementary material



Supplementary Figure 1. Scanning electron microscopy (SEM) images of the silica-encapsulated SA samples. Images were taken at 1000x magnification. Figures a, b and c, correspond to silica-encapsulated SA samples prepared in water at (1:1), (1:0.5), and (1:0.25) ratio, respectively. Figures d, e and f, correspond to silica-encapsulated SA samples prepared in acetone at (1:1), (1:0.5), and (1:0.25) ratio, respectively.

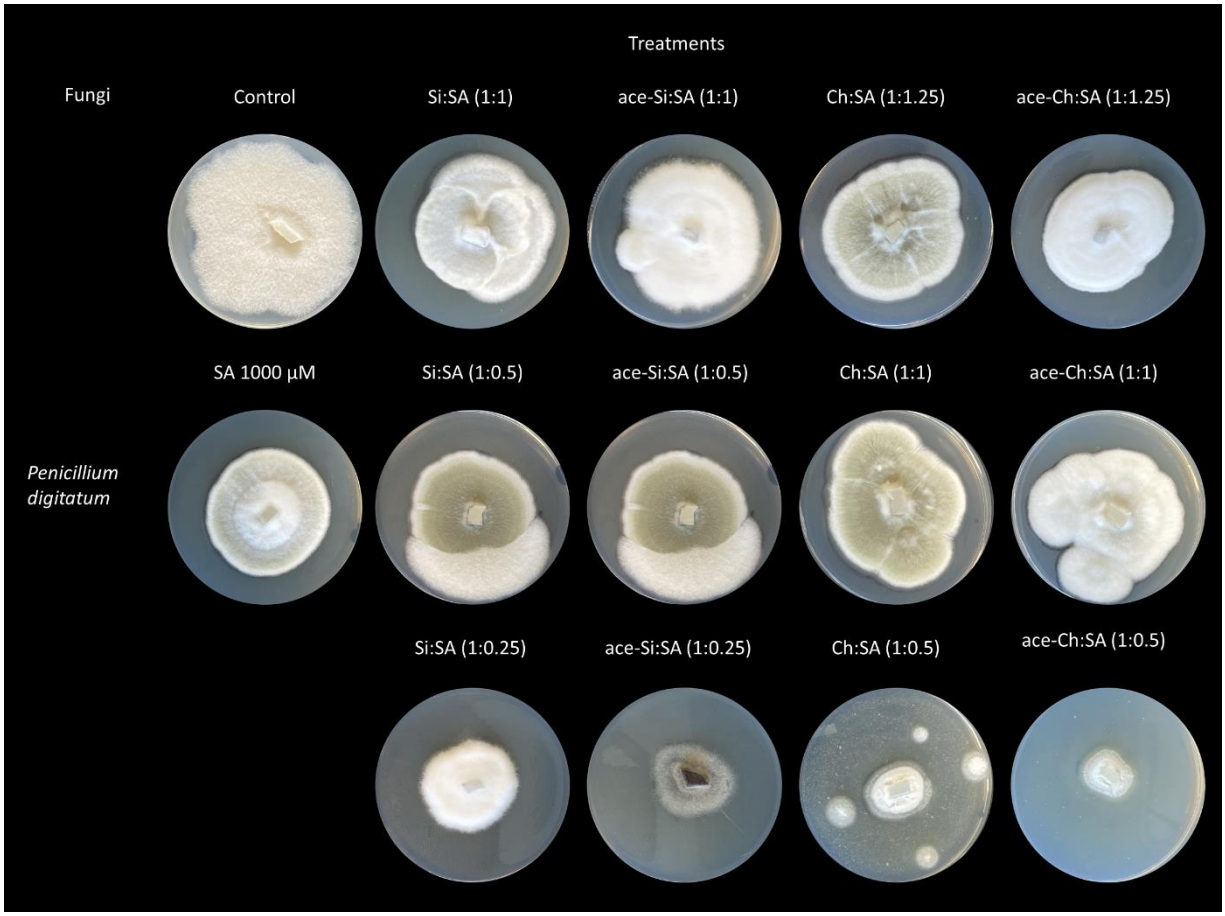


Supplementary Figure 2. Scanning electron microscopy (SEM) images of the chitosan-encapsulated SA samples. Images were taken at 1000x magnification. Figures a, b and c, correspond to chitosan-encapsulated SA samples prepared in water at (1:1.25), (1:1), and (1:0.5) ratio, respectively. Figures d, e and f, correspond to chitosan-encapsulated SA samples prepared in acetone at (1:1.25), (1:1), and (1:0.5) ratio, respectively.

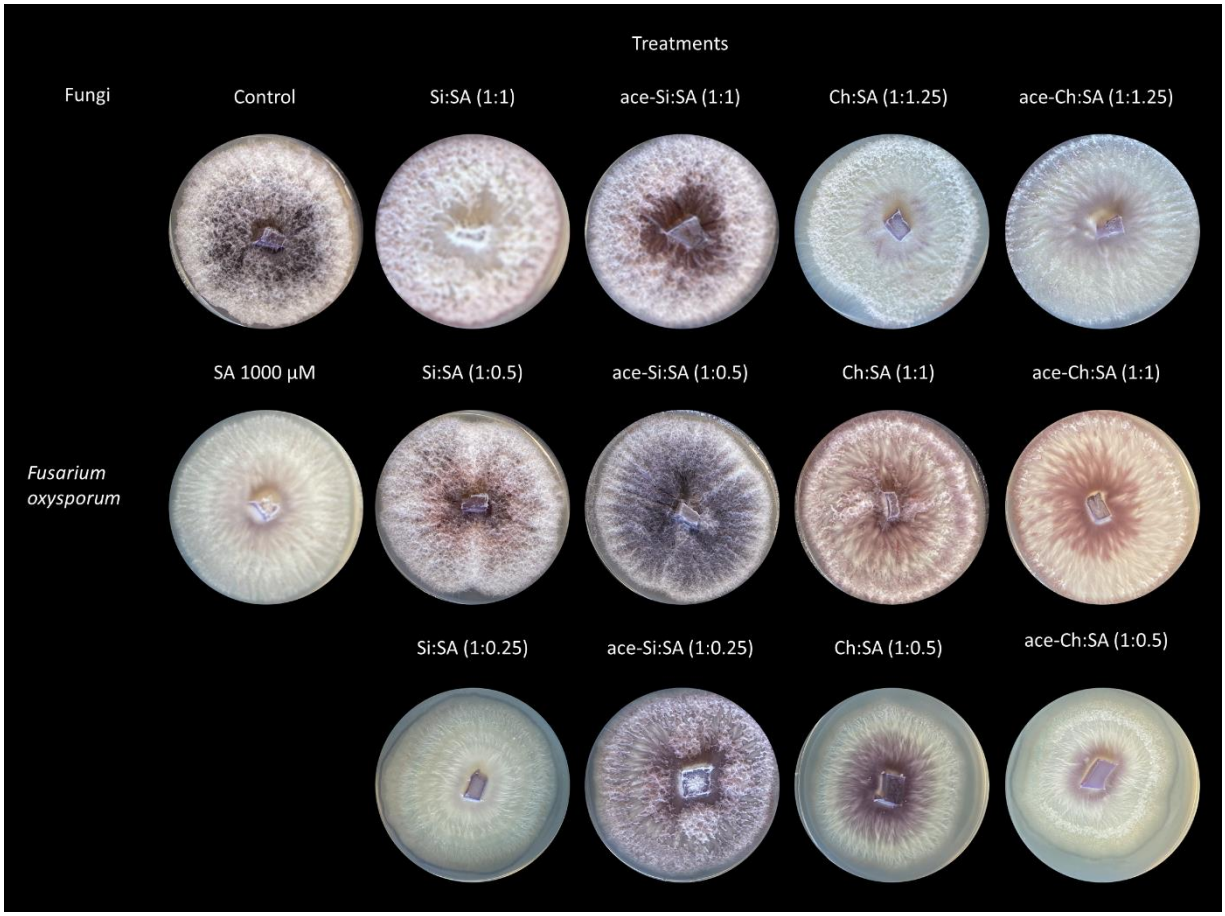


Supplementary Figure 3. Thermal analysis of the silica/chitosan-encapsulated SA samples performed in acetone and water.

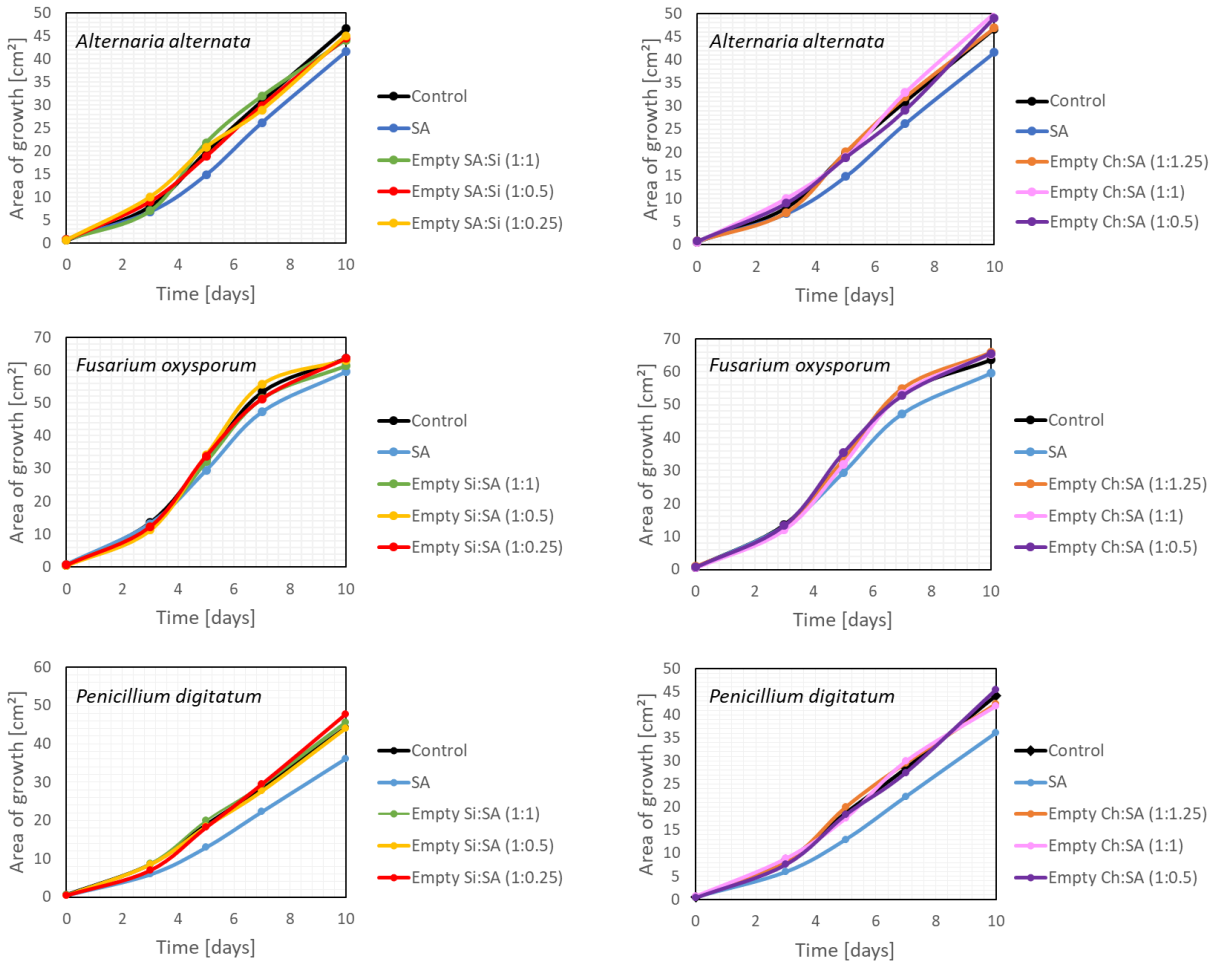
Supplementary Figure 4. Antifungal effects of silica/chitosan-encapsulated SA samples performed in water and acetone at 1000 µM on *Alternaria alternata*.



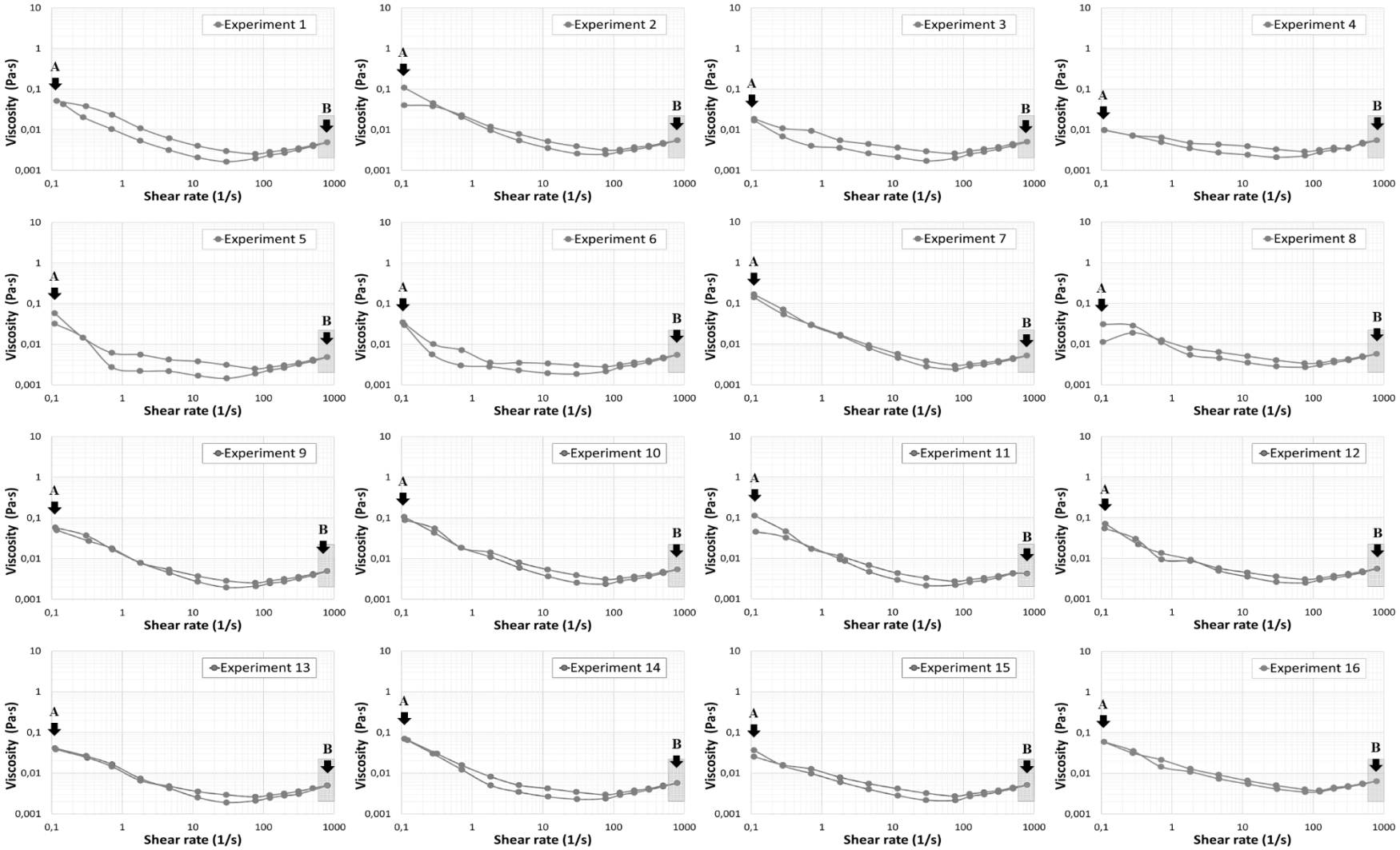
Supplementary Figure 5. Antifungal effects of silica/chitosan-encapsulated SA samples performed in water and acetone at 1000 µM on *Penicillium digitatum*.



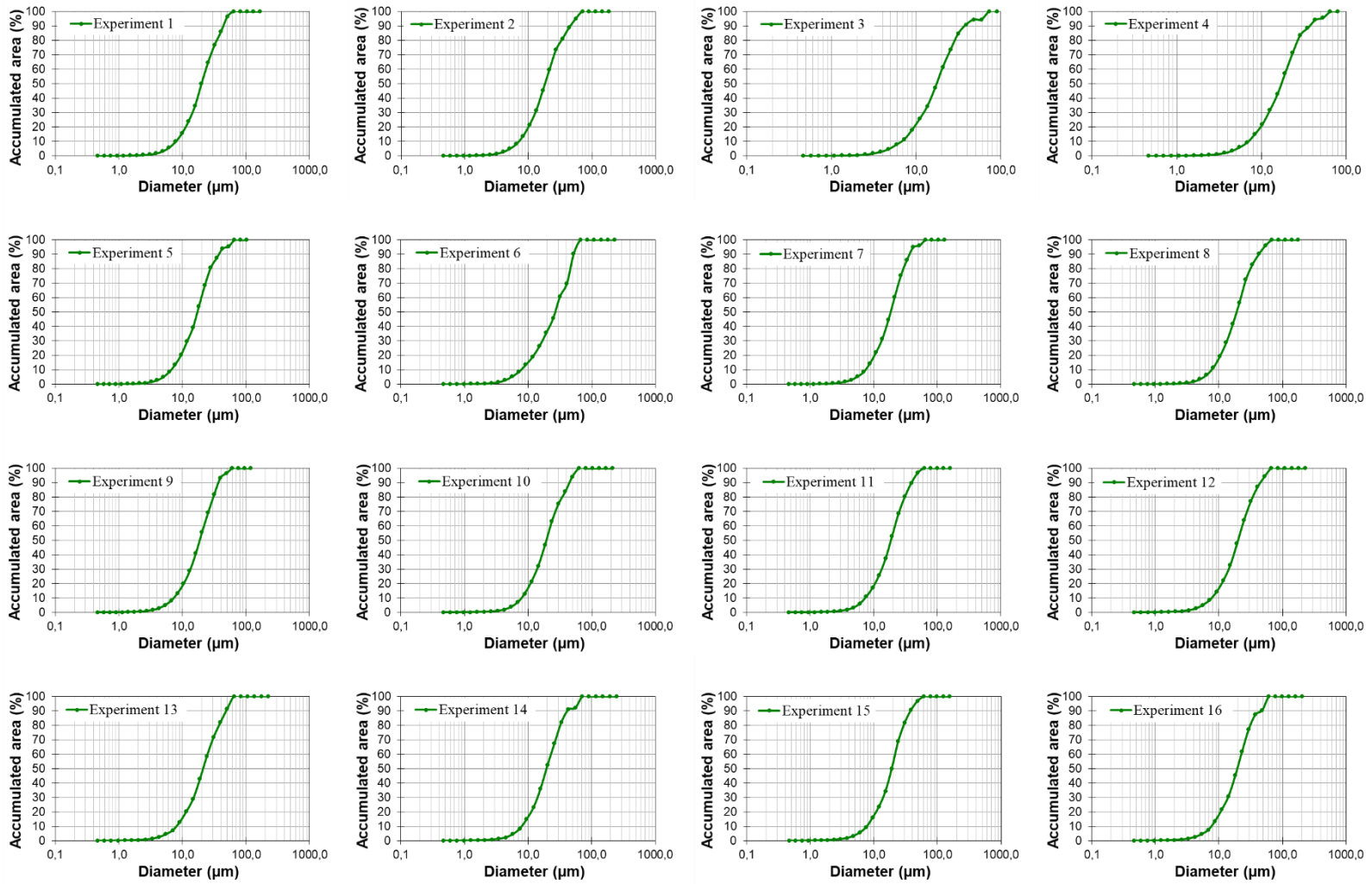
Supplementary Figure 6. Antifungal effects of silica/chitosan-encapsulated SA samples performed in water and acetone at 1000 µM on *Fusarium oxysporum*.



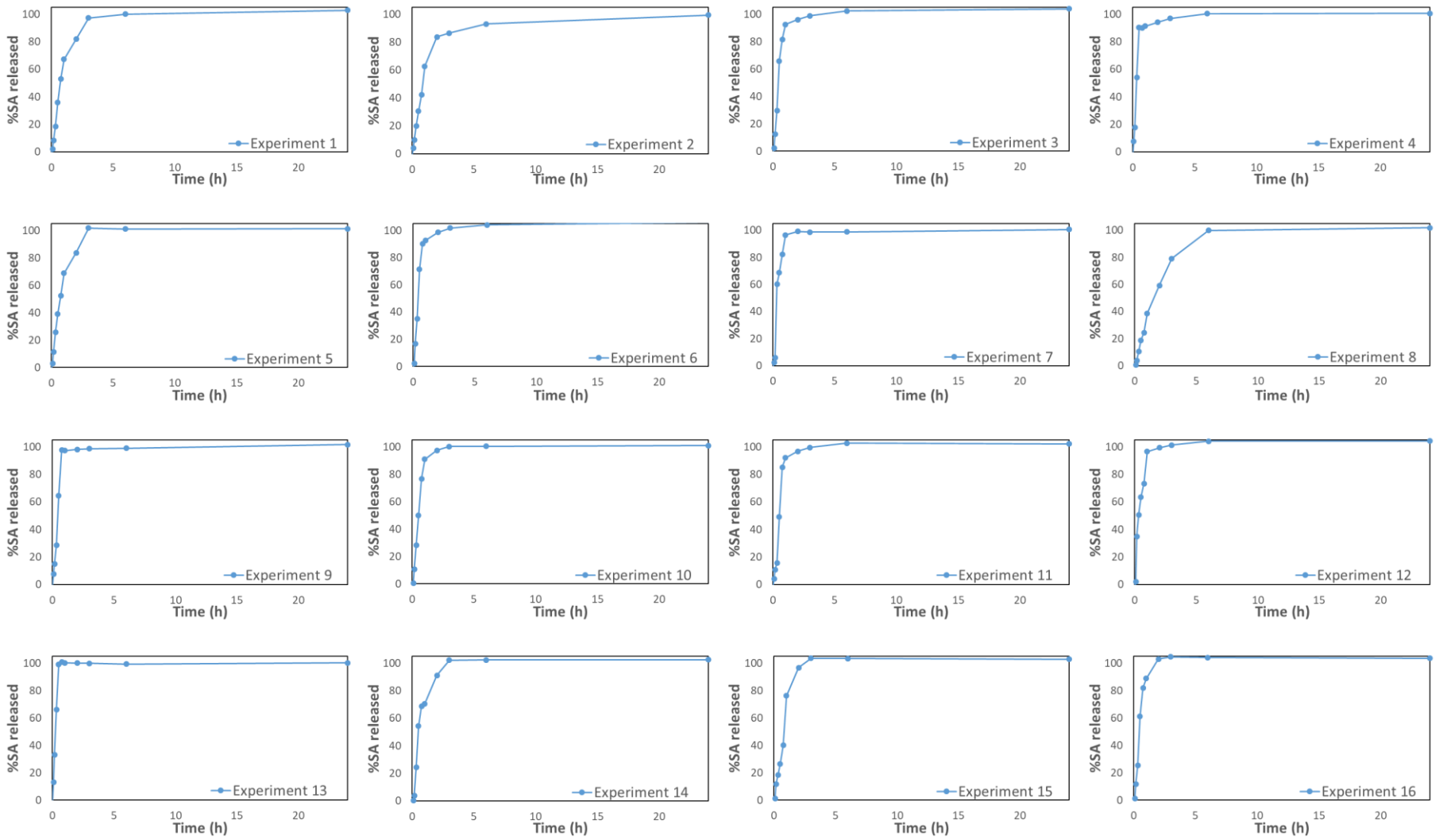
Supplementary Figure 7. Micelial growth rate of *Alternaria alternata*, *Fusarium oxysporum* and *Penicillium digitatum* treated with capsules without SA at 1000 µM.



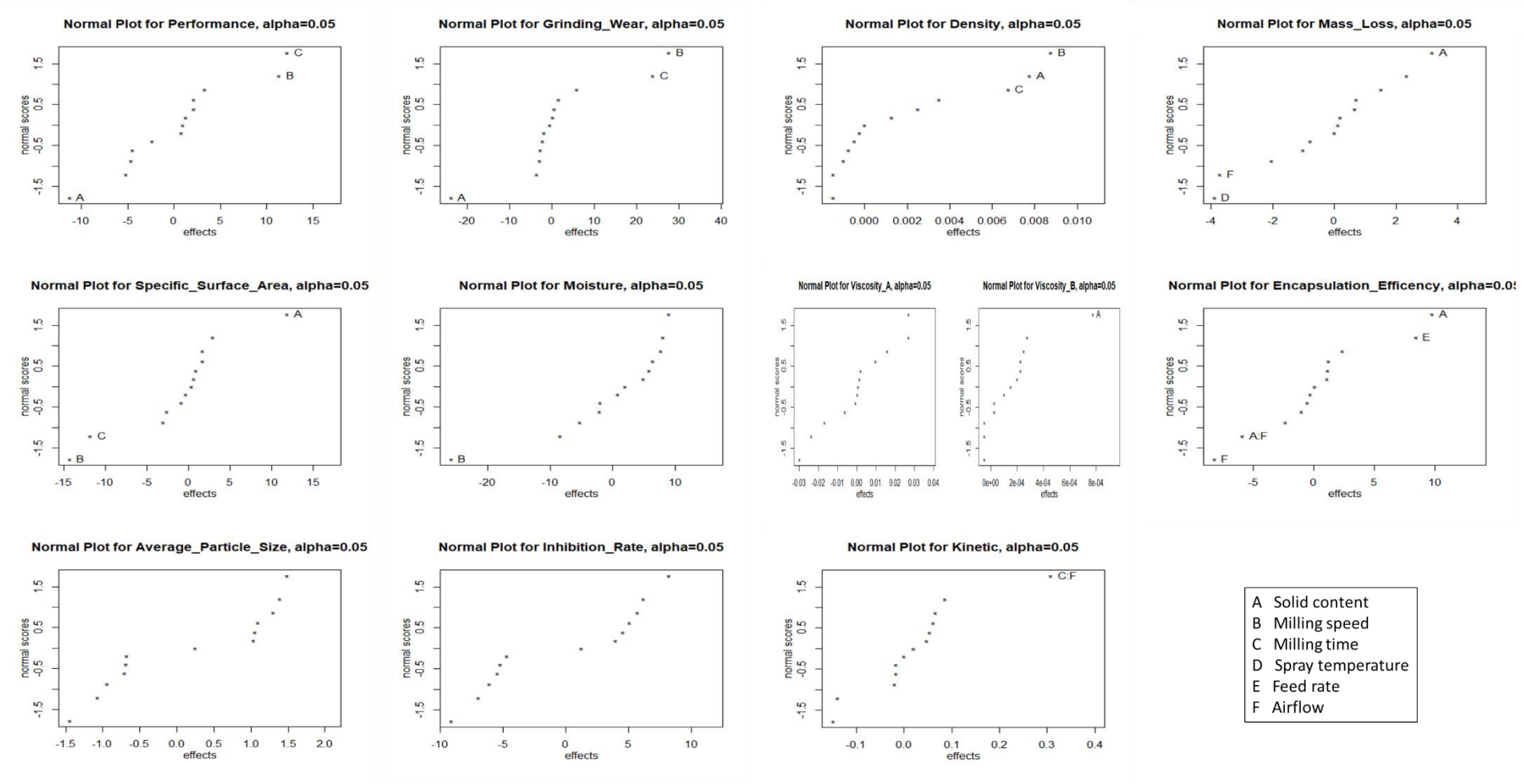
Supplementary Figure 8. Viscosity flow behaviour of the 16 experiments. A and B points viscosity values are shown in Table 4. The grey area corresponds to the optimal parameter range for the slurry to enable the atomization process.



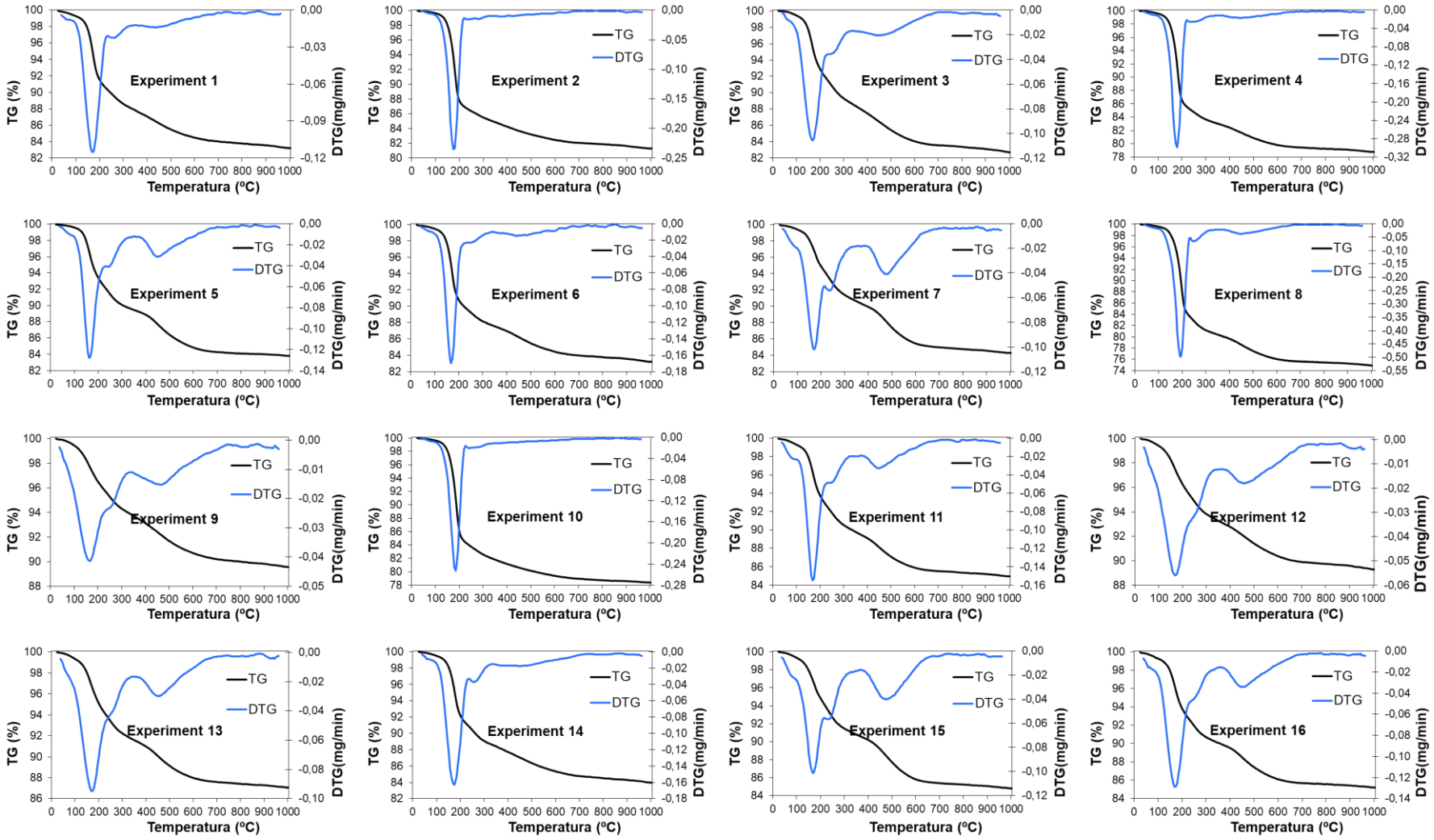
Supplementary Figure 9. Capsule size distributions of the 16 experiments. Diameters values (D_{50}) are shown in Table 4.



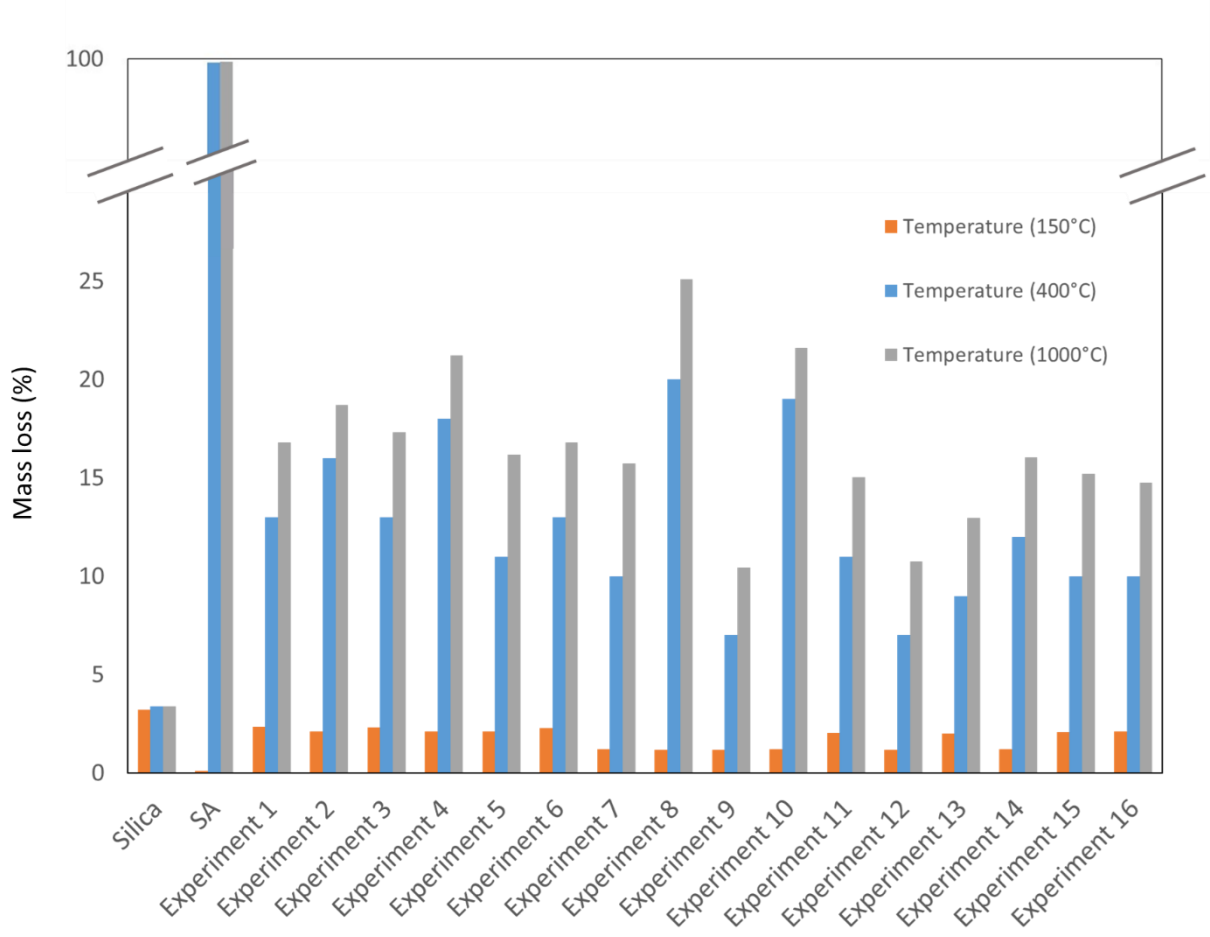
Supplementary Figure 10. SA release from the 16 experiments.



Supplementary Figure 11. Normal plots for experimental responses.



Supplementary Figure 12. TG curves of the 16 experiments. The percentages of mass loss are shown in Table 4.



Supplementary Figure 13. Mass loss of the 16 experiments. Loss mass values are represented at 150°C, 400°C and 1000°C, with color bars of orange, blue, and grey, respectively.

CHAPTER 3

ENCAPSULATION REDUCES THE DELETERIOUS EFFECTS OF SALICYLIC ACID TREATMENTS ON ROOT GROWTH AND GRAVITROPIC RESPONSE

Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., and Clausell-Terol, C. (2022). *International Journal of Molecular Sciences* **23**, 14019. https://doi.org/10.3390/ijms232214019

Key words for the tittle: DR5::GFP auxin sensor – Encapsulation process – Indole acetic acid

International Journal of *Molecular Sciences*

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Encapsulation Reduces the Deleterious Effects of Salicylic Acid Treatments on Root Growth and Gravitropic Response

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Abstract: The role of salicylic acid (SA) on plant responses to biotic and abiotic stresses is well documented. However, the mechanism by which exogenous SA protects plants and its interactions with other phytohormones remains elusive. SA effect, both free and encapsulated (using silica and chitosan capsules), on *Arabidopsis thaliana* development was studied. The effect of SA on roots and rosettes was analysed, determining plant morphological characteristics and hormone endogenous levels. Free SA treatment affected length, growth rate, gravitropic response of roots and rosette size in a dose-dependent manner. This damage was due to the increase of root endogenous SA concentration that led to a reduction in auxin levels. The encapsulation process reduced the deleterious effects of free SA on root and rosette growth and in the gravitropic response. Encapsulation allowed for a controlled release of the SA, reducing the amount of hormone available and the uptake by the plant, mitigating the deleterious effects of the free SA treatment. Although both capsules are suitable as SA carrier matrices, slightly better results were found with chitosan. Encapsulation appears as an attractive technology to deliver phytohormones when crops are cultivated under adverse conditions. Moreover, it can be a good tool to perform basic experiments on phytohormone interactions.

Keywords: DR5::GFP auxin sensor; encapsulation process; indole acetic acid

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Citation: Sampedro-Guerrero, J.; Vives-Peris, V.; Gomez-Cadenas, A.; Clausell-Terol, C. Encapsulation Reduces the Deleterious Effects of Salicylic Acid Treatments on Root Growth and Gravitropic Response. *Int. J. Mol. Sci.* **2022**, *23*, 14019. https://doi.org/10.3390/ijms232214019

Academic Editor: Esther M. González

Received: 29 September 2022
Accepted: 11 November 2022
Published: 14 November 2022

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1. Introduction

Physiological processes that allow the correct development of plants are controlled by biomolecules [1]. Among them, plant growth regulators (PGRs) are active substances that can have a natural origin, such as the phytohormones, but also can be chemically synthesized. The phytohormones, which are low molecular weight organic compounds of endogenous origin [2], are distributed in different plant tissues and, in small quantities, are capable of modulating morphogenetic and physiological processes [3]. Moreover, phytohormone effects are complex because their action, in some cases, is indirect and the signal can initiate in a plant tissue far from where the final effect is observed [4]. It is important to determine the role of phytohormones and their interactions since PGRs are commonly used to modify developmental patterns and growth rates in seeds, shoots and roots [5], with high economic and agronomic benefits. Climate change is enhancing the incidence and intensity of abiotic stresses in the fields, giving rise to a complicate scenario where crops are gradually reducing yields and fruit qualities [6,7]. PGRs have a remarkable potential to induce plant responses to stress, contributing to the adaptation of crops to adverse environments [8].

Salicylic acid (SA), fully recognized as a PGR in the 1990s [9], has an aromatic benzene ring in its structure and belongs to the family of phenolic compounds [10]. SA participates in key biological processes such as plant development, antioxidant system, nitrogen

Abstract

The role of the salicylic acid (SA) on the induction of systemic acquired resistance is well known. Moreover, literature reports the participation of SA in the plant response to biotic and abiotic stresses. Despite this basic studies, the mechanism by which exogenous SA protects plants, as well as the interactions of SA with other phytohormones, such as auxins, still remains elusive. Similarly, determining the SA suitable dosage to probe on in-vitro plants systems are not yet well developed. For these reason, the main objective of this work was to study the SA behaviour both free and encapsulated on *Arabidopsis thaliana* plants, and its effect in plants development. The effect of SA on roots and rosettes of Arabidopsis plants was analysed at different doses after treatments with free and encapsulated SA (using silica and chitosan capsules), determining their affectation through their morphological characteristics and hormones internal levels changes. The results showed that the free SA treatment affected length and growth rate of the roots, the size of the rosettes, and modified the normal gravitropism of the roots, both at low and high doses, due to changes in endogenous SA and IAA levels, while, the encapsulated samples affected the plants at the highest doses. The dose limit to use in free SA treatments is 1 µM, however, in the encapsulates samples treatments, it is in the range of 50 to 100 µM, depending on the parameter evaluated. Encapsulation allowed a controlled release of the SA, reducing the amount of hormone available in the media and the uptake by the plant, avoiding or decreasing the deleterious effects of the SA. Therefore, encapsulation is an effective method to determinate physical and chemical effects of a determinate hormone, in this case the SA, and could be an attractive technology to applicate when an adverse conditions or a stress affect an important crop, using the appropriate amount, evaluating first in the laboratory and then in the field.

1. Introduction

Physiological processes that allow the correct development of plants are controlled by biomolecules [1]. Among them, plant growth regulators (PGRs) are active substances that can have a natural origin, such as the phytohormones, but also can be chemically synthesized. The phytohormones, which are low molecular weight organic compounds of endogenous origin [2], are distributed in different plant tissues and, in small quantities, are capable of modulating morphogenetic and physiological processes [3]. Moreover, phytohormone effects are complex because their action, in some cases, is indirect and the signal can initiate in a plant tissue far from where the final effect is observed [4]. It is important to determine the role of phytohormones and their interactions since PGRs are commonly used to modify developmental patterns and growth rates in seeds, shoots and roots [5], with high economic and agronomic benefits. Climate change is enhancing the incidence and intensity of abiotic stresses in the fields, giving rise to a complicate scenario where crops are gradually reducing yields and fruit qualities [6,7]. PGRs have a remarkable potential to induce plant responses to stress, contributing to the adaptation of crops to adverse environments [8].

Salicylic acid (SA), fully recognized as a PGR in the 90s [9], has an aromatic benzene ring in its structure and belongs to the family of phenolic compounds [10]. SA participates in key biological processes such as plant development, antioxidant system, nitrogen metabolism and photosynthesis regulation, among others [11]. In addition, it is an important component of plant tolerance to biotic stresses [12–14]. SA has an import role on plant responses to abiotic stresses in cooperation with other phytohormones such as jasmonic acid (JA) and abscisic acid (ABA), in a controlled cross-talk that allows the plant to adapt to drought, highlight, high temperatures and high salinity [15,16]. The capacity of exogenous SA of inducing stress tolerance mechanisms has been deeply studied. In fact, different application methods, such as the addition to the nutrient solution or irrigation water, spraying or soaking the seeds have been tested [17,18]. A good example is the protective effect of SA against salt stress, where the addition of SA is capable of dispelling the toxicity symptoms in many plant species, restoring membrane potential and improving photosynthetic capacity and antioxidant protection [19,20]. When considering a SA treatment, several aspects must be taken into account, such as the SA concentration, the plant species, the age of the plant and the duration of the treatment [21]. Actually, choosing the optimal dose of SA is an important point to consider because, a small amount will be rapidly absorbed by the plant, decreasing its protective effect over time and, on the contrary, a large amount will cause stress in the plant [22].

Encapsulation is an efficient solution to control the release of SA and, therefore, the applied concentration. Encapsulation is a relatively new technology where an active agent is loaded into a carrier matrix [23] of different nature, often polymer-based. The benefits of this process are many, such as: a) active agent protection during the storage and the application process, b) decrease in the amount of active agent required, and c) controlled release of the encapsulated molecule [24,25]. The encapsulated active agents can be small or large molecules, such as proteins, drugs or dyes, and capsules are generally organic or inorganic polymers, fatty acids or lipids [26]. In the last years, various polymeric shells had been developed, for example: polyuria, polysaccharides such as chitosan and alginate, aliphatic polyesters and amorphous silica [27], which are efficient to formulate capsule-active agent mixtures, using an aqueous system without changing room temperature [28]. It has been recently shown that chitosan and amorphous silica are effective carriers of plant-derived substances [29], immobilizing and releasing them in a controlled manner [30].

The aim of this work was to study the differences among treatments with free SA and encapsulated SA (in silica or chitosan), in *Arabidopsis thaliana* plants. The effect of SA concentration was studied, demonstrating that free and encapsulated SA affect plants in different ways. The encapsulation of SA using both capsules proved to be an effective method to reduce the negative effects of the phytohormone accumulated in roots and rosettes through its controlled release and its correct delivery to the plant. The

results provide a desirable encapsulated product that can be used in agriculture to mitigate the adverse effects of different stresses on plants.

2. Materials and Methods

Fig.1. shows a summary of the methodology used in this work: 1) formulation of treatments and Arabidopsis seed sown, 2) analysis of SA effect on root and rosette growth and root gravitropism, 3) extraction and quantification of phytohormones, 4) visualization of the auxin-specific reporter gene DR5 in roots, and 5) statistical and PCA analyses.

2.1. Materials and plant growth conditions. Salicylic acid (SA), pyrogenic amorphous silica HDK® S13 (Si) and chitosan DG CHI 0.20 g/ml and 85% deacetylated (Ch) were purchased from Sigma-Aldrich (St. Louis, USA), AOXIN (Shanghai, China) and WACKER (Barcelona, Spain), respectively. *Arabidopsis thaliana* wild-type (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK), and *Arabidopsis thaliana DR5::GFP* line was obtained from the Arabidopsis Biological Resource Center (Columbus, USA). Seeds were surface sterilized with 1% v/v sodium hypochlorite and 0.01% v/v Tween 20 solution for 10 min with moderate incubation, washed in triplicate with sterile distilled water, and sown in 9 x 15 cm petri dishes containing Murashige and Skoog medium 0.5% (Duchefa, Haarlem, The Netherlands), sucrose 1% (Merck Millipore, Darmstadt, Germany) and European Bacteriological Agar (Condalab, Madrid, Spain). Seeds were germinated and petri dishes were vertically arranged (Figure 1) in growth chambers (SANYO MLR-350, Sakata, Gunma, Japan) for 5 days under 16 h light/8 h dark cycles at 22.5°C and 60% relative humidity. After this period, plants were transferred to the media containing the different SA treatments (see point 2.2.) and kept in the same growing conditions for different periods.

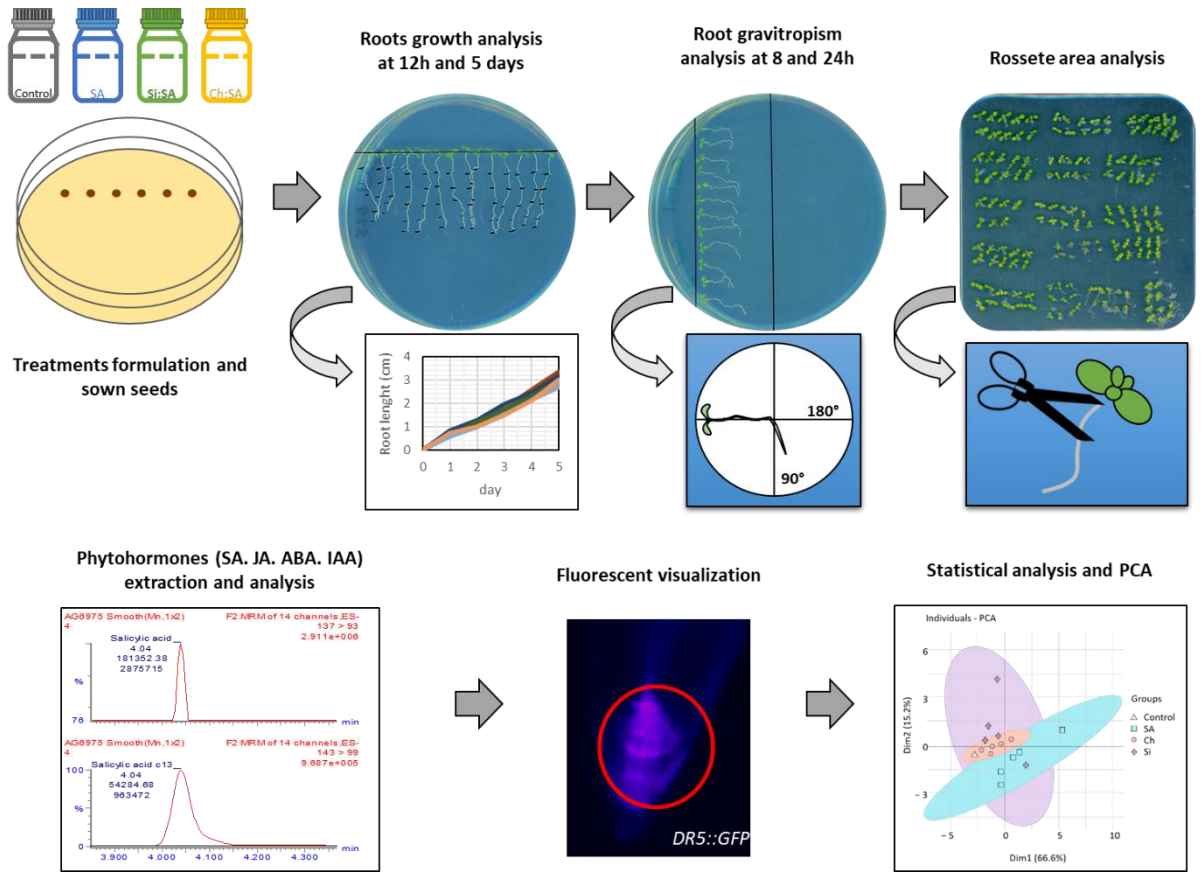

Figure 1. Experimental method developed to determine the effect of free and encapsulated SA (Si:SA and Ch:SA) in *Arabidopsis thaliana* plants.

2.2. SA treatment conditions. The following concentrations of SA were used for treatments: 1, 10, 50, 100 and 500 μM. For each SA concentration, in addition to non-encapsulated SA (referred to as free SA), 1:0.25 ratio of Si:SA and 1:0.5 ratio of Ch:SA encapsulated samples were obtained by spray-drying the aqueous suspensions prepared by planetary mixing (Fritsch, Pulverisette®) of the specific amounts of SA with silica and chitosan, respectively, following the experimental procedure detailed in a previous publication [29]. In brief, Si:SA sample was prepared by mixing the respective amount of SA with 320 ml distilled water (15 min at 120 rpm), adding the amorphous silica stepwise and homogenizing the mixture (1 h at 180 rpm). Ch:SA was prepared by mixing 138.6 ml of distilled water and 1.4 ml of acetic acid (5 min at 150 rpm) and by adding, in successive steps, 4.2 g of chitosan (15 min at 210 rpm), 1.4 ml of tween 80 (15 min at 210 rpm), the appropriate amount of SA pre-dissolved in dichloromethane (15 min at 210 rpm) and 2.1 g of TPP-Na pre-dissolved in 137.9 ml of distilled water (1 h at 210 rpm). Spray drying was performed in a SD-06 spray drier (Lab Plant, UK), with a standard 0.5 mm nozzle and the following standard conditions: inlet temperature 150°C, spray flow 10

ml/min, drying air fan 80% and compressed air pressure 1.5 bar. Encapsulated samples and free SA were mixed with the culture medium and poured in petri dishes.

2.3. Determination of root growth, rosette area and root gravitropism. After transferring plants to the different treatments, petri dishes were scanned with an Epson perfection v600 photo scanner, and root length measured by Image J software using the obtained images (Fig. 3i). Dishes were scanned each hour until 24 hours and each day until 5 days, calculating root length from the images. 5-day growth plants were removed from the dish, and their rosettes (separated from their roots) were scan. Finally, for the gravitropism test, dishes were tilted 90°. Every hour (from 0h to 24h) the dishes were scanned, maintaining the same inclination. Root angle changes were measured by the same Image J software.

2.4. Extraction and phytohormones analysis. After transferring plants to the different treatments, plants were grown for 4 weeks in 90° tilted dishes. Then, plants were sampled and both rosettes and roots frozen with liquid nitrogen and stored until further analysis. Extraction and analysis were carried out as described in [31] with few modifications. Briefly, 0.2 g of plant tissue were extracted with 1 ml of acetonitrile 50% in a ball mill (MillMix20, Domel Železniki, Slovenija) after spiking with 2.5 ng of [$^{2}H_{5}$]-indole acetic acid (IAA) and 25 ng of the following molecules: [$^{13}C_{6}$]-SA, dehydro jasmonic acid (DHJA) and [$^{2}H_{6}$]-ABA. Extracted samples were sonicated and centrifuged to remove debris. Then, 1 ml of the sample was charged in an "Oasis HLB 1 cc Vac Cartridge, 30 mg (Waters, Mildford, USA)" column with 500 µl of acetonitrile 30%, collecting the eluent. Phytohormones SA, JA, ABA, and IAA were determined in rosettes and roots by high performance liquid chromatography coupled online to a triple quadrupole mass spectrometer (Micromass, Manchester, UK) through an orthogonal Z-spray electrospray ion source [32].

2.5. Fluorescence analysis. The DR5::GFP sensor system has been widely used to study the auxin response because it contains regulatory elements suitable for inferences about auxin levels. 5-day-old *Arabidopsis thaliana DR5::GFP* plants were transferred to different treatments. After 5 days, whole plants were taken from the medium, placed on microscope slides and visualized under the microscope with a 40× objective. Fluorescence images were acquired by a "Nikon Eclipse 80i fluorescent microscope (MicroscopyU, Melville, New York, USA)", equipped with an epifluorescence GFP filter. Fluorescence intensity and exposure time were at 30% and 200 ms, respectively. Images were treated with plugin FIJI from Image J software.

2.6. Statistical analysis and principal component analysis (PCA). Treatments consisted of three replications and, at least, ten plants for each replication. SPSS version 21 software was used for statistical analysis and one-way analysis of variance test (Anova) with Bonferroni correction to determine significant differences between treatment groups at $p \leq 0.05$. Correlation matrix graphic and

Individual – PCA/Variables – PCA were constructed using R package corrplot [33] and R package factoextra [34] – R package FactoMineR [35], respectively.

3. Results and Discussion

3.1. Encapsulation decreases the SA negative effect on primary root growth

Phytohormones are essential molecules that control the correct development of plants through a regulated homeostasis which may be disrupted by the exogenous application of PGRs [36]. SA works as a plant defense activator and a growth regulator. However, applications of SA to concentrations greater than 1 mM inhibit seed germination and plant growth [37]. To control the release of SA and reduce its negative effect, encapsulated samples were developed in a previous study [29].

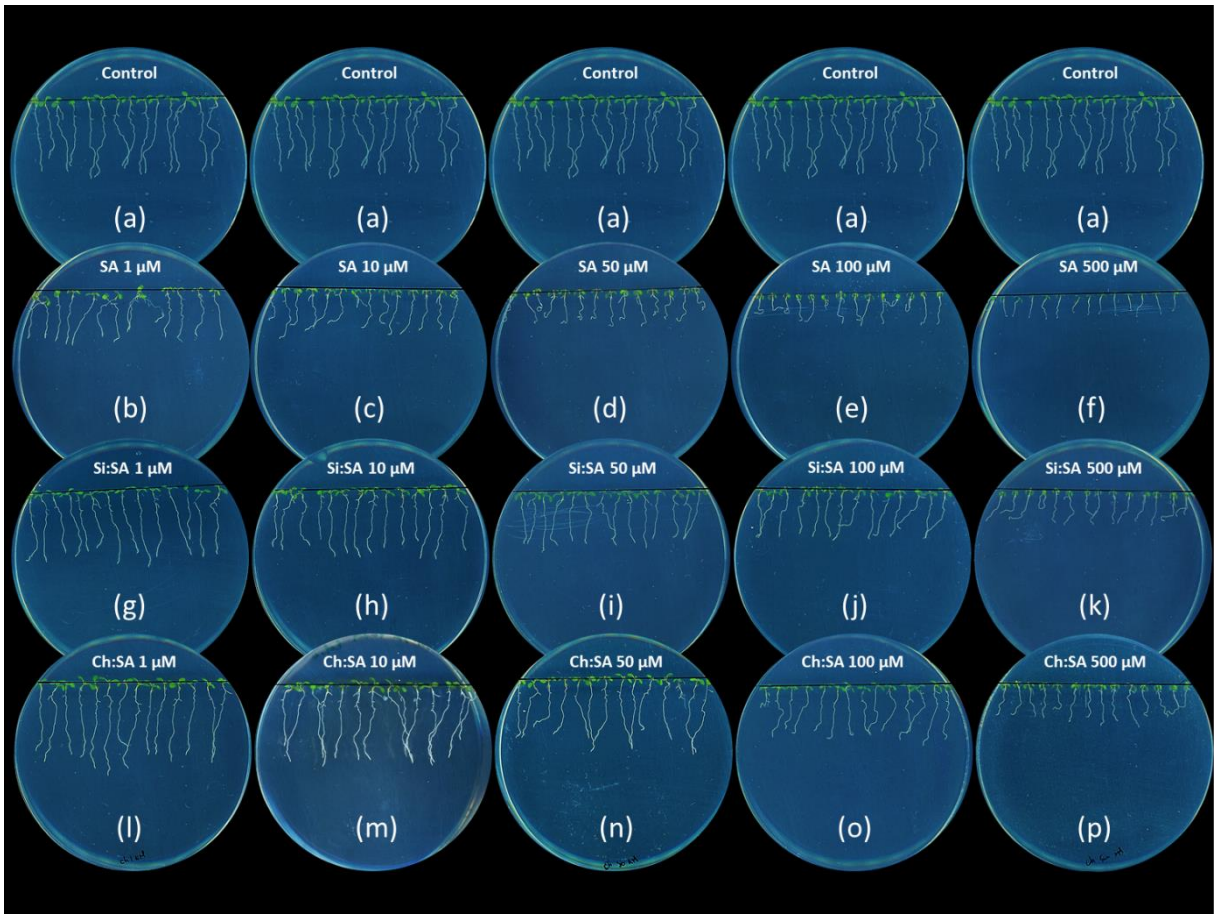

Figure 2. Effect of free SA, Si:SA and Ch:SA on root growth in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and pictures were taken 5 days later.

The samples selected for their best results inhibiting fungal growth were those with the lowest ratio tested with both capsules: 1:0.5 for Ch:SA and 1:0.25 for Si:SA. In this work, doses of SA (either free or encapsulated), ranging from 1 to 500 µM, were tested to measure their effect on Arabidopsis root growth (Figure 2). Data indicate that after 5 days of treatment, a significant decrease in the length of the primary root was observed in free-SA treated plants in all the doses tested (Fig. 2b–f). Plants treated with SA encapsulated with Si:SA had a root length similar to that of the control plants at doses of 1 and 10 µM (Figure 2g–k) and those treated with Ch:SA at doses of 1, 10 and 50 µM (Figure 2i–p). Intermediate lengths (shorter that controls but longer than free SA treated plants) were found at higher doses for both capsules.

Growth rate of the Arabidopsis roots was calculated for each treatment and dose, a value that depends on the root tip elongation [38]. Growth rate in roots of control plants was, approximately, 0.7 cm on the 1st day and 0.4 cm on the rest (2nd to 5th day) (Figure 3 and Supplementary Table 1). The first day of analysis, root growth rate in plants treated with free SA was similar to that of controls only at 1 µM dose. However, from the 2nd day and until the end of the test, growth rate was considerably reduced (Figure 3a). Final root length was 1.485 cm at the 1 µM dose (compared to the 2,393 cm of control roots), and much shorter in those plants treated with higher free SA concentrations. In other plant systems, constantly-applied free SA had a strong effect on primary root growth, due to inhibition of cell elongation at low doses (≤ 100 µM) [39] and complete stoppage at high doses (≥ 100 µM), as occurs in other dicots as bean [40] and cucumber [41]. Interestingly, plants treated with Si:SA and Ch:SA at the lowest doses had a root growth rate similar to control plants (Figure 3b-c), and when the three treatments were compared (free SA, Si:SA and Ch:SA) throughout the test period, it was observed that, in general, the encapsulation process (regardless of the capsule used) reduce the adverse effect of the SA on root growth (Figures 1, 2 and Supplementary Table 1).

The difference in root length among plants treated with the different products could be due to the progressive release of SA from the capsules [42], decreasing the amount of SA in the medium (available then to the plant). When treating with free SA, the amount of molecule available to the plant in the first moments coincides with the total dose, quickly stressing the plant and causing the death of root cells [43]. In this regard, the results of the root growth test performed from 0 to 12 hours (Supplementary Figure 1) corroborate the results shown in Figure 3. As can be noticed, the least toxic treatment was again Ch:SA, compared to Si:SA and free SA. The experiment confirmed that free SA becomes toxic very rapidly in the plant. No effects on growth rate was observed when plants were treated with only the Si and Ch capsules (Supplementary Figure 2).

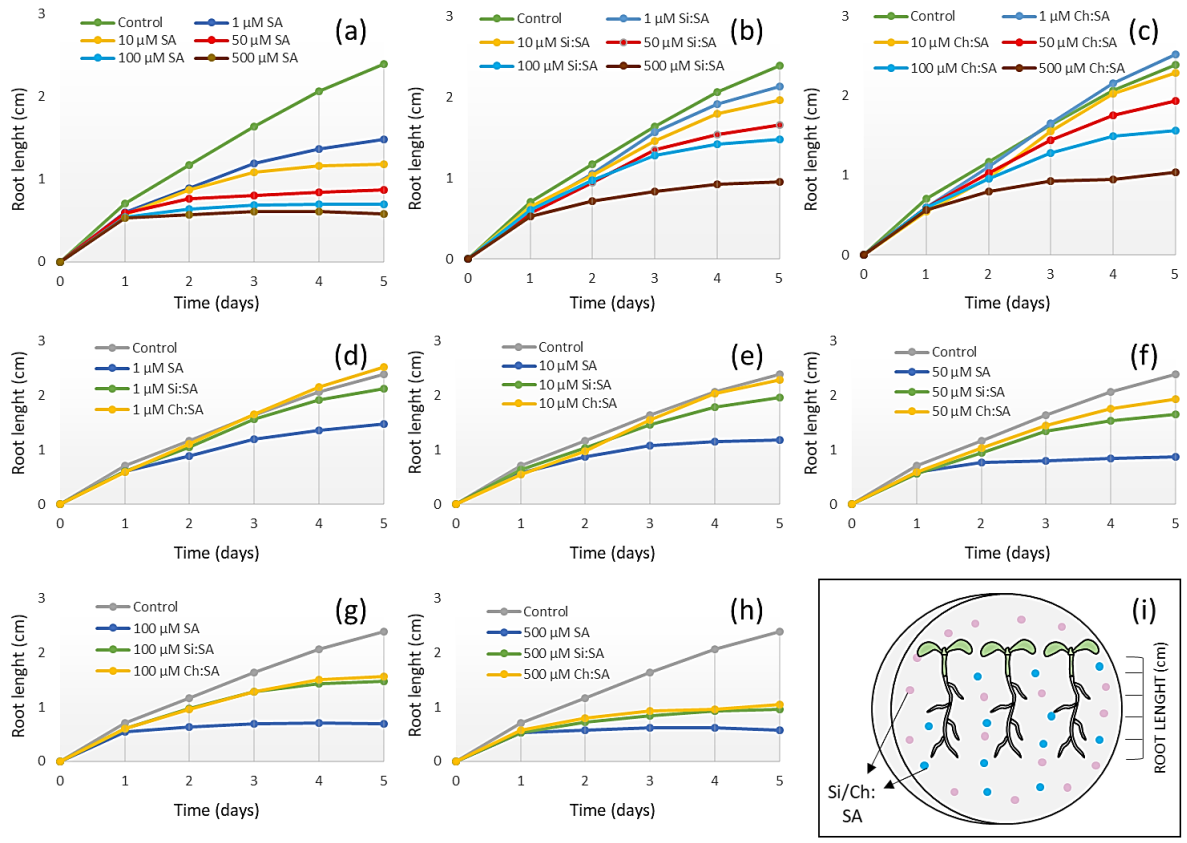

Figure 3. Effect of free SA, Si:SA and Ch:SA on root growth in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and root length measured daily (scheme i). Graphs (a), (b) and (c) compare root length among the doses at each treatment, and graphs (d), (e), (f), (g) and (h) compare root length among the treatments at each dose.

3.2. The encapsulation of SA allows the correct development of the rosette

The effect of SA on rosette size is an important parameter of healthy plants and the rosette area, in early stages, is proportional to biomass [44]. In fact, results show that SA treatment affects the rosette size in a dose-dependent manner, being rosettes smaller as free SA concentration increases, (Figure 4, Supplementary Figure 3). The constitutive response to high concentrations of SA may cause morphological alterations, such as dwarfism [45]. As shown in the Supplementary Figure 4 and quantified in Figure 4a, rosettes of plants treated with the Si:SA showed a size similar to those of the Control at concentrations of 1 and 10 µM. However, size decreased as the concentration of encapsulated SA increased (50, 100 and 500 µM). Ch:SA was even better as rosettes of treated plants were as large as controls at 1, 10 and 50 doses. Comparison among treatments reveals that rosettes treated with free SA had a very small size, even at the lowest doses (area was approximately half of that of control plants, Figure 4b–f). However, encapsulation decreases the adverse effects on rosettes. Rosette and root results

are consistent since high concentrations of SA available in the growth medium, increase the SA absorbed by the plant [46], which is possibly transported to the rosette and accumulated in the aerial tissues until plants are intoxicated. No effects on the rosette size were observed when plants were treated with only the Si and Ch capsules (Supplementary Figure 4).

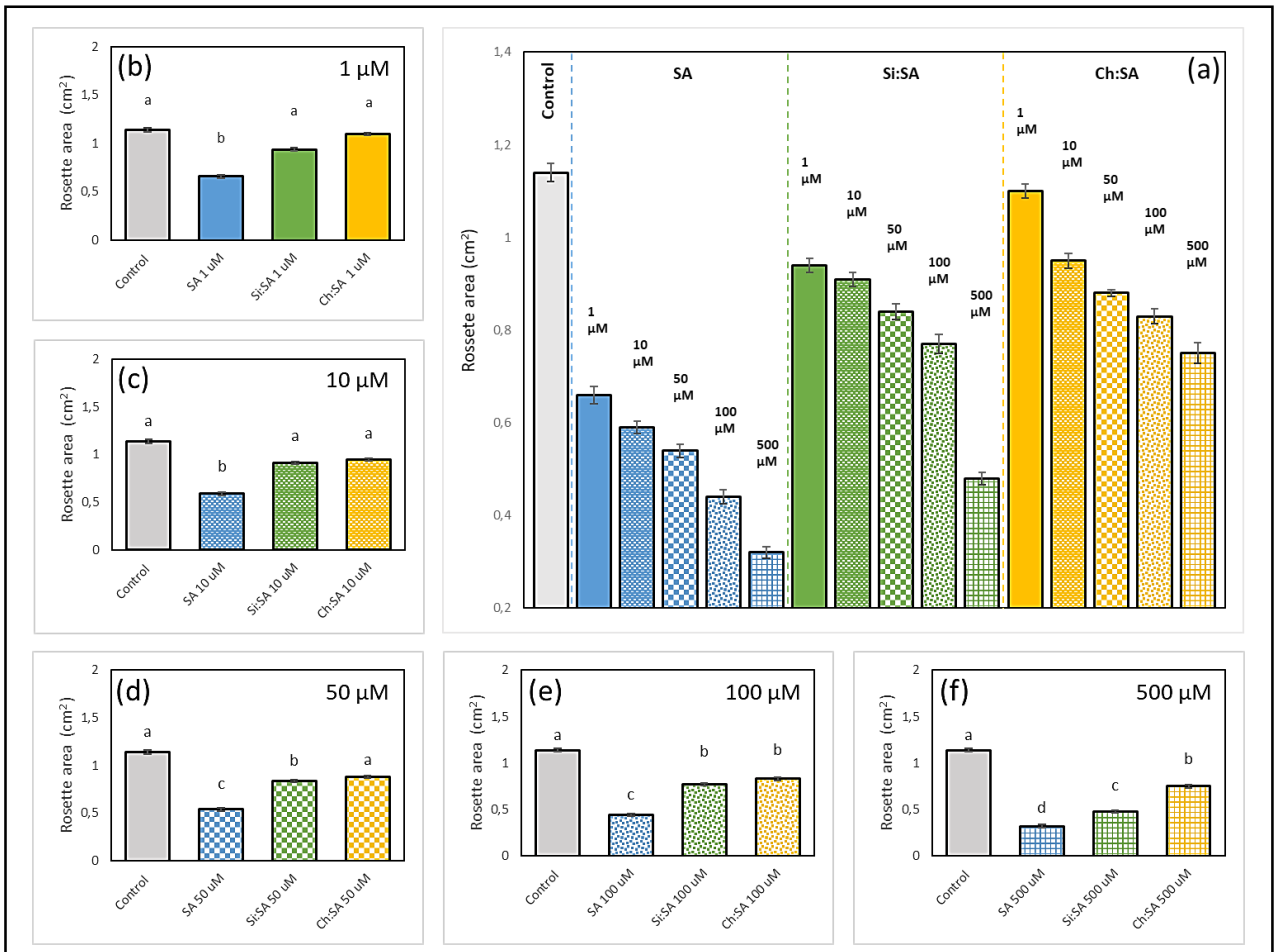

Figure 4. Effect of free SA, Si:SA and Ch:SA on rosette size in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and size was measured 5 days later. Graph (a), depicts rosette area for the three treatments and at all doses, and graphs (b), (c), (d), (e) and (f) compare rosette area among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

3.3. The encapsulation of SA prevents alteration of root gravitropism

Roots are able to feel and respond to gravity changes, always growing in the direction of the gravity vector, what is referred as positive gravitropism [47,48]. As depicted in Figure 5 and supplementary

Figure 5a, plants treated with free SA at the lowest doses showed a similar root gravitropism to the plants Control, 8 hours after the plant orientation change. However, roots lost the capacity of reorienting when plants were treated with free SA at 50 or 100 µM. At the highest dose (500 µM), roots became mostly agravitropic (unable to turn).

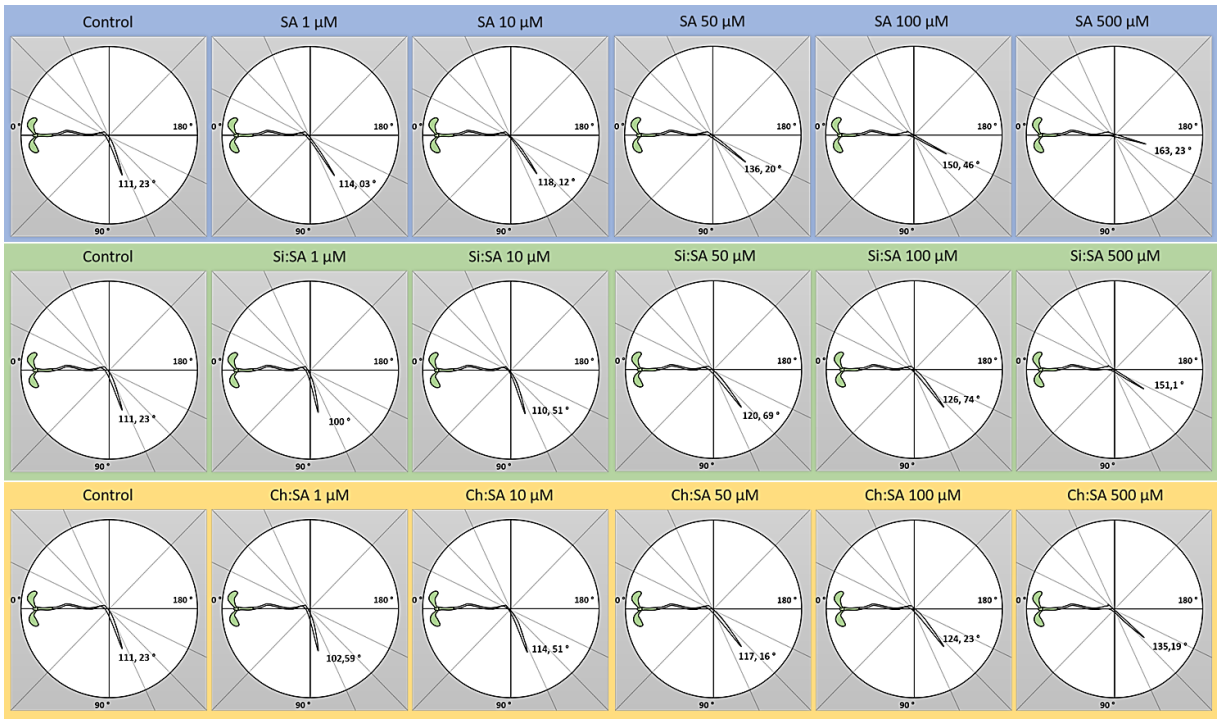

Figure 5. Effect of free SA, Si:SA and Ch:SA on root reorientation in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and root angle measured 8 hours later.

As shown in Supplementary Figure 6b, the same conclusions can be drawn 24 hours after the plant rotation. Previous reports showed that exogenous SA controls the root change orientation in an IAA crosstalk network [49] and reduces the root orientation angle in a dose-dependent manner, evidencing that SA has a negative effect on gravitropism [50,51]. However, the altered gravitropism response was significantly reverted when treating plants with the encapsulated hormone. Plants treated with SA encapsulated with any of the two capsules showed gravitropic roots (Figure 5 and Supplementary Figure 5a). After 24 hours of changing angle orientation, plant treated with the 1, 10 and 50 µM doses of Si:SA had a root orientation identical to Controls (Supplementary Figure 5b). Treatments with Ch:SA were still less aggressive considering this parameter and only plants treated with the highest dose had problems to change their angle orientation (Supplementary Figure 5b). These data show that exogenous SA alters the root gravitropism response in Arabidopsis plants until abolish it at high doses. We

hypothesise that this treatment induces changes in gene expression, especially in those genes related to auxin synthesis and transport that subsequently alter root patterning and growth direction [52]. However, encapsulation decreases SA deleterious effects on root gravitropism. No effects on the gravitropism response were observed when plants were treated with only the Si and Ch capsules (Supplementary Figure 6a-b).

3.4. The encapsulation of SA modulates endogenous SA accumulation in plants

A profile of phytohormones: SA, JA, ABA and IAA was obtained after 28 days of treatment, both from roots and rosettes. The most affected plants, with small tidied up rosettes and short and agravitropic roots, were those treated with free SA at 100 and 500 µM doses (Figure 6e-f).

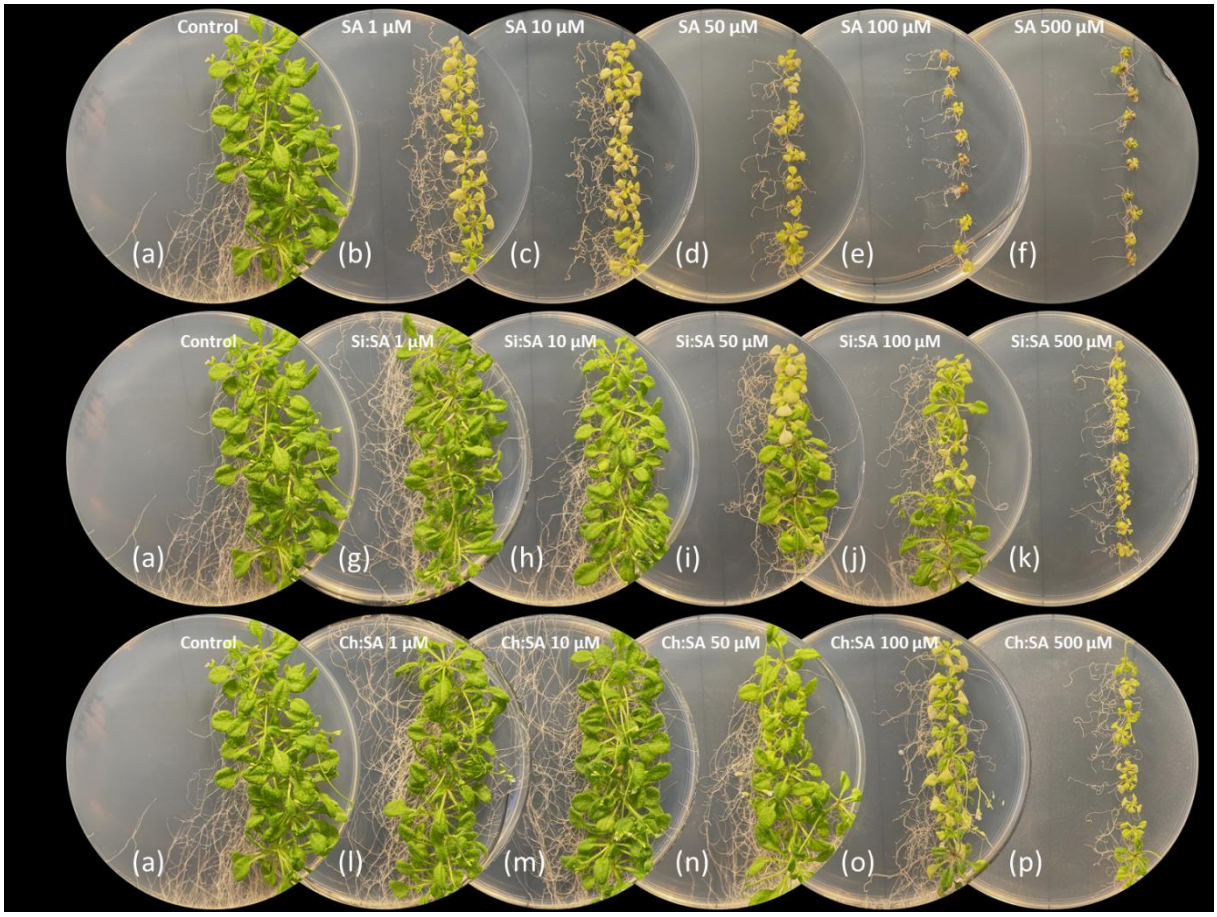

Figure 6. Effect of free SA, Si:SA and Ch:SA on plant performance in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and pictures were taken 28 days later.

These results agree with sections 3.1, 3.2 and 3.3 where the increase in SA concentration impaired plant growth. The negative effect of SA was reduced by the encapsulation process as reported before (Figures 2-4-5). These results confirm that the capsules have an optimal design as carriers and shields of SA, as well as for its gradual release, controlling the potential toxicity of the phytohormone [53]. Plants had higher levels of endogenous SA both in roots and rosettes after the treatment with free SA. However, treatments with the encapsulated SA importantly reduced these increased levels of endogenous SA. In detail, significant increases in the endogenous SA in rosettes was obtained after treating plants with all doses of free SA and only at the doses of 50, 100 and 500 µM for both treatments with encapsulated samples (Figure 7).

Endogenous SA levels in roots of the treated plants followed a similar pattern and roots treated with free SA had the highest values of endogenous SA, followed by those treated with Si:SA, and Ch:SA, for doses of 1, 10, 50, 100 µM (Figure 8b-c-d-e). At the 500 µM dose, no differences in root SA concentrations were detected among plants under the different treatments. However, it is important to highlight that plants grew differently depending on the treatment (Figure 6f-k-p). Hormones are important signals involved in the regulation of the cell division and size in plants [54,55]. Therefore, we propose that exposition to free SA increases endogenous hormone levels from the beginning of the experiment and this early increase causes short roots and small and pale rosettes. Encapsulation is able to control the early uptake of SA, limiting the amounts of hormone that roots absorb and translocation to the rosette. This regulation of the endogenous SA levels reduces the damaging effect of the treatments and, therefore, has less effect on plant phenotypes.

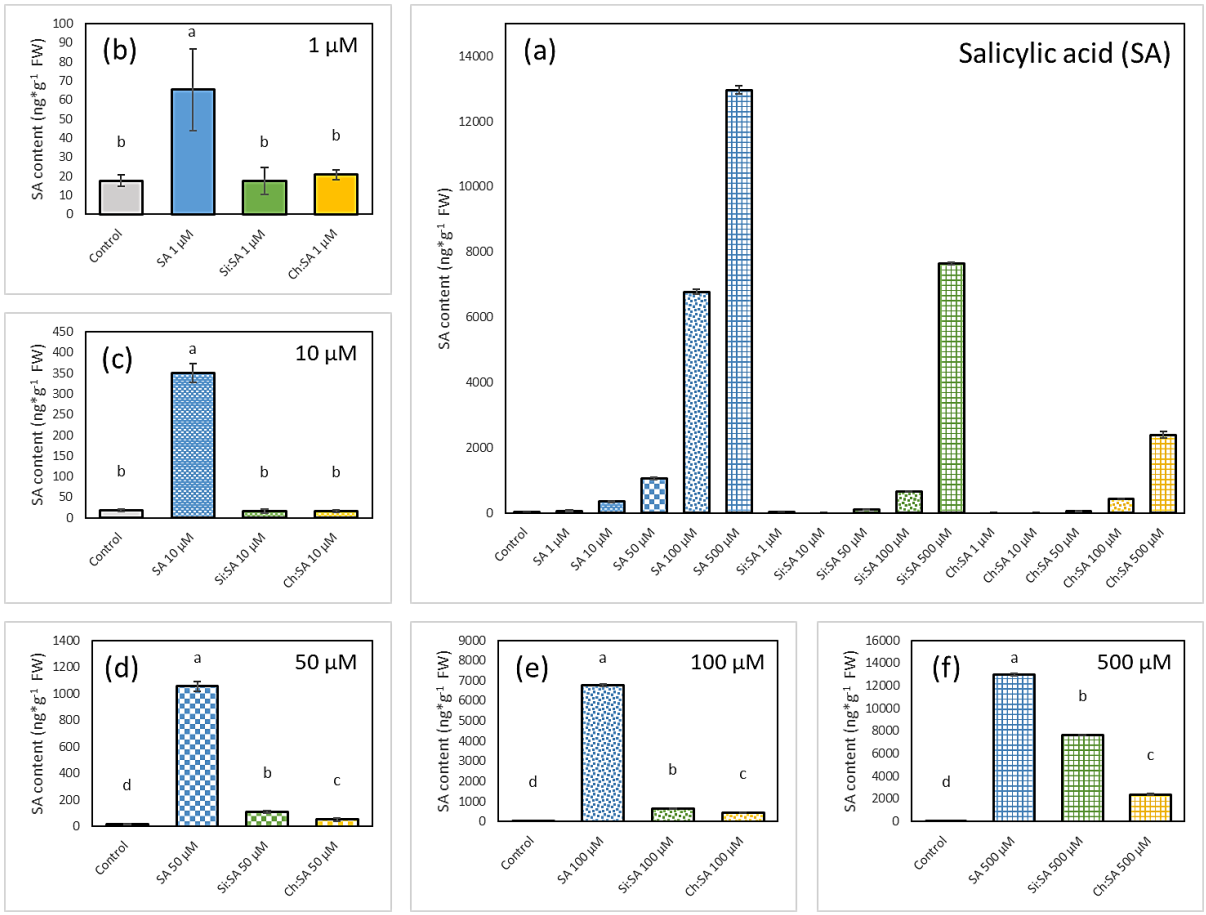

Figure 7. Effect of free SA, Si:SA and Ch:SA on endogenous SA levels in rosettes of Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 28 days later. Graph (a) depicts SA levels in the three treatments at all doses, and graphs (b), (c), (d), (e) and (f) compare SA levels among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

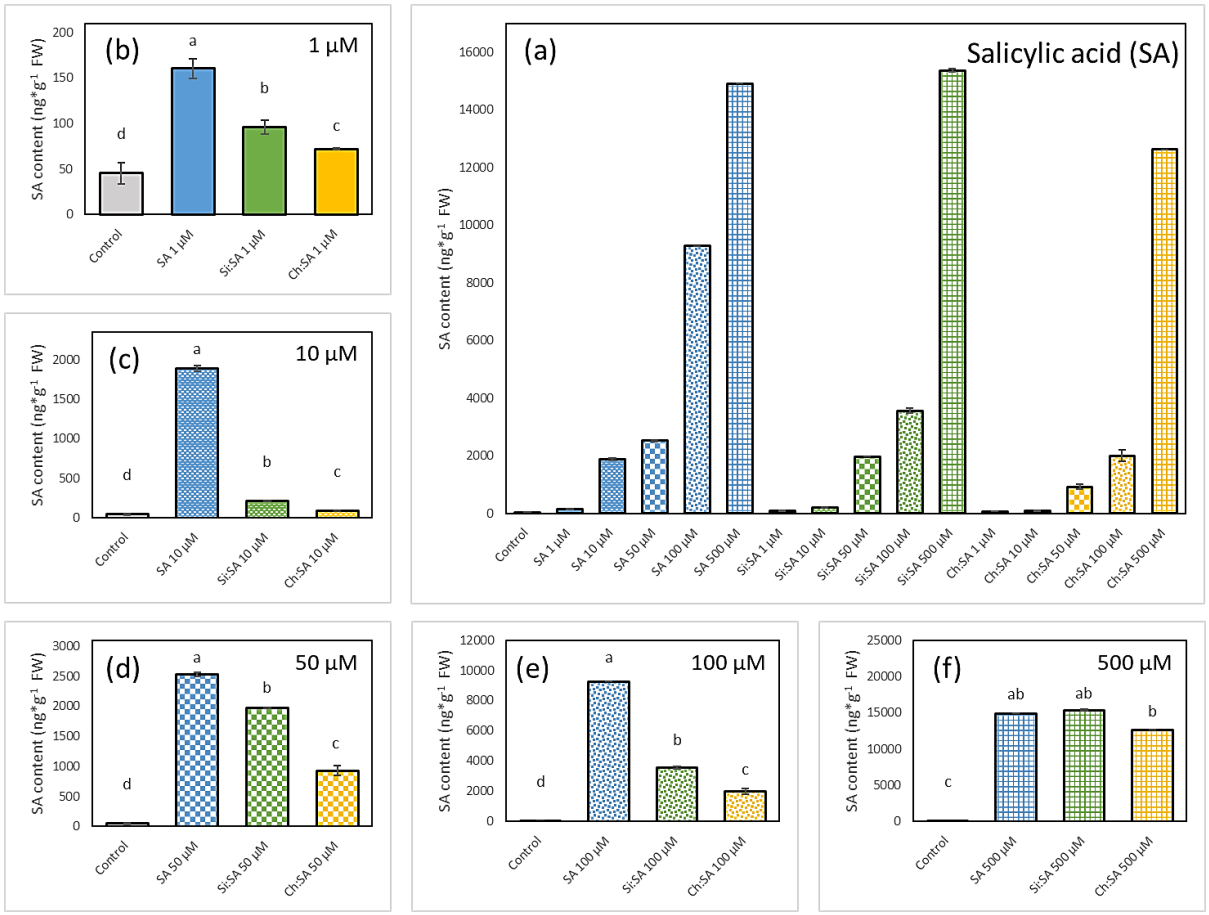

Figure 8. Effect of free SA, Si:SA and Ch:SA on endogenous SA levels in roots of Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 28 days later. Graph (a) depicts SA levels in the three treatments at all doses, and graphs (b), (c), (d), (e) and (f) compare SA levels among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

3.5. The encapsulation of SA modulates endogenous IAA accumulation in roots

The IAA is the main auxin that regulates root elongation and several developmental processes in plants, such as tissue differentiation, cell division, response to different pathogens, etc. [56,57]. Endogenous levels of IAA in the roots showed a noticeable depletion with the increase in the dose of free SA, reaching barely detectable values in plants treated with the 500 µM SA (Figure 9a). However, in plants treated with the encapsulated SA, fluctuant values were observed compared to the control plants (Figure 9b-c). In fact, SA regulates root growth together with IAA in a balanced pathway, which could be altered by the change in the levels of any of them (in this case the SA levels) and modify the root response [58], as roots are highly sensitive to fluctuations of the IAA levels [59].

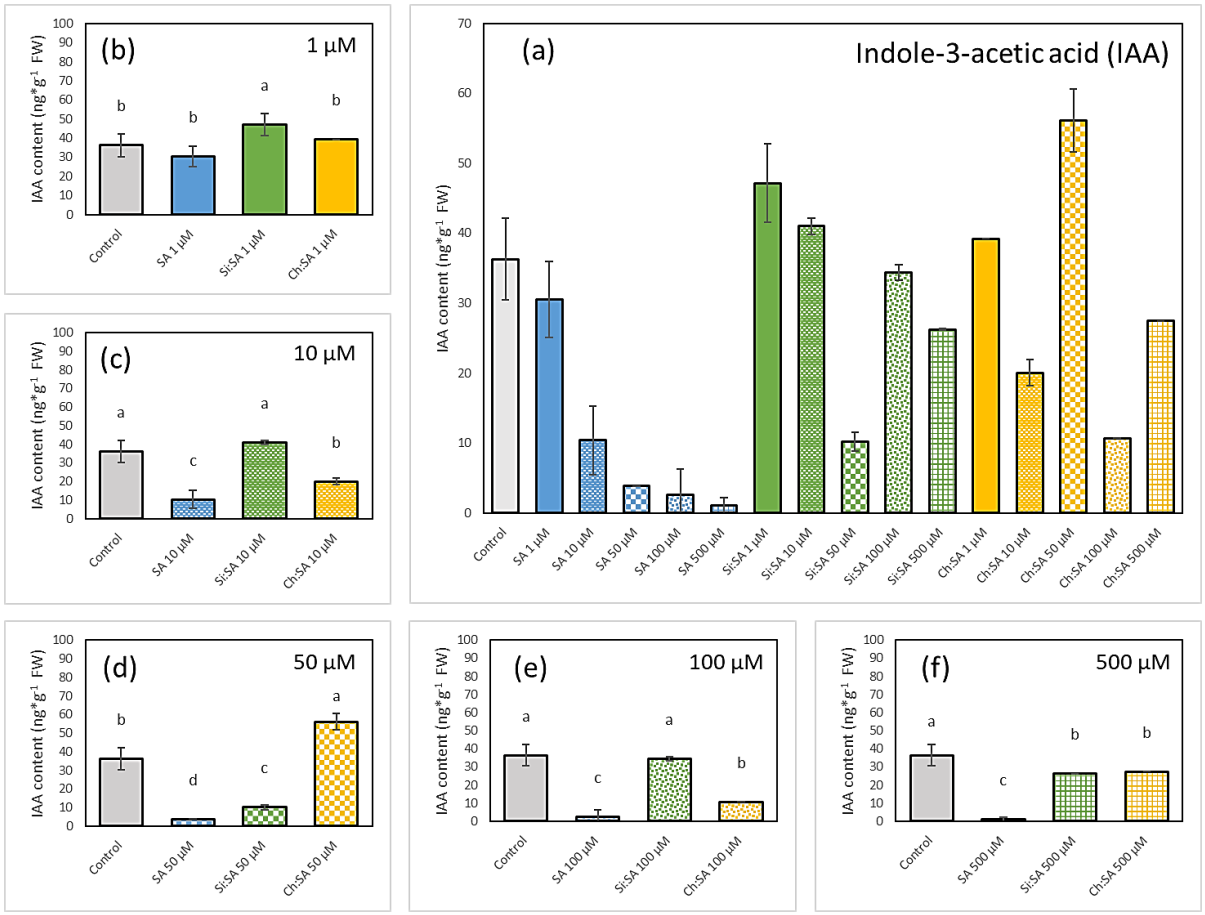

Figure 9. Effect of free SA, Si:SA and Ch:SA on endogenous IAA levels in roots of Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 28 days later. Graph (a) depicts IAA levels in the three treatments at all doses, and graphs (b), (c), (d), (e) and (f) compare IAA levels among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

To confirm the effect of SA treatments on IAA levels, a reporter line of *Arabidopsis thaliana* "DR5::GFP" was used [60]. The DR5 system allows monitoring auxin levels, especially in the root tip since this group of cells show important accumulation of IAA in response to any stimulus [61]. In general IAA levels in roots (Figure 9) correspond with the activity of the DR5::GFP auxin sensor in the quiescent centre (Figure 10) although some differences are observed, probably due to the specific cells monitored in the DR5 system (the quiescent centre) versus the bulk levels in the whole primary root detected in the analytical assay.

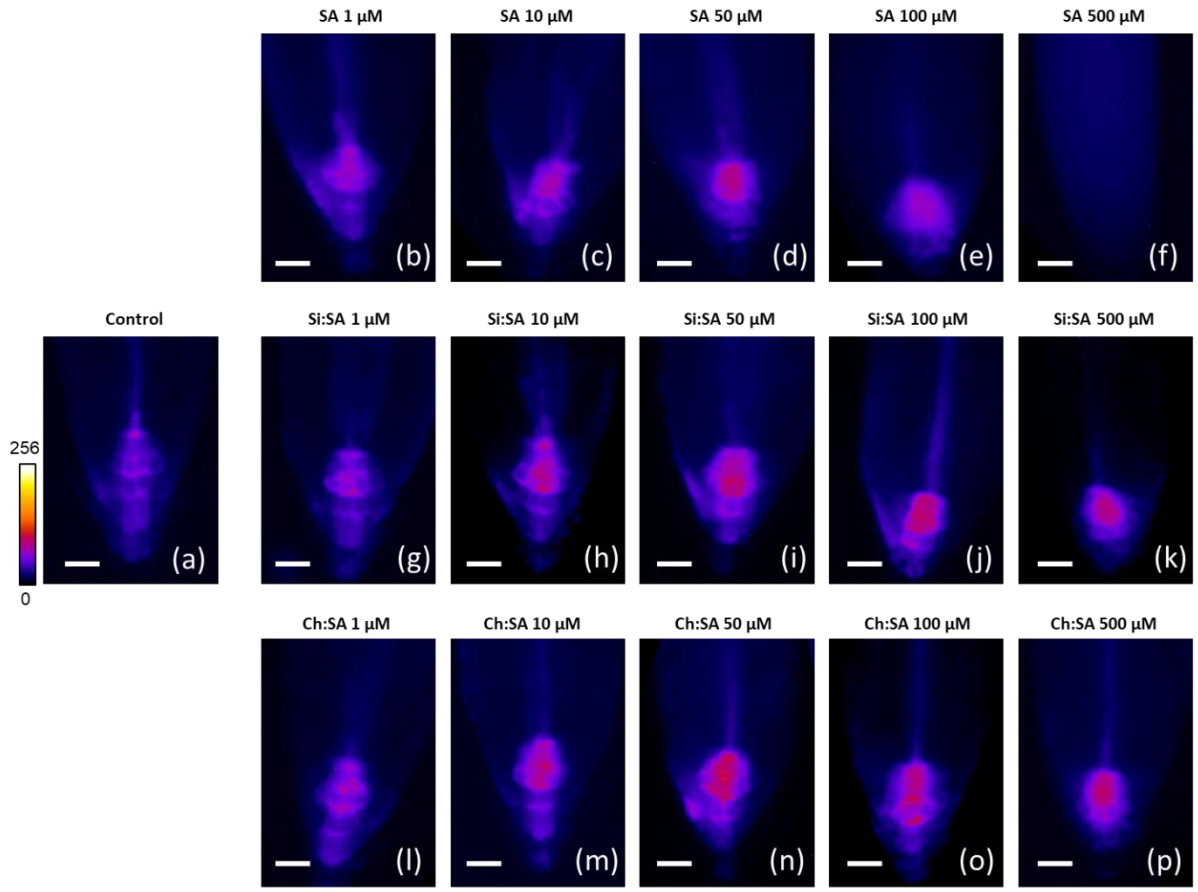

Figure 10. Effect of free SA, Si:SA and Ch:SA on root fluorescence in *Arabidopsis thaliana* DR5::GFP plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 5 days later. The scale represents 100 µm.

As depicted in Figure 10a, a progressive increase in the activity of DR5::GFP in plants treated with 1, 10 and 50 µM SA doses were observed. Interestingly, in plants treated with Si:SA and Ch:SA, fluorescence still increases at the 100 µM dose (Figure 10j-o) but had a slight decrease at the 500 µM dose (Figure 10k-p). However, in plants treated with free SA, fluorescence decrease was observed at the 100 µM dose (Figure 10e) and any activity could be found at the 500 µM dose (Figure 10f). This may be due to the fact that high doses of SA (≥ 100 µM) reduce auxin levels, decreasing the activity of the DR5::GFP auxin sensor in the quiescent centre [62]. Therefore, it seems that an effect of SA can be the suppression of the auxin flow from the stem to the root tip. This relationship between SA and IAA is related to their functions, well described in the literature, suggesting that the gradient of IAA plays an essential role in the correct dynamics of the root growth [63]. In fact, the encapsulated samples prevented IAA levels from declining further at doses of 100 and 500 µM, allowing roots to growth in contrast with roots treated with free SA. The different treatments with SA cause small changes in the

levels of JA and ABA in a random way that did not allow to identify any pattern of regulation (Supplementary Figure 7 and Supplementary Figure 8).

3.6. General comparison of free SA vs encapsulated SA treatments

Results of root growth, root angle, rosette area and phytohormone levels (SA, JA, ABA, IAA) from the roots were evaluated in a Principal Component Analysis (PCA) plot to reduce the dimensionality of our datasets and avoid losing important information [64]. To establish the relation among the variables analysed in the treatments, a plot of variables was made to correlate the importance of each variable in the main component [65]. According to the results shown in the Variables-PCA plot (Supplementary Figure 9a), there is a positive correlation and a good quality representation of the variable in the principal component (cos2) among root growth per day, root angle at 8h and SA content, grouped in the same direction, and with a less cos2, root growth per hour and the IAA content had a positive correlation. However, a negative correlation is observed among rosette area, JA content and ABA content, since these variables are represented on the opposite side to the origin and also plotted in the opposite quadrants (Supplementary Figure 9a). According to these results, SA and IAA levels explain the direct relationship between SA levels and the affection in the roots of the treated plants. In the same way, a correlation matrix was made to highlight the importance of the variables in the two main components. The results matrix showed that all variables were found in the first principal component (Dim1), except that of JA content which is found in the second principal component (Dim2) (Supplementary Figure 9b). This graph shows that the variables are perfectly represented by only two main components in this case, Dim.1 and Dim.2 [66].

The PCA is also capable of evaluating the correlation among several treatments, growth parameters, and internal phytohormone levels in plants [67]. The individual PCA of the treatments and the different doses of SA revealed that the two main components covered approximately 81.8% of the total variance (66.6% and 15.2% for Dim1 and Dim2, respectively) (Figure 11). In the first Dim1, the treatments with free SA are grouped, observing a large separation between the doses of free SA and Si:SA at 100 and 500 µM doses, so free SA and encapsulated SA at these doses caused important morphological changes in plants. Moreover, these negative effects become stronger when the SA doses increase and raise point distribution variability of Free SA and Si:SA treatments at highest doses values in the PCA (Figure 11). On the other hand, in the Dim2, the treatment with Si:SA at doses of 1, 10 and 50 µM and those of Ch:SA at all doses are grouped, which indicates that plants were not seriously affected (as these values are not different from those of the Control with a similar profile and less variability), placing them within a range in which the plant is affected but still is able to develop.

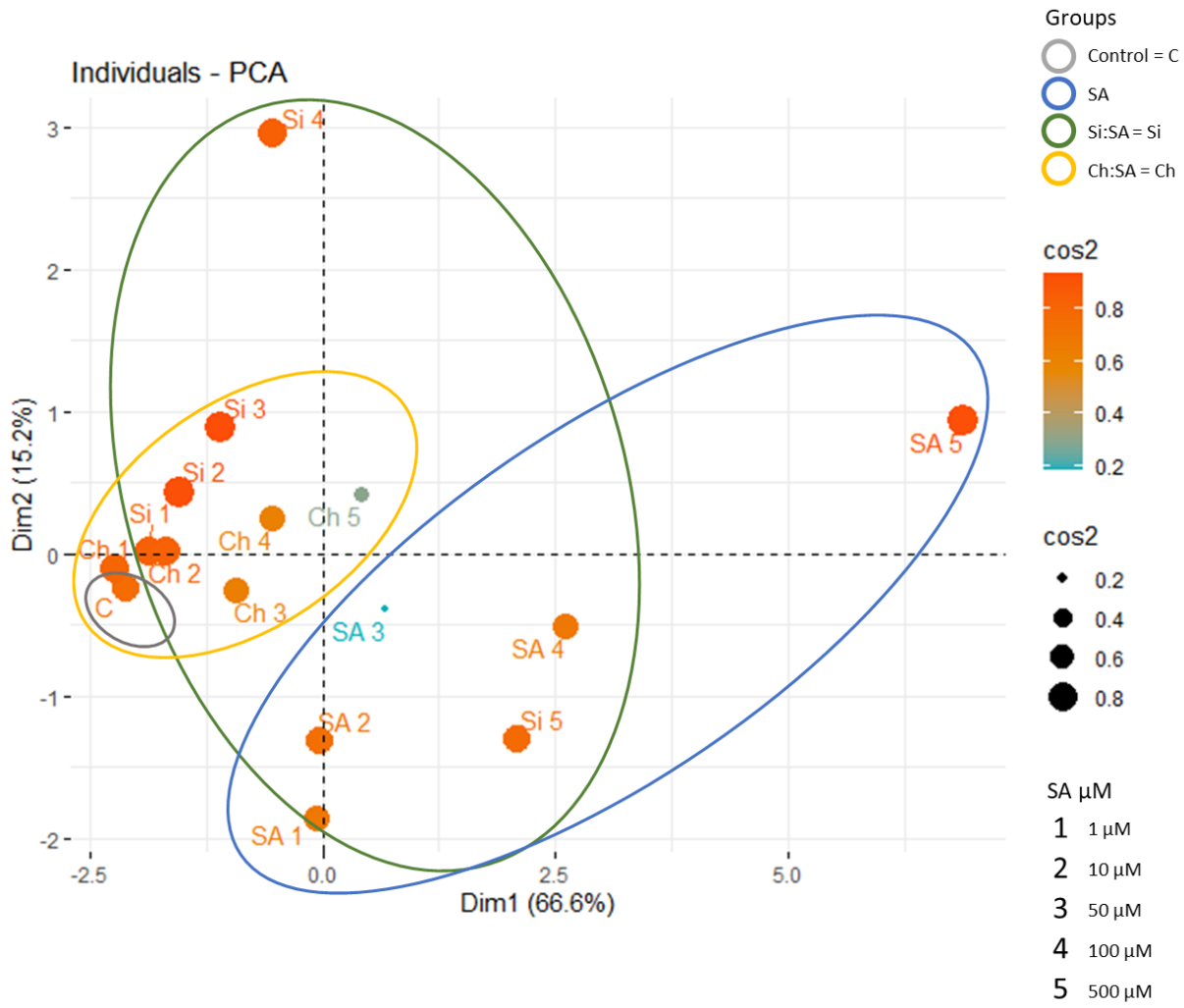

Figure 11. Individual-PCA of Col-0 Arabidopsis plants treated with free SA, Si:SA and Ch:SA at 1, 10, 50, 100 and 500 µM doses.

4. Conclusions

The experiments performed demonstrate that encapsulation prevents the uncontrolled release of SA and decrease the adverse pleotropic effects of the free SA treatment on plant physiology. Plants are able to take up free SA when it is available in the medium. This rapid uptake affects the structure and length of the roots, and the size and architecture of the rosettes in a concentration-dependent manner, due to both the high SA accumulation in both tissues and the decrease in the IAA accumulation and activity, especially in the root. However, changes in IAA levels in root germ cells due to fast uptake of SA, can be prevented by its encapsulation, reducing the amount available for the plants. SA encapsulation with silica or chitosan results in a controlled release of SA and, therefore, in less negative effects (when compared with free SA), avoiding that plants suffer impaired physiological and morphological

responses. Encapsulated samples, at lowest doses, have no impact on treated plants, being Ch:SA the least harmful. At the highest doses (100 and 500 µM) plants are more damaged, because the amount of SA is excessive. Differences among treatments are consistent with the PCA, showing that encapsulation is a useful method to control deleterious SA effects. When comparing capsules, Ch has less impact on treated plants, which maintain a relative normal development, and allows to work with highest doses in comparison with the Si capsule. Future work will be aimed at studying the effect of encapsulated hormones on plants under conditions that emulate the climate change to evaluate the positive effect that encapsulated sample can have on plant tolerance to biotic and abiotic stress conditions. In these future studies, the system developed in this work with Arabidopsis growing in controlled conditions can be a key tool to decipher the optimal capsules, hormone dose range and optimal plant growth conditions before extrapolating the experimental design to the greenhouse or fields.

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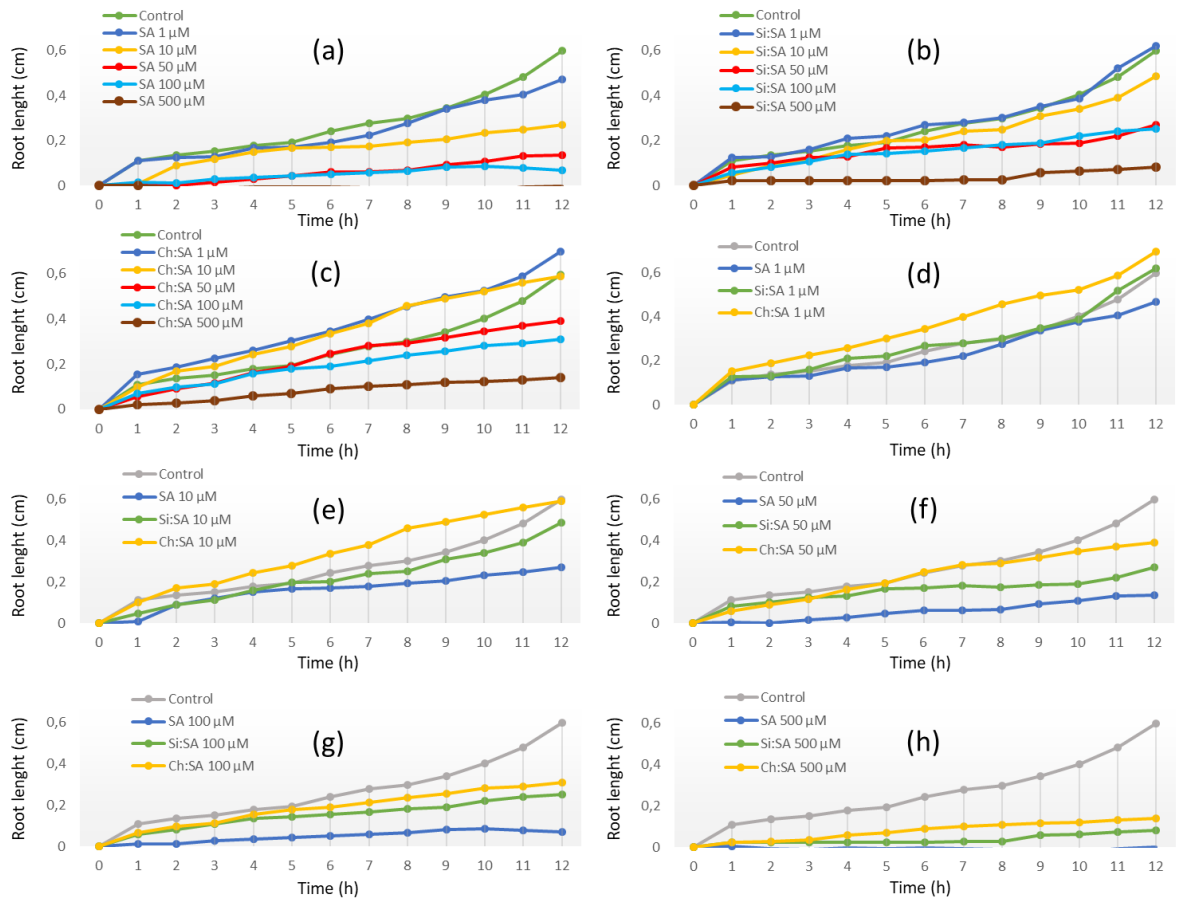
Supplementary material

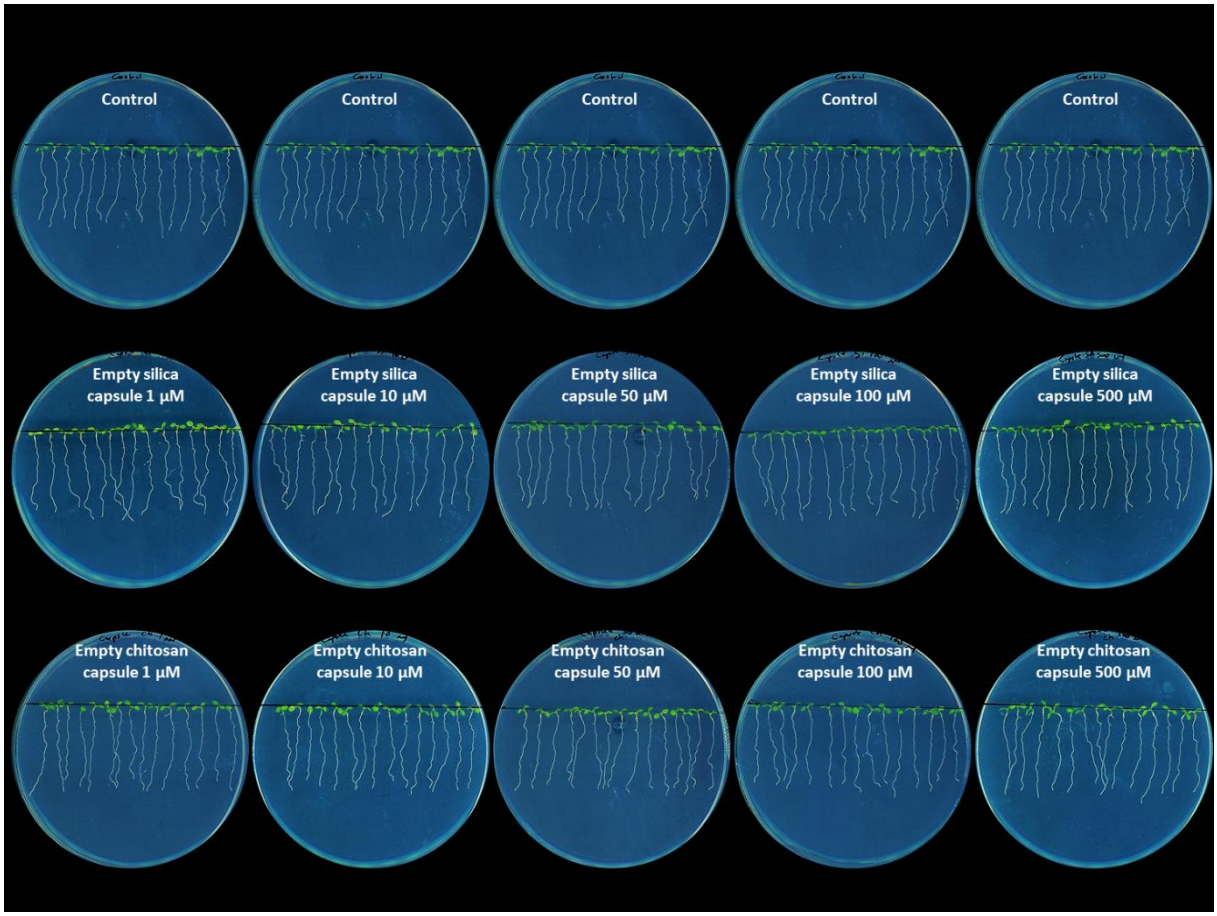
Supplementary Table 1. Root length of Col-0 Arabidopsis plants treated with free SA, Si:SA and Ch:SA. 5-day-old plants were transferred to media containing the different SA treatments and root length measured daily. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

	TREATMENTS					
		Doses				
Days	**Control**	**1 µM**	**10 µM**	**50 µM**	**100 µM**	**500 µM**
SA						
1	0.701 ± 0.07^{a}	0.603 ± 0.10^{ab}	0.572 ± 0.15^{b}	0.593 ± 0.11^{b}	0.543 ± 0.11^{bc}	0.530 ± 0.09^{c}
2	1.162 ± 0.09^{a}	0.894 ± 0.11^{bc}	0.875 ± 0.09^{c}	0.766 ± 0.10^{d}	0.635 ± 0.20^{de}	0.567 ± 0.10^{e}
3	1.639 ± 0.14^{a}	1.190 ± 0.18^{bc}	1.081 ± 0.25^{c}	0.804 ± 0.16^{de}	0.685 ± 0.23^{e}	0.612 ± 0.13^{f}
4	2.059 ± 0.16^{a}	1.364 ± 0.19^{b}	1.158 ± 0.25^{c}	0.839 ± 0.15^{d}	0.700 ± 0.21^{de}	0.610 ± 0.12^{e}
5	2.393 ± 0.19^{a}	1.485 ± 0.22^{bc}	1.185 ± 0.27^{c}	0.866 ± 0.16^{d}	0.692 ± 0.22^{e}	0.578 ± 0.13^{f}
Si:SA						
1	0.701 ± 0.07^{a}	0.601 ± 0.10^{ab}	0.640 ± 0.07^{ab}	0.563 ± 0.09^{ab}	0.605 ± 0.07^{ab}	0.526 ± 0.09^{bcd}
2	1.162 ± 0.09^{a}	1.055 ± 0.11^{ab}	1.028 ± 0.10^{ab}	0.947 ± 0.11^{b}	0.967 ± 0.07^{cd}	0.715 ± 0.14^{d}
3	1.639 ± 0.14^{a}	1.565 ± 0.18^{ab}	1.463 ± 0.19^{ab}	1.348 ± 0.11^{b}	1.276 ± 0.09^{c}	0.838 ± 0.20^{d}
4	2.059 ± 0.16^{a}	1.917 ± 0.20^{a}	1.792 ± 0.25^{ab}	1.537 ± 0.09^{c}	1.423 ± 0.08^{de}	0.920 ± 0.17^{e}
5	2.393 ± 0.19^{a}	2.132 ± 0.19^{a}	1.968 ± 0.21^{ab}	1.653 ± 0.08^{b}	1.477 ± 0.13^{c}	0.954 ± 0.18^{d}

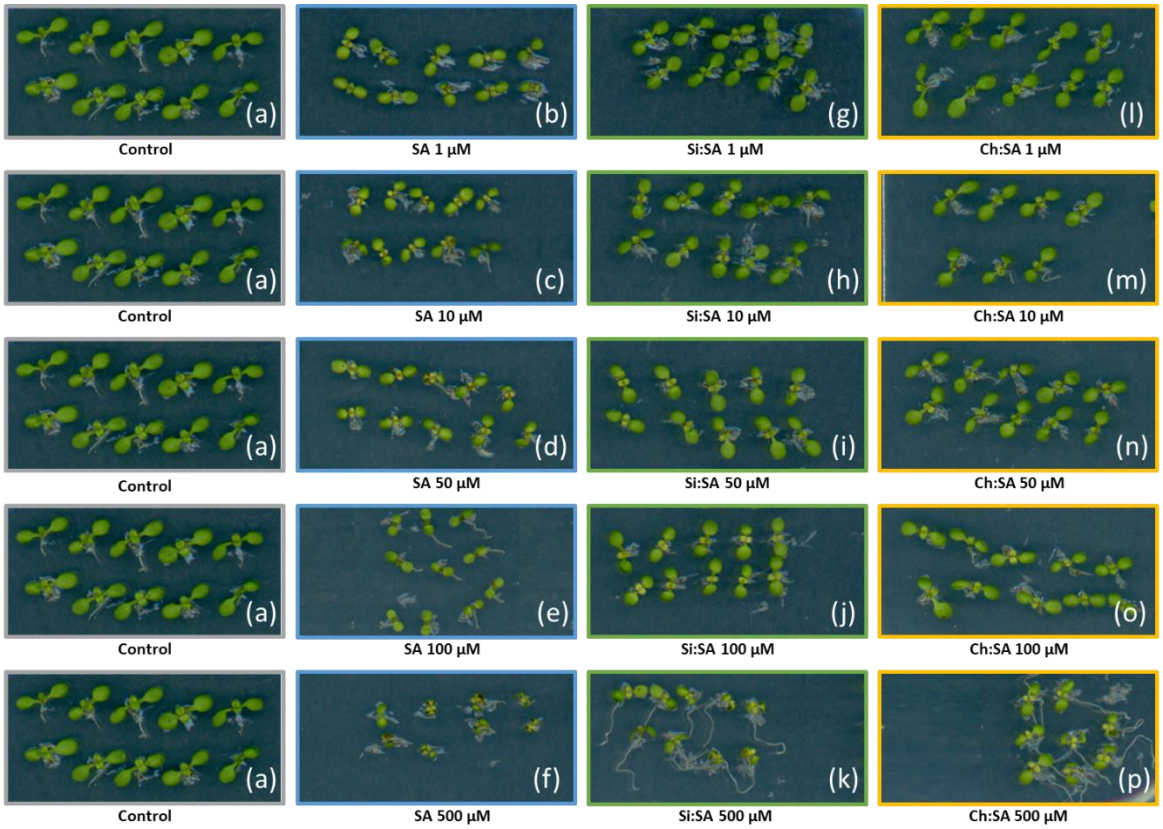
Ch:SA						
1	0.701 ± 0.07^{a}	0.597 ± 0.05^{ab}	0.549 ± 0.10^{ab}	0.591 ± 0.13^{ab}	0.596 ± 0.08^{ab}	0.567 ± 0.08^{ab}
2	1.162 ± 0.09^{a}	1.109 ± 0.09^{a}	0.971 ± 0.09^{ab}	1.032 ± 0.14^{ab}	0.954 ± 0.17^{b}	0.794 ± 0.11^{c}
3	1.639 ± 0.14^{a}	1.650 ± 0.13^{a}	1.555 ± 0.08^{ab}	1.447 ± 0.23^{b}	1.280 ± 0.25^{c}	0.930 ± 0.17^{d}
4	2.059 ± 0.16^{a}	2.158 ± 0.18^{a}	2.031 ± 0.17^{ab}	1.754 ± 0.32^{b}	1.497 ± 0.30^{c}	0.950 ± 0.19^{d}
5	2.393 ± 0.19^{a}	2.521 ± 0.26^{a}	2.290 ± 0.20^{a}	1.942 ± 0.40^{ab}	1.561 ± 0.27^{b}	1.039 ± 0.18^{c}



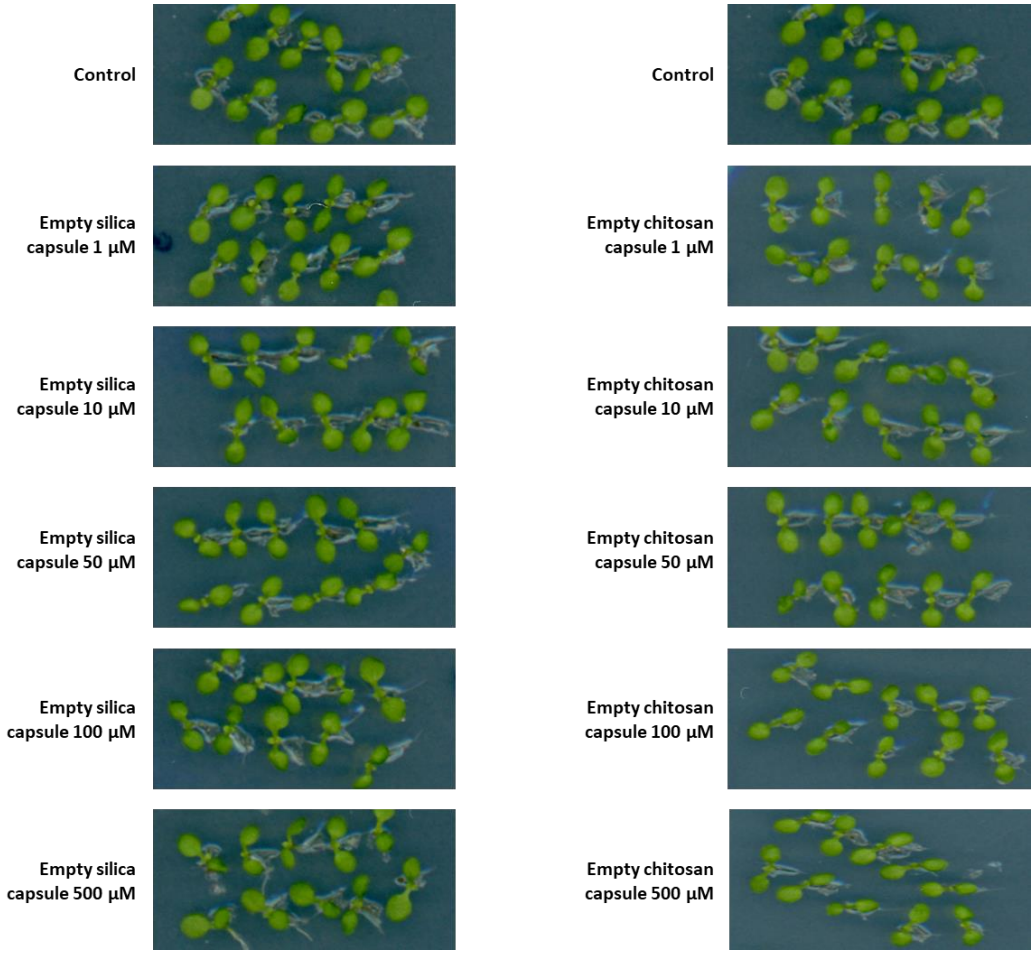
Supplementary Figure 1. Effect of free SA, Si:SA and Ch:SA on root growth in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and root length measured each hour (0 to 12 hours). Graphs (a), (b) and (c) compare root length among the doses at each treatment, and graphs (d), (e), (f), (g) and (h) compare root length among the treatments at each dose.



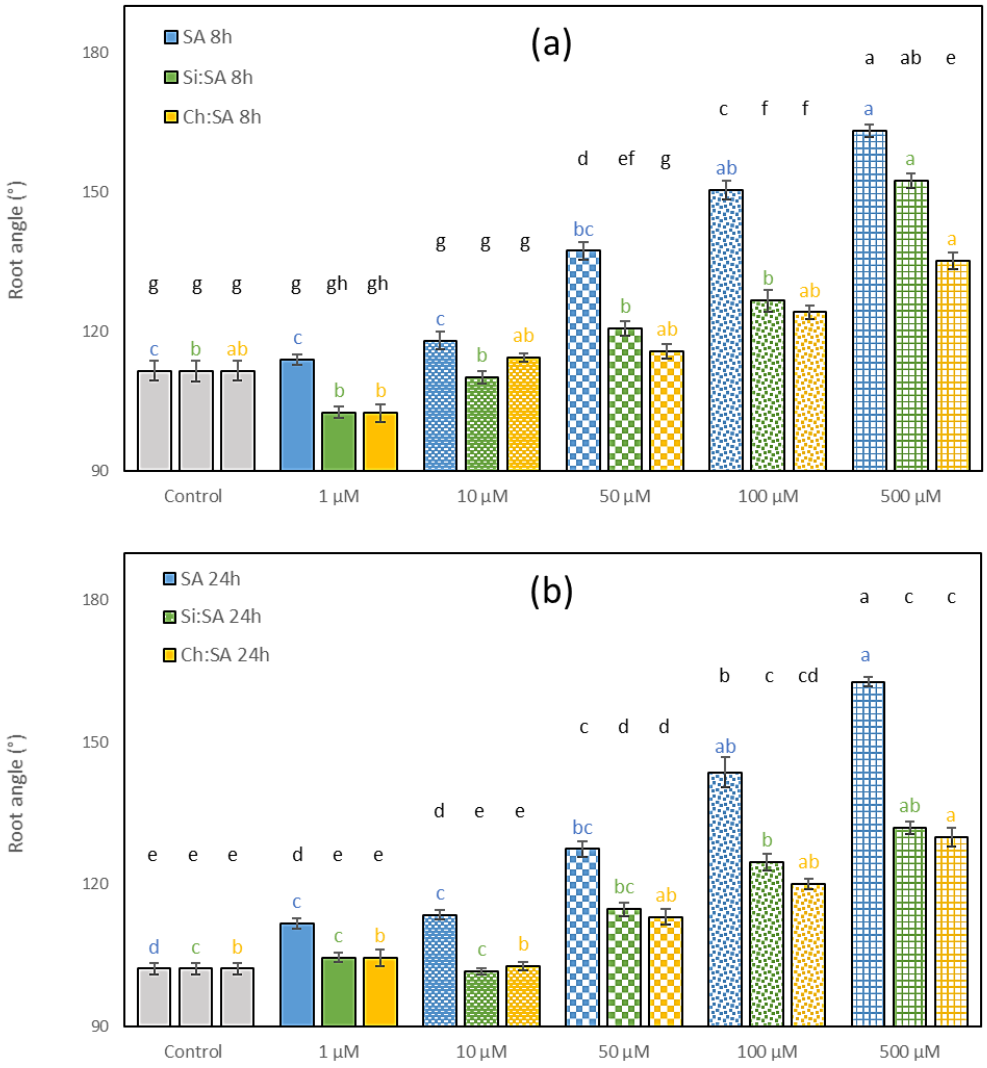
Supplementary Figure 2. Effect of empty silica and chitosan capsules on root growth in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the empty capsules and pictures were taken 5 days later.



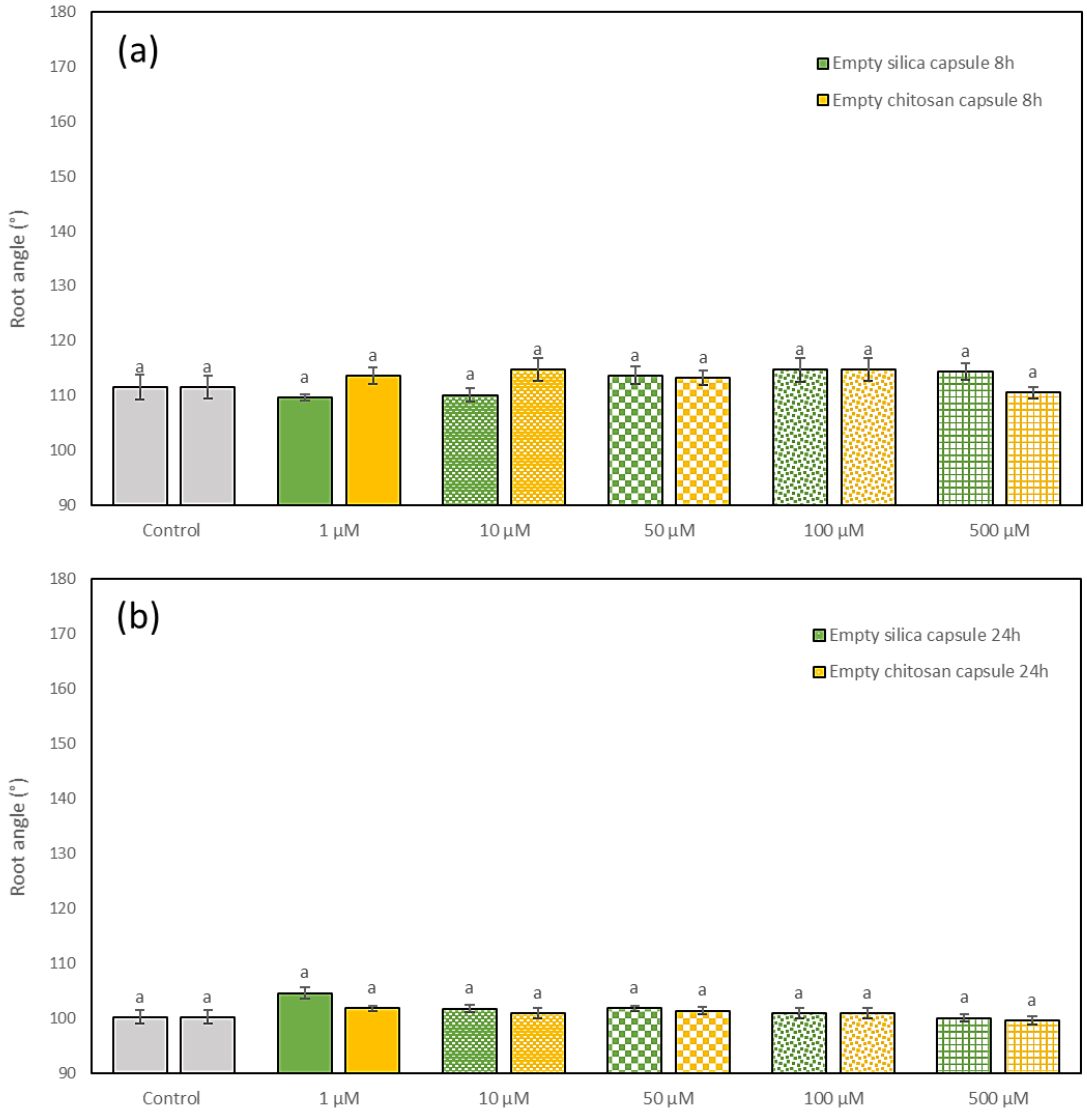
Supplementary Figure 3. Effect of free SA, Si:SA and Ch:SA on rosette size in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and pictures were taken 5 days later.



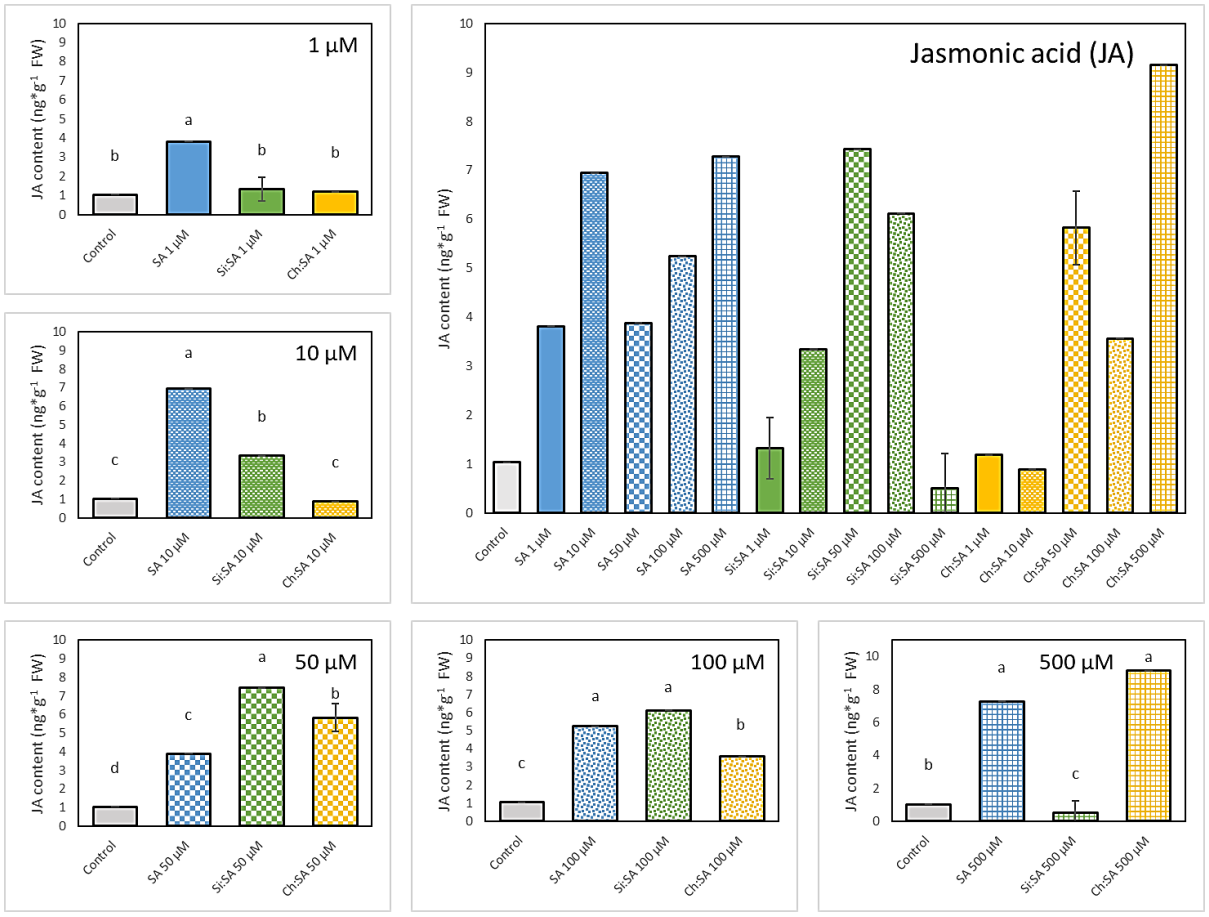
Supplementary Figure 4. Effect of empty silica and chitosan capsules on rosette size in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the empty capsules and pictures were taken 5 days later.



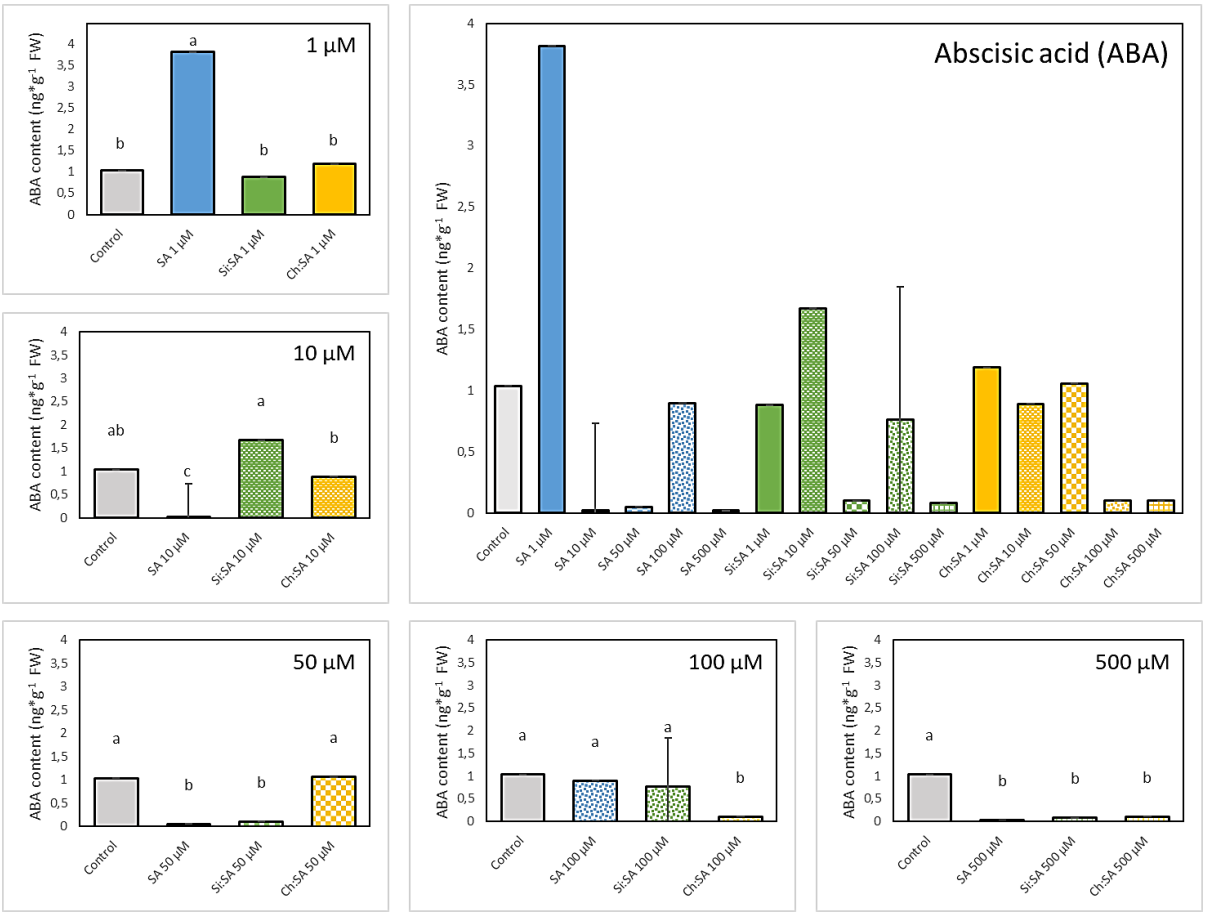
Supplementary Figure 5. Effect of free SA, Si:SA and Ch:SA on root reorientation in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and root angle measured 8 and 24 hours later. Different letters indicate significant differences among treatment groups at $p \leq 0.05$. The colored letters are comparisons of dose and Control in that particular treatment, and the black letters are comparisons of dose and Control among all treatments.



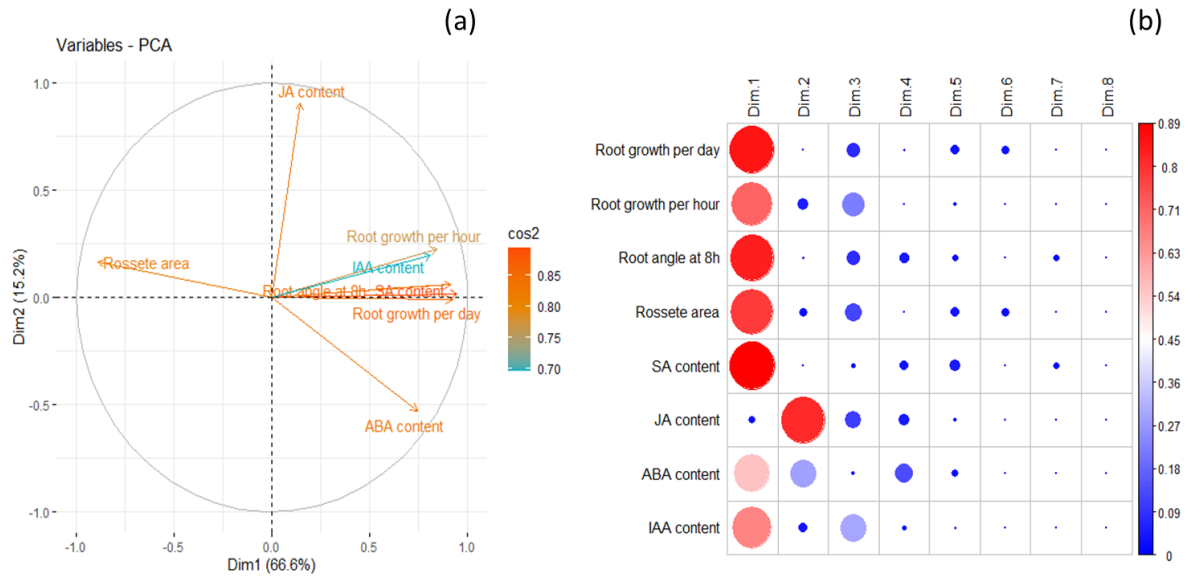
Supplementary Figure 6. Effect of empty silica and chitosan capsules on root reorientation in Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the empty capsules and root angle measured 8 and 24 hours later. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.



Supplementary Figure 7. Effect of free SA, Si:SA and Ch:SA on endogenous JA levels in roots of Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 28 days later. Graph (a) depicts JA levels in the three treatments at all doses, and graphs (b), (c), (d), (e) and (f) compare JA levels among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.

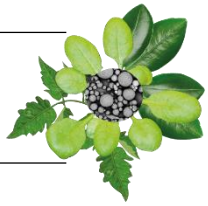


Supplementary Figure 8. Effect of free SA, Si:SA and Ch:SA on endogenous ABA levels in roots of Col-0 Arabidopsis plants. 5-day-old plants were transferred to media containing the different SA treatments and plant hormones measured 28 days later. Graph (a) depicts ABA levels in the three treatments at all doses, and graphs (b), (c), (d), (e) and (f) compare ABA levels among the treatments at each dose. Different letters indicate significant differences among treatment groups at $p \leq 0.05$.



Supplementary Figure 9. Correlation graphics of variables measured in Col-0 Arabidopsis plants treated with free SA, Si:SA and Ch:SA at 1, 10, 50, 100 and 500 µM doses. Graph (a) depicts the Variables-PCA plot where, in the cos2 color scale, red denotes a good representation of the variable in the principal component and blue that the variable is not perfectly represented, and graph (b) depicts correlation variables matrix.

CHAPTER 4



THE EFFECTIVENESS OF ENCAPSULATED SALICYLIC ACID AS A TREATMENT TO ENHANCE ABIOTIC STRESS TOLERANCE STEMS FROM MAINTAINING PROPER HORMONAL HOMEOSTASIS

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Key words for the tittle: Auxins – DR5::GFP – In vitro – Plant adaptation – RT-qPCR

Unpublished

Abstract

Climate change is a major threat to agriculture, inducing significant abiotic stresses that adversely affect both the quantity and quality of crop yields. Phytohormones play pivotal roles within the intricate stress response system of plants. One promising solution to improve plant resilience under adverse conditions is the application of exogenous salicylic acid (SA). While SA application offers benefits in plant stress response, its negative effects on growth and development are a concern. Encapsulation with protective materials like amorphous silica and chitosan have demonstrated a controlled release of SA, minimizing detrimental impacts on Arabidopsis plant growth. In this work, we elucidate the physiological and biochemical mechanisms behind this protective mechanism. We employed in vitro cultivation of Arabidopsis, comparing plant responses to both free and encapsulated SA under conditions of salt or mannitol stress, combined or not with high temperature (30°C). Plants treated with encapsulated SA displayed an enhanced tolerance to these stresses that was due, at least in part, to the maintenance of physiological endogenous SA levels, which in turn regulate indole-3-acetic acid (IAA) homeostasis. The activity of the Arabidopsis "DR5::GFP" reporter line supported this finding. Unlike plants treated with free SA (with altered DR5 activity under stress), those treated with encapsulated SA maintained similar activity levels to control plants. Moreover, stressed plants treated with free SA overexpressed genes involved in the SA biosynthesis pathway, leading to increased SA accumulation in roots and rosettes. In contrast, plants treated with encapsulated SA under stress did not exhibit increased expression of EDS1, PAL1, and NPR1 in roots, or of PAL1, PBS3, and NPR1 in rosettes. This indicates that these plants likely experienced lower stress levels, possibly because the encapsulated SA provided sufficient defense activation without triggering pleiotropic effects. Our findings demonstrate that encapsulated SA enhances plant tolerance to adverse conditions, offering a viable strategy for sustaining agricultural productivity in the face of climate change.

1. Introduction

Plants are constantly exposed to a range of unfavourable abiotic environmental conditions, including drought, heat, cold, nutrient deficiencies, and excessive levels of salt or toxic metals in the soil [1]. Abiotic stresses stand as the responsible of a crop yield reduction by over 50% at global scale, generating substantial economic losses [2]. Among these stresses, water scarcity, high salinity, and high temperatures exert the most significant impact due to their widespread influence on plant growth and productivity. The main causes of osmotic stress in plants are drought and salinity which reduce the water potential of the environment [3]. Osmotic stress causes several effects on plants, including the inhibition of cell elongation [4], closure of stomata [5], decreased photosynthetic activity [6], and interruption of ion uptake [7]. Heat stress disrupts the cell membrane structure breaking ion balance

within plant cells, hampers plant growth and development, and leads to sterility and decreased yield [8,9]. Individually or in combination, these stresses induce oxidative damage and the formation of reactive oxygen species (ROS) that can even cause cell death [10]. Additionally, stress triggers physiological and metabolic alterations at various stages of plant development. For example, it can affect the germination process or inhibit photosynthesis in seedlings [11].

To ameliorate the negative effects caused by these stresses, plants have developed several strategies, such as modifying metabolic pathways [12], optimizing nutrient and water uptake [13], accumulating specific metabolites [14], among others. Most of these responses lead are mediated by phytohormone signalling [15]. The primary stress-related phytohormones include abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonates (JAs), and salicylic acid (SA) [16]. Additionally, other phytohormones such as cytokinins (CKs), ethylene, gibberellins (GAs), brassinosteroids, and strigolactones, significant influence on plant adaptation to unfavourable conditions [17]. During abiotic stress, IAA is involved in promoting root growth away from soil areas with high levels of salinity [18], and mediates hypocotyl elongation in plants under heat stress [19,20]. ABA regulates lateral root formation in response to salt stress [21,22] and activates SnRK2-type protein kinases to induce stomatal closure under osmotic stress [23]. Additionally, ABA improves seedling performance (and survival) during heat stress [24,25]. JAs interact with IAA during heat stress and also are involved in stomatal closure [26].

SA plays a multifaceted role in mediating plant defense mechanisms against environmental stresses [27]. As a phenolic compound, SA not only regulates plant growth and development [28], but also governs fundamental physiological processes such as photosynthesis [29], nitrogen metabolism [30], proline metabolism [31], glycine betaine production [32], the antioxidant defense system [33], and the regulation of plant-water relations [34]. It also has a role as a protective agent under abiotic stresses [35]. Treatments with exogenous SA can induce an acclimation effect, enabling plants to adapt to various types of abiotic stress [36]. Improvement in rice yield under high-temperature conditions has been reported when plants were treated with exogenous SA [37], as well as an enhancement in salt tolerance of pepper, cucumber, and soybean [38]. During osmotic stress, SA regulates the synthesis of osmolytes, triggering the accumulation of amino acids and their derivatives (especially proline and glycine betaine), soluble sugars, and polyamines [39]. In *Triticum aestivum*, the application of exogenous SA improved osmotic stress tolerance by increasing the proline content [40].

Currently, three main strategies are employed to enhance the effect of exogenous SA in plants, particularly in response to abiotic stresses: i) spraying, ii) priming, and iii) controlled release, which involves encapsulation [41]. Encapsulation of phytohormones is a technique that remains relatively unexplored, with only a few studies utilizing this approach for JAs, ABA, IAA, GAs, CKs, and SA [42]. Encapsulation entails the utilization of shell materials as carriers containing the phytohormones, allowing for controlled release [43]. The coating material should primarily provide protective properties

while also possessing other desirable characteristics such as flexibility, stability, strength, and permeability [44]. Moreover, the outer layer needs to be adaptable to various scenarios while maintaining durability against harsh conditions. Previous research conducted by Sampedro-Guerrero et al. 2022 [45] involved treating *Arabidopsis thaliana* plants with SA encapsulated in two coating materials, silica and chitosan. Compared to free SA treatment, encapsulated SA treatment alleviated the negative effects observed in plants.

In this context, the objective of this study consists of elucidating the physiological and biochemical mechanisms underlying the protective effect of encapsulated SA on stressed plants. This objective was accomplished by comparing and evaluating several physiological, biochemical and genetic parameters in Arabidopsis plants cultivated under salt stress, mannitol stress, and their combination with heat stress, treated or not with free SA or encapsulated SA.

2. Materials and Methods

Figure 1 illustrates the flow diagram employed in this study: i) formulation of the treatments, sowing of Arabidopsis seeds and subsequent transfer of 4-day-old plants; ii) analysis encompassing measurements of root length, rosette area, and secondary root density for both plant cultivations; iii) extraction and quantification of endogenous phytohormones; iv) visualization of the auxin-specific reporter gene (DR5::GFP) in roots; v) quantification of SA-related genes expression; and vi) statistical analysis.

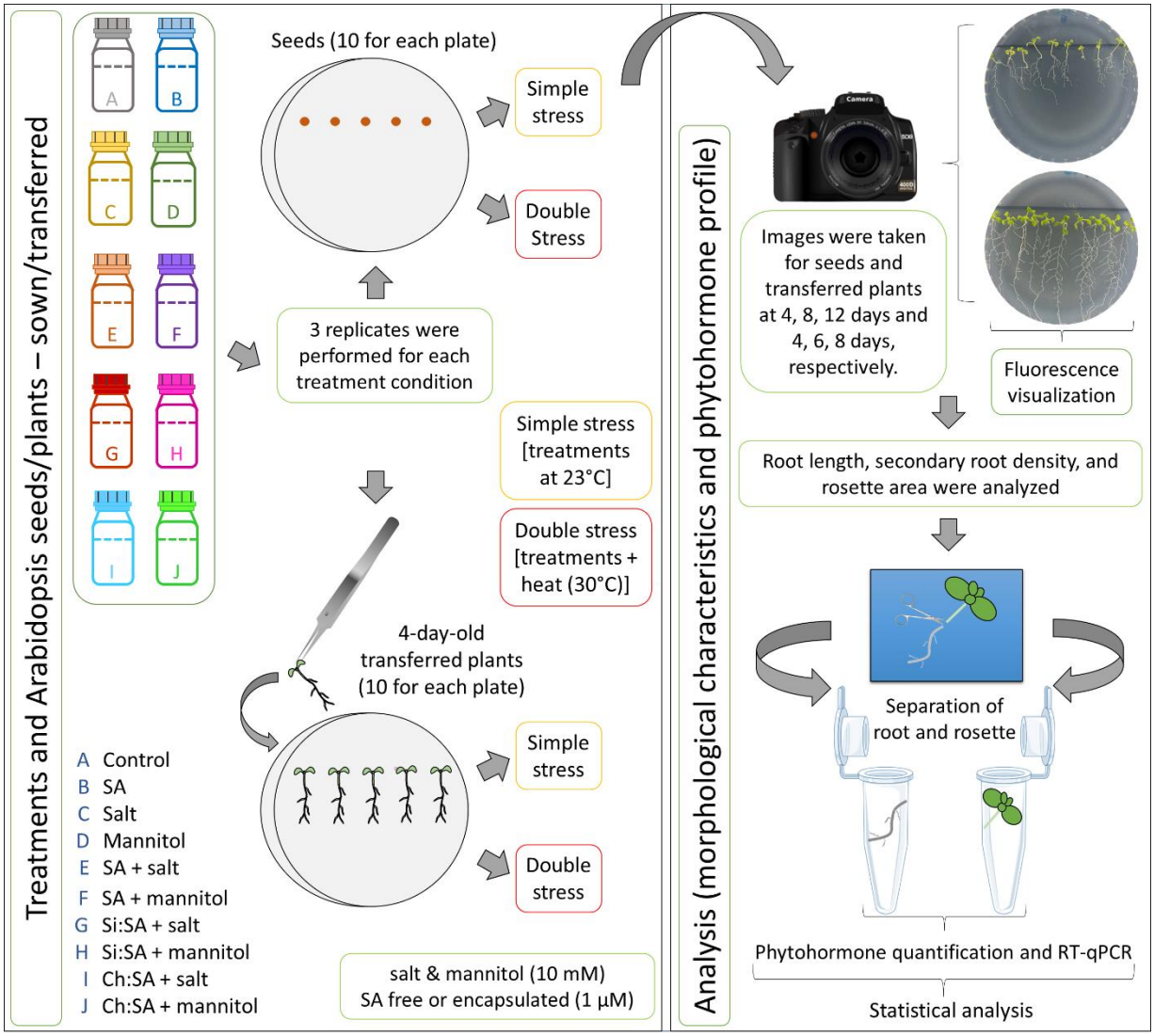

Figure 1. Experimental method used to study the protective effect of encapsulated SA in Arabidopsis stressed plants.

2.1. Materials. In this study, the following resources were used: pyrogenic silica HDK®S13 (Si) of WACKER (Barcelona, Spain), chitosan DG CHI0.20 g/mL and 85% deacetylated (Ch) of AOXIN (Shanghai, China) and Salicylic acid (SA) of Sigma-Aldrich (St. Louis, MO, USA). The formulation of the plant media was based on Murashige & Skoog Medium (MS) 0.5% (Duchefa Biochemie), sucrose 1% (D(+)-Sucrose for molecular biology, PanReac AppliChem), and agar at 1% (European Bacteriological Agar; Condalab). The media was poured onto petri dishes (9×15 cm) in a horizontal laminar flow cabinet (ASTEC MICROFLOW, North Somerset, UK), and then, conserved at 4°C.

2.2. Plant material and growth conditions. *Arabidopsis thaliana* Columbia ecotype (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK), and those of the DR5::GFP line from the Arabidopsis Biological Resource Center (Columbus, OH, USA). Seeds underwent surface sterilization using a 1%v/v sodium hypochlorite and 0.01%v/v Tween 20 solution for 10 minutes. Afterward, they were rinsed three times with sterile distilled water and then sown in the petri dishes with the media. Petri dishes were vertically arranged in growth chambers (SANYOMLR-350, Sakata, Gunma, Japan) under 16 h light/8 h dark cycles at 23°C (normal conditions) or 30°C (heat stress conditions).

2.2.1. Treatment formulation and conditions. Table 1 outline the different treatment conditions. The encapsulated SA samples were prepared according to the methodology outlined by Sampedro-Guerrero et al. (2022) [46]. Briefly, Si:SA (1:0.25 w/w) and Ch:SA (1:0.5 w/w) ratios were obtained through spray-drying (SD-06 spray drier, Lab Plant, UK) of an aqueous suspension prepared using specific quantities of Si and Ch. The suspensions were homogenized using the planetary mixing technique (Pulverisette®, Fritsch, IDar-Oberstein, Germany). For heat stress, all treatments described in Table 1 were applied at 30°C. Supplementary Table 1 presents the treatments that served as control conditions for the empty capsules and encapsulated SA samples.

Table 1. Description of treatments applied to *Arabidopsis thaliana* plants.

Treatment	Description	Concentration
Control	Murashige & Skoog (MS)	-
SA	free SA	1 µM
Salt	Salt	10 mM
Mannitol	Mannitol	10 mM
SA + salt	free SA and salt	1 µM/10 mM
SA + mannitol	free SA and mannitol	1 µM/10 mM
Si:SA + salt	Encapsulated SA in silica and salt	1 µM/10 mM
Si:SA + mannitol	Encapsulated SA in silica and mannitol	1 µM/10 mM
Ch:SA + salt	Encapsulated SA in chitosan and salt	1 µM/10 mM
Ch:SA + mannitol	Encapsulated SA in chitosan and mannitol	1 µM/10 mM

2.2.2. Morphological characteristics analysis of Arabidopsis plants. Two different methodologies were used for plant cultivation: i) used seeds and ii) used 4-day-old transferred plants. For i), 10 seeds were sown in each treatment. Photographs were taken on the 4th, 8th, and 12th days after sowing. For ii) 10 seeds were sown in MS media to facilitate their growth. On the 4th day after sowing, seedlings were transferred to dishes containing the different treatments (Figure 1). Subsequently, photographs were taken on the 4th, 6th, and 8th days after transplantation. These experiments were replicated three times, and photographs were taken using a camera (Sony DSC-H300) and analyzed with ImageJ 1.53t software to measure root length and rosette area, while EZ-Rhizo 2.5.5.1 software was used to determine secondary root density.

2.3. Extraction and phytohormones quantification. Endogenous phytohormone levels were determined at each sampling day. Plants were sampled, and both the rosettes and roots were promptly frozen with liquid nitrogen for storage until further analysis. Extraction and analysis procedures followed the methodology previously outlined [47], with some minor adjustments. Specifically, 0.2 g of plant tissue was homogenized with 1 mL of 50% acetonitrile using a ball mill (Millmix20, Domel Železniki, Slovenia), after spiking with 2.5 ng of [$^{2}H_{5}$]-indole acetic acid (IAA) and 25 ng of the following molecules: [$^{13}C_{6}$]-SA, dehydro jasmonic acid (DHJA), and [$^{2}H_{6}$]-ABA. The extracted samples underwent sonication and centrifugation to remove any debris. Subsequently, 1 mL of the sample was loaded onto an "Oasis HLB 1 cc Vac Cartridge, 30 mg (Waters, Mildford, CT, USA)" column with 500 µL of 30% acetonitrile, and the eluent was collected. The phytohormones SA, JA, ABA, and IAA were quantified in both rosettes and roots using high-performance liquid chromatography coupled online with a triple quadrupole mass spectrometer (Micromass, Manchester, UK) via an orthogonal Z-spray electrospray ion source [48].

2.4. Fluorescence Analysis. Four-day-old *Arabidopsis thaliana* DR5::GFP plants were initially grown in MS media before being transferred to various treatment conditions. Following 4 days of treatment, the entire plants were removed from the growth medium and mounted on microscope slides for examination under a confocal microscope. Fluorescence images of the root were captured using a Leica DMi8 microscope (Leica Microsystems cms GmbH, Wetzlar, Germany). The green fluorescent protein (GFP) was excited using a 488nm laser line and detected within the range of 493-553 nm. Z series images were obtained at 1-µm intervals using a 40× objective lens. The serial images were acquired using Leica Confocal software (V 2.61). Subsequently, the acquired images underwent processing using the FIJI plugin from the Image J software, and relative fluorescence levels were calculated.

2.5. Gene expression analysis. RNA was extracted from fresh frozen tissue, comprising both the rosettes and roots, using the RNeasy extraction kit (Qiagen, Germany). The RNA was treated with the RNAase-Free DNase Set (Qiagen, Germany) to remove any residual DNA. Subsequently, RNA

concentration and quality were assessed using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA), with absorbance ratios measured at 260/280 nm and 260/230 nm. Following this, 1 µg of extracted RNA was reverse transcribed into cDNA using the Primescript RT Reagent Kit (Takara, Japan). RT-qPCR analysis was conducted using an ABI Step One detection system (Applied Biosystems, CA, USA). The reaction mixture included 1 µl of retrotranscribed cDNA, 5 µl of SYBR Green/ROX (Thermo Scientific, USA), 1 µl of a 10 µM mix of each gene-specific primer pair, and 3 µl of RT-qPCR Grade Water (Thermo Scientific, USA) in a final volume of 10 µl per reaction. The temperature amplification curve involved an initial preincubation of 10 min at 95°C, followed by 40 amplification cycles. Each cycle comprised denaturation for 10 s at 95°C, annealing for 10 s at 60°C, and extension for 20 s at 72°C. The resulting data were analyzed using StepOne Software v2.3, and relative expression was calculated using the REST Software [49]. Actin (ACT) and tubulin (TUB) served as housekeeping genes to normalize gene expression levels. The primers employed for gene expression analysis are provided in Supplementary Table 2.

2.6. Statistical analysis. Each treatment comprised three replications, with a minimum of ten plants for replication. Statistical analysis was conducted using SPSS version 21 software. A one-way analysis of variance test (ANOVA) with Bonferroni correction was employed to determine significant differences between treatment groups at a significance level of $p \leq 0.05$. Additionally, a two-tailed Student´s t-test was used for phytohormone and RT-qPCR analysis. In the case of the latter, significance levels are indicated by *, ** and ***, denoting $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, in comparison to control plants values.

3. Results and Discussion

3.1. Treatments with encapsulated SA limit changes in morphological characteristic in stressed Arabidopsis plants

3.1.1. Root length

Plant roots act as first sensors to recognize adverse conditions, becoming the primary tissue affected during abiotic stress [50]. Plants exposed to stress conditions (salt or mannitol stress, combined or not with heat stress) exhibited reduced root length compared to control plants (Figures 2, 3, 4 and 5). These reductions were evident from six days after the onset of the stress treatments in seeds and from eight days after this point in transferred plants (Supplementary Figure 1). Osmotic stress directly triggers alterations in the root architecture, leading to growth inhibition, characterized by reduced root meristem size and slower root growth [51]. The combination of free SA with either salt or mannitol, along with the additional application of heat stress, exacerbated root shortening in plants. (Supplementary Figure

1). The disruption of auxin balance caused by increased SA concentration and its subsequent accumulation [45,52] may explain the observed root shrinkage.

Conversely, Arabidopsis plants treated with SA encapsulated with any of the two materials assayed demonstrated improved stress tolerance, occasionally even exceeding the root length of control plants (Supplementary Figure 1). In a study conducted by Aazami et al. [53], it was found that encapsulated SA protects *Vitis vinifera* plants under osmotic stress by enhancing their antioxidant defense system and preserving their morphological characteristics. Additionally, a separate investigation on *Leymus chinensis* showed harmful effects from excessive gibberellic acid (GA), highlighting the potential of controlled release technology to prevent exogenous GA treatment from affecting seed germination [54].

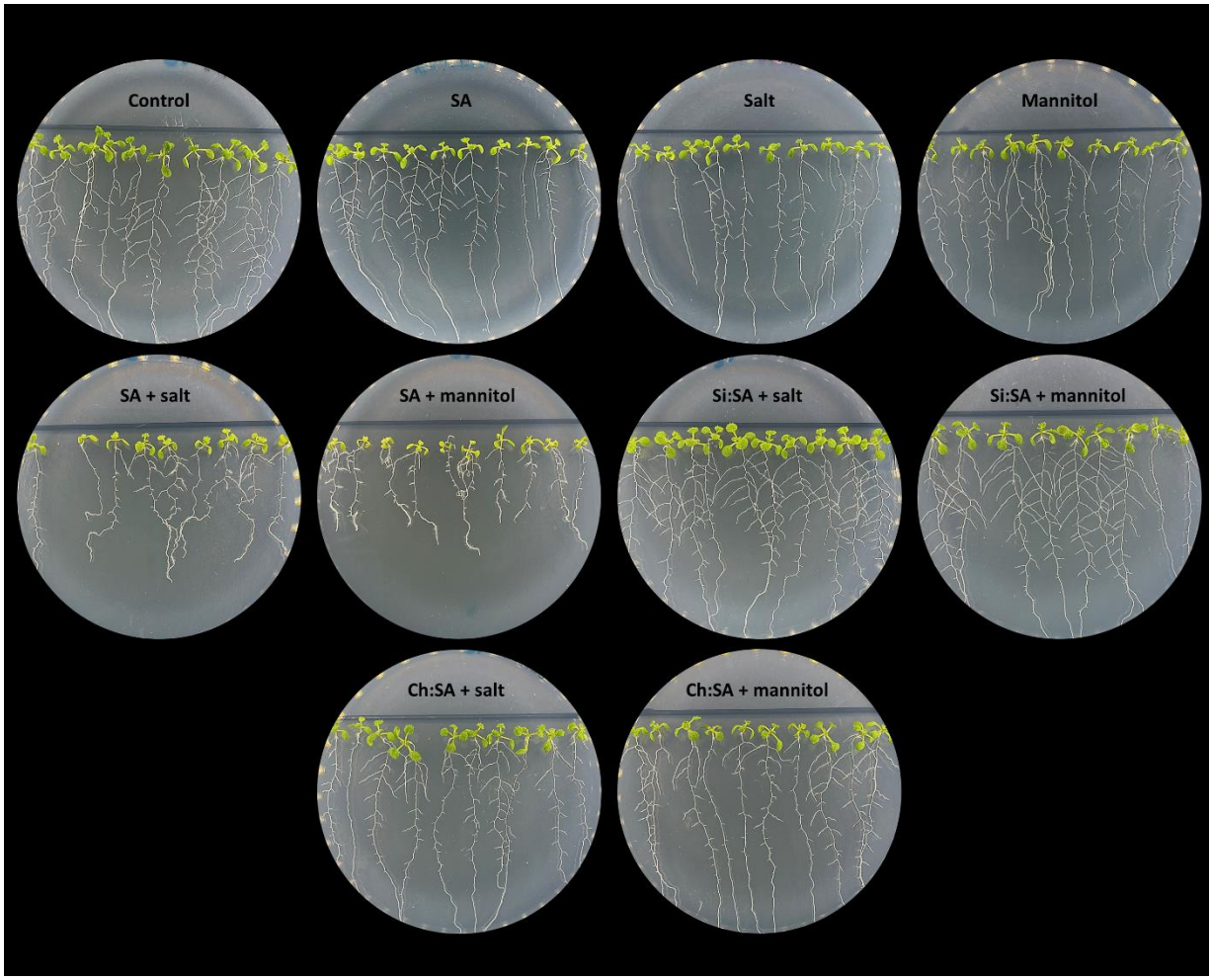

Figure 2. Effect of free SA, Si:SA and Ch:SA on morphological characteristics of *Arabidopsis thaliana* seeds under salt or mannitol stress at regular temperature (23°C) (simple stress conditions). Plant growth parameters were evaluated using photographs taken 12 days after sowing.

Seeds and transferred plants treated with Si:SA, Ch:SA, Si and Ch, cultivated under control conditions, showed no differences in root length compared to non-treated plants (Supplementary Figure 2). The controlled release mechanism suggests a possible gradual uptake of SA by the plant, thus maintaining a balance between defense and growth and preventing the development of short roots, as illustrated in Figures 2, 3, 4, and 5.

3.1.2. Rossete area

In both plant cultivation systems, the application of free SA, salt, or mannitol, individually or combined with heat stress, resulted in a decrease in rosette area compared to control plants (Figures 2, 3, 4, and 5). Furthermore, it was found that combining SA with any stressor significantly worsened the negative impacts on rosette size. Prior studies have demonstrated that exposure to osmotic stress (25 mM of mannitol or higher concentrations) reduced Arabidopsis rosette size by 50% without compromising overall plant survival [55].

Conversely, seeds and transferred plants treated with encapsulated SA maintained rosette area comparable to that of control plants, both under single and combined stress conditions (Supplementary Figure 3). During stress, SA serves as a versatile regulator of plant development, impacting cell growth in both positive and negative ways [28]. Research indicates that SA influences rosette leaf growth in a dose-dependent manner [45]. Low concentrations ($\leq$ 50 μM) of SA stimulate the growth of *Matricaria chamomilla* rosette by 32%, while high concentrations ($\geq$ 250 μM) inhibit growth by 40% [56].

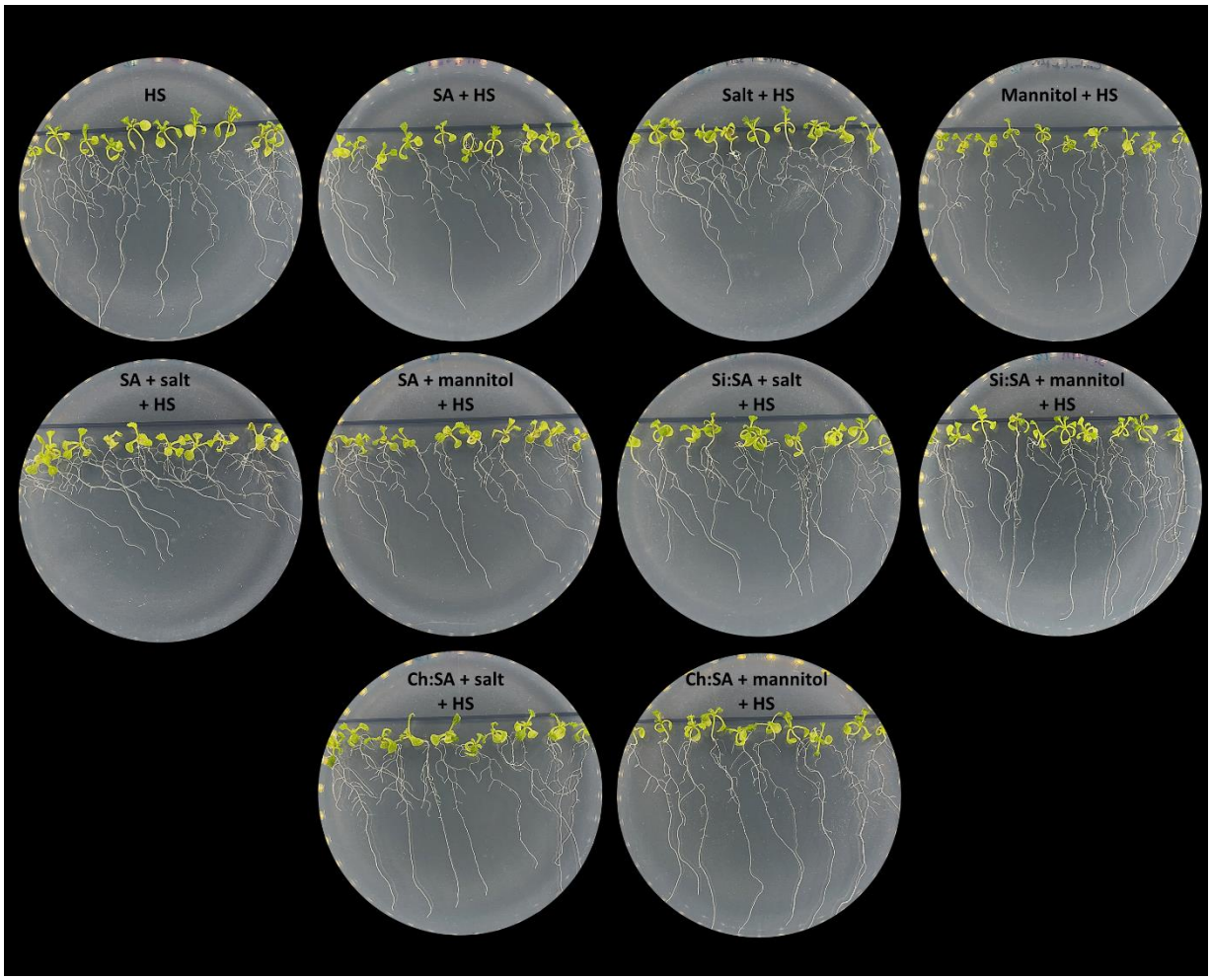

Figure 3. Effect of free SA, Si:SA and Ch:SA on morphological characteristics of *Arabidopsis thaliana* seeds under salt or mannitol stress combined with heat (30°C) (double stress conditions). Plant growth parameters were evaluated using photographs taken 12 days after sowing.

Transferred plants were more severely affected by osmotic and heat double stress than seeds. This difference could be attributed to the inherent adaptability of seeds to cope with heat stress, as SA signalling promotes basal thermotolerance in Arabidopsis [57]. Seed basal thermotolerance refers to the ability of seeds to resist moderate temperatures without losing their viability to germinate [58]. Research has determined that 95% of seeds subjected to heat stress (47°C) were able to germinate, indicating a high capacity of Arabidopsis seeds to adapt to negative conditions [57]. Unlike seeds, transferred plants require the induction of acquired thermotolerance, achieved through the production of heat shock proteins (HSPs) or stress-protective molecules that stabilize membranes [59].

Notably, rosette size remained unaffected in both seeds and transferred plants treated with Si:SA and Ch:SA, without applied stress conditions, compared to their respective control plants. Similarly, plants treated with Si and Ch capsules showed no differences in rosette size compared to control plants (Supplementary Figure 2).

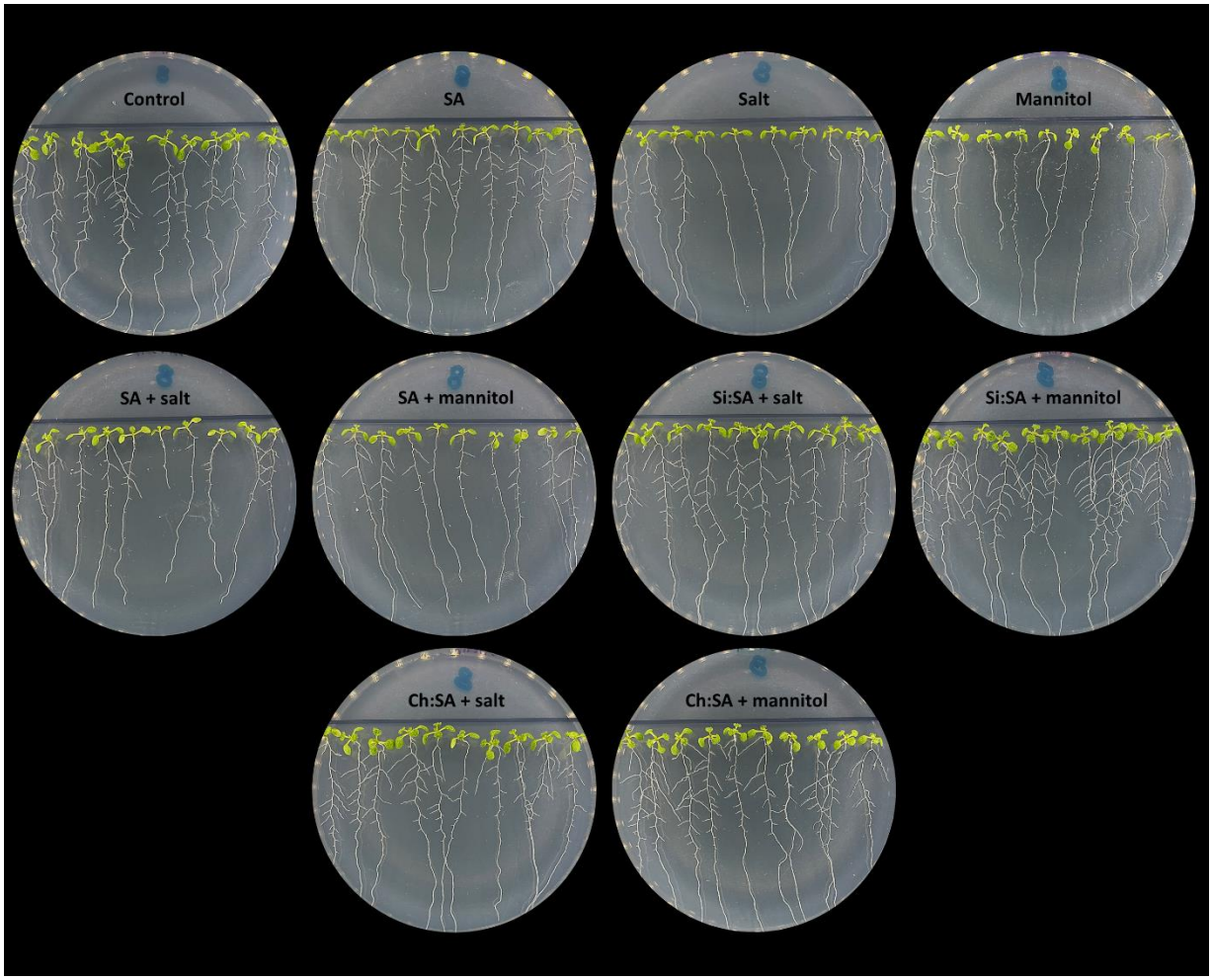

Figure 4. Effect of free SA, Si:SA and Ch:SA on morphological characteristics of 4-day-old transferred *Arabidopsis thaliana* plants under salt or mannitol stress at regular temperature (23°C) (simple stress conditions). Plant growth parameters were evaluated using photographs taken 8 days after transferring.

3.1.3. Secondary root density

Interestingly, secondary root density declined in both plant cultivation systems upon salt and mannitol treatments (Supplementary Figure 4). Osmotic stress disrupts the cellular balance of Ca^{2+} and K^{+} ions in plants [60], compromising root architecture and length [61]. Arabidopsis roots are particularly

sensitive to changes in ion concentrations, which can subsequently affect secondary root development [19].

Exogenous application of SA in Arabidopsis has been shown to stimulate the formation of lateral roots [62]. However, plants under simple stress and treated with free SA decreased the number of secondary roots in comparison to control plants for both cultivation systems (Supplementary Figure 4). The altered root morphology observed in plants treated with free SA is likely due to the differential effects of low-concentration exogenous SA on primary and lateral root development. As depicted in Figures 2 and 4, the SA treatment along with the stress conditions can inhibit primary root growth and simultaneously affect the formation of secondary roots, consistent with the findings of Pasternak, et al. (2019) [52].

Once again, both encapsulated SA treatments reverted the negative effects of salt and mannitol stress in both plant cultivation experiments (Supplementary Figure 4).

Under combined conditions of osmotic stress and heat, plants in both cultivation systems exhibited a decreased density of secondary roots (Figures 3 and 5). Notably, seeds subjected to double stress displayed a slight yet statistically significant increase in secondary root number compared to control plants (Supplementary Figure 4). However, transferred plants experienced a more pronounced reduction in secondary roots under the same stress conditions. This disparity in response suggests that the combination of heat and osmotic stress triggers a restructuring of the cell wall in transferred plants, potentially involving altered biosynthesis and coordination of key components crucial for secondary root development [63].

Furthermore, 4-day-old transferred plants subjected to double stress showed no significant differences in secondary root density across the various treatments. This suggests that these plants may prioritize main root growth under combined stress conditions. On the other hand, neither seeds nor transferred plants treated with Si:SA, Ch:SA or capsules (Si or Ch), displayed any changes in secondary root density compared to their respective controls under non-stress conditions (Supplementary Figure 2).

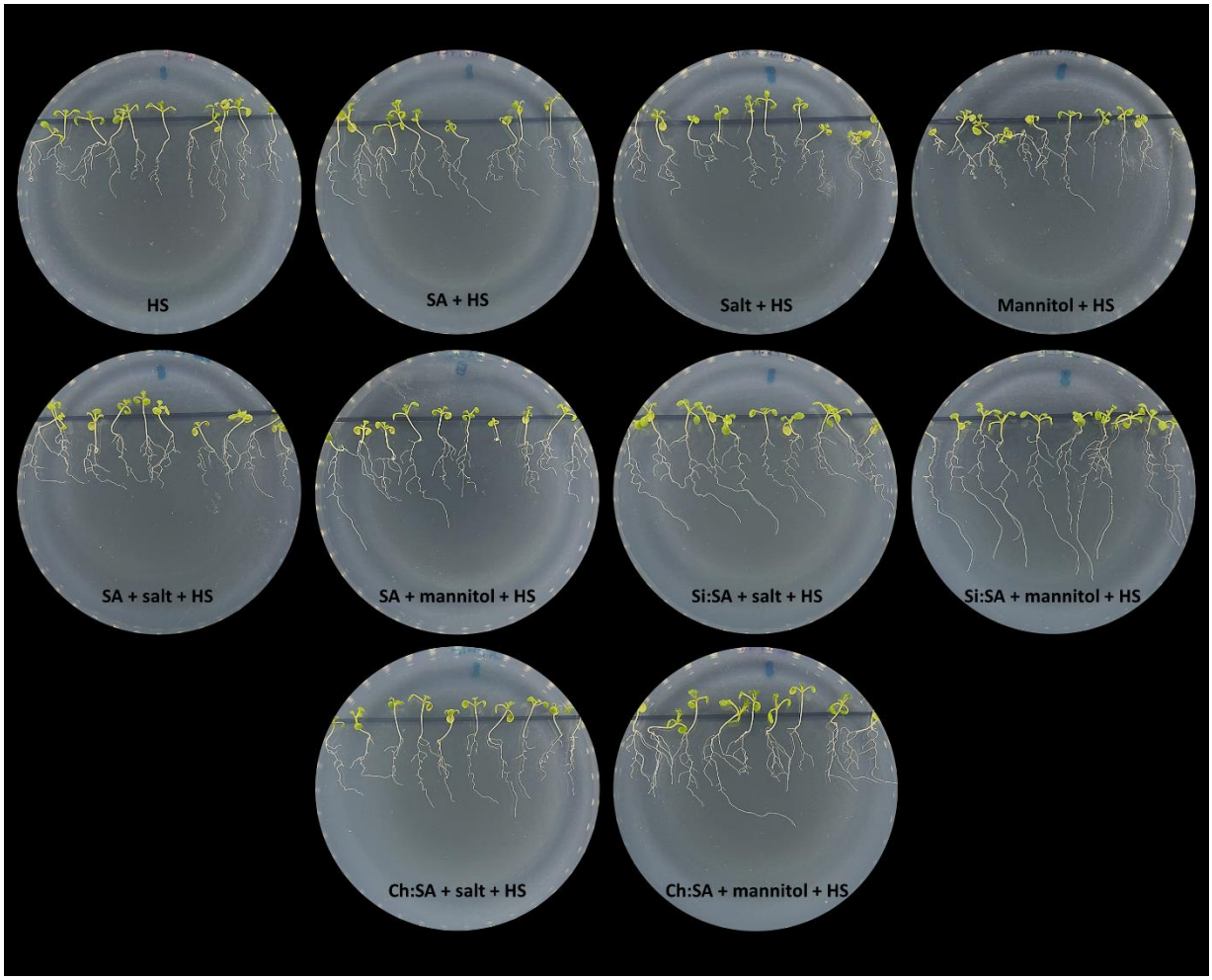

Figure 5. Effect of free SA, Si:SA and Ch:SA on morphological characteristics of 4-day-old transferred *Arabidopsis thaliana* plants under salt or mannitol stress combined with heat (30°C) (double stress conditions). Plant growth parameters were evaluated using photographs taken 8 days after transferring.

3.2. Phytohormone interactions are crucial for *Arabidopsis thaliana* adaptation

3.2.1. SA and IAA interaction promotes plant tolerance against osmotic stress

Phytohormone profiles were obtained from both plant cultivation systems under simple stress conditions (salt or mannitol). Notably, significant changes were observed in seeds on the 12th day and in transferred plants on the 8th day. Analysis revealed elevated endogenous SA and decreased JA levels in plants treated with free SA, salt/mannitol, or their combination, compared to control plants (Figure 6 and Supplementary Figures 5-6). The observed increase in SA content might be attributed to the plant adaptive response to the stress imposed by salt or mannitol in the medium. Additionally, as expected. free SA treatment specifically led to a notable increase in endogenous SA levels within roots, accompanied by a decrease in IAA levels, as reported by Caarls, et al. [64].

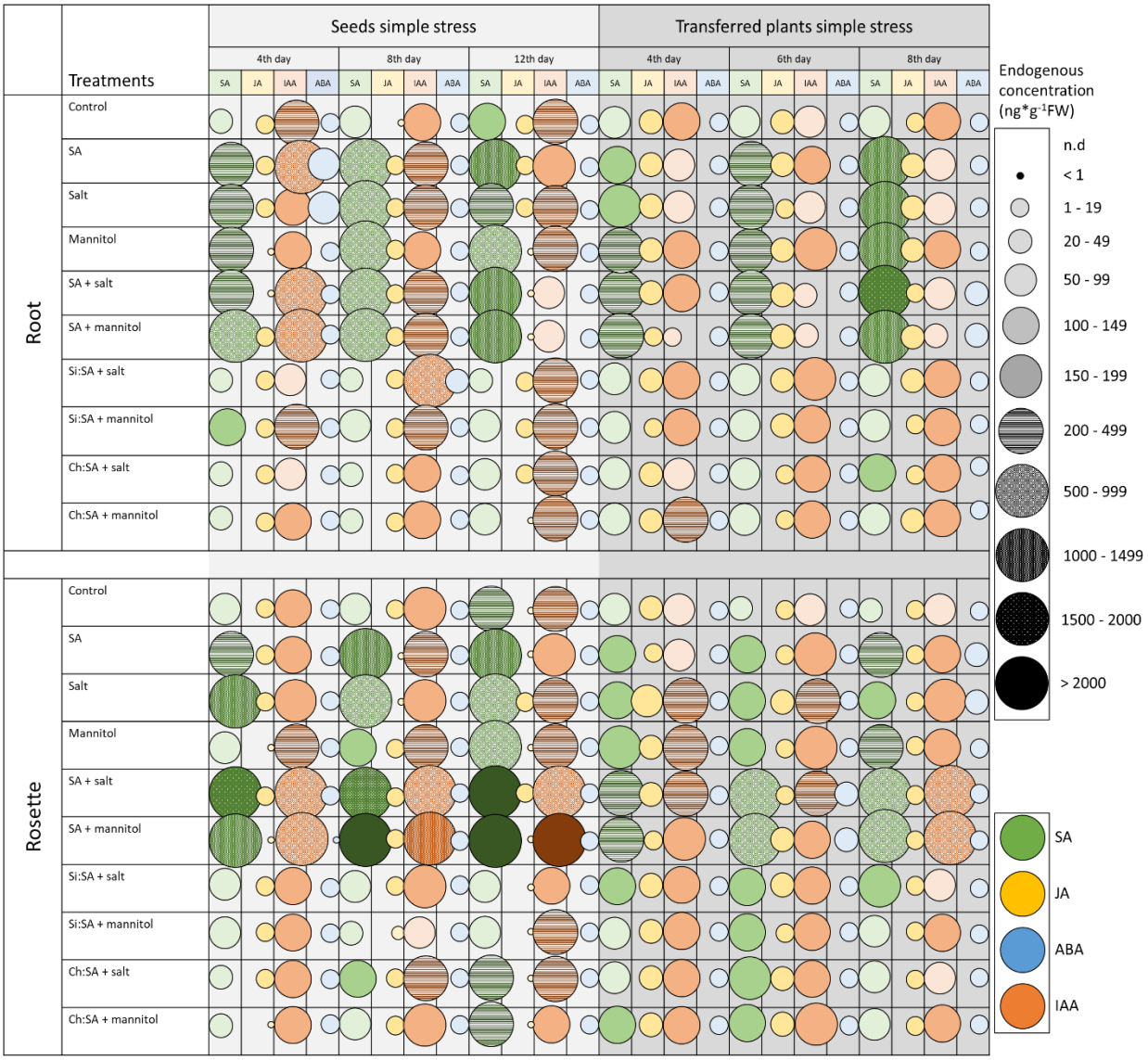

Figure 6. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants under salt and mannitol stress at regular temperature (23°C) (simple stress conditions). Analyses were performed on the two plant cultivation systems: seeds and 4-day-old transferred plants.

Conversely, increases in IAA levels were observed in rosettes despite the high SA levels. This suggests that IAA accumulation might be triggered by SA binding to the A subunits of protein phosphatase 2A (PP2A), leading to hyperphosphorylation of an IAA transporter [65]. The established SA-IAA crosstalk suggests that elevated concentrations of SA may influence root development by reducing IAA transport to this organ concomitantly with its accumulation in aerial tissues [66,67]. Interestingly, seeds and transferred plants across all ten treatments displayed low ABA levels in both roots and rosettes. An

increase in SA negatively regulates ABA through an antagonistic response [68], which explains the observed reduction in endogenous ABA levels (Figure 6 and Supplementary Figures 5-6).

In contrast, roots in both plant cultivation systems treated with Si:SA and Ch:SA exhibited decreased SA levels compared to plants treated with free SA, salt/mannitol, or their combination (Figure 6). This suggests that encapsulated SA moderates the plant SA uptake, regulating SA accumulation, and therefore, controlling IAA endogenous synthesis. Maintaining the balance between SA and IAA consequently allows plants to adapt to the applied stress. Figure 6 also shows a similar effect in the rosette compared to the root for encapsulated treatments, except for seeds treated with Ch:SA, which exhibited higher SA levels than those treated with Si:SA. The observed variance might be due to the slower release of SA from chitosan compared to silica [46]. This slower release could limit SA uptake by the plant to a level that promotes defense responses, but potentially insufficient to stimulate optimal growth through IAA synthesis.

3.2.2. SA, ABA and IAA interaction promotes plant tolerance against combined osmotic and heat stresses

Figure 7 depicts the phytohormone profiles of roots and rosettes from all treatments and their respective controls under heat stress, as observed in both plant cultivation experiments. In line with previous observations under simple stress conditions, significant effects were found in seeds on the 12th day and in transferred plants on the 8th evaluation day. Notably, the trends for endogenous SA and IAA levels mirrored those in section 3.2.1, but with a pronounced increase attributed to heat stress. Plants treated with free SA exhibited the highest levels in each treatment (free SA and its combinations with salt or mannitol) under heat stress, consistent with their behaviour under simple stress conditions. Likewise, plants treated with encapsulated SA (Si:SA and Ch:SA) exhibited levels similar to those of control plants, suggesting that although they may be affected by heat stress, the controlled release of SA helps mitigate the negative effects induced by the double stress. SA enhances thermotolerance and facilitates recovery across different growth stages, from seeds to mature plants [57].

Furthermore, the application of Si:SA and Ch:SA combined with mannitol and heat stress led to an increase in ABA levels in plants (Figure 7 and Supplementary Figures 7-8) that suggests that SA levels are sufficiently elevated to confer plant protection without disrupting the homeostasis of other phytohormone levels. ABA plays a crucial role in regulating responses to heat stress and is necessary at various developmental stages to induce thermotolerance [69]. In Arabidopsis, ABA triggers the activation of transcription factors that contribute to thermotolerance acquisition [70]. Interestingly, JA levels remained similar in both plants systems, suggesting a potential negative response to the observed SA increase (Figure 7 and Supplementary Figures 7-8).

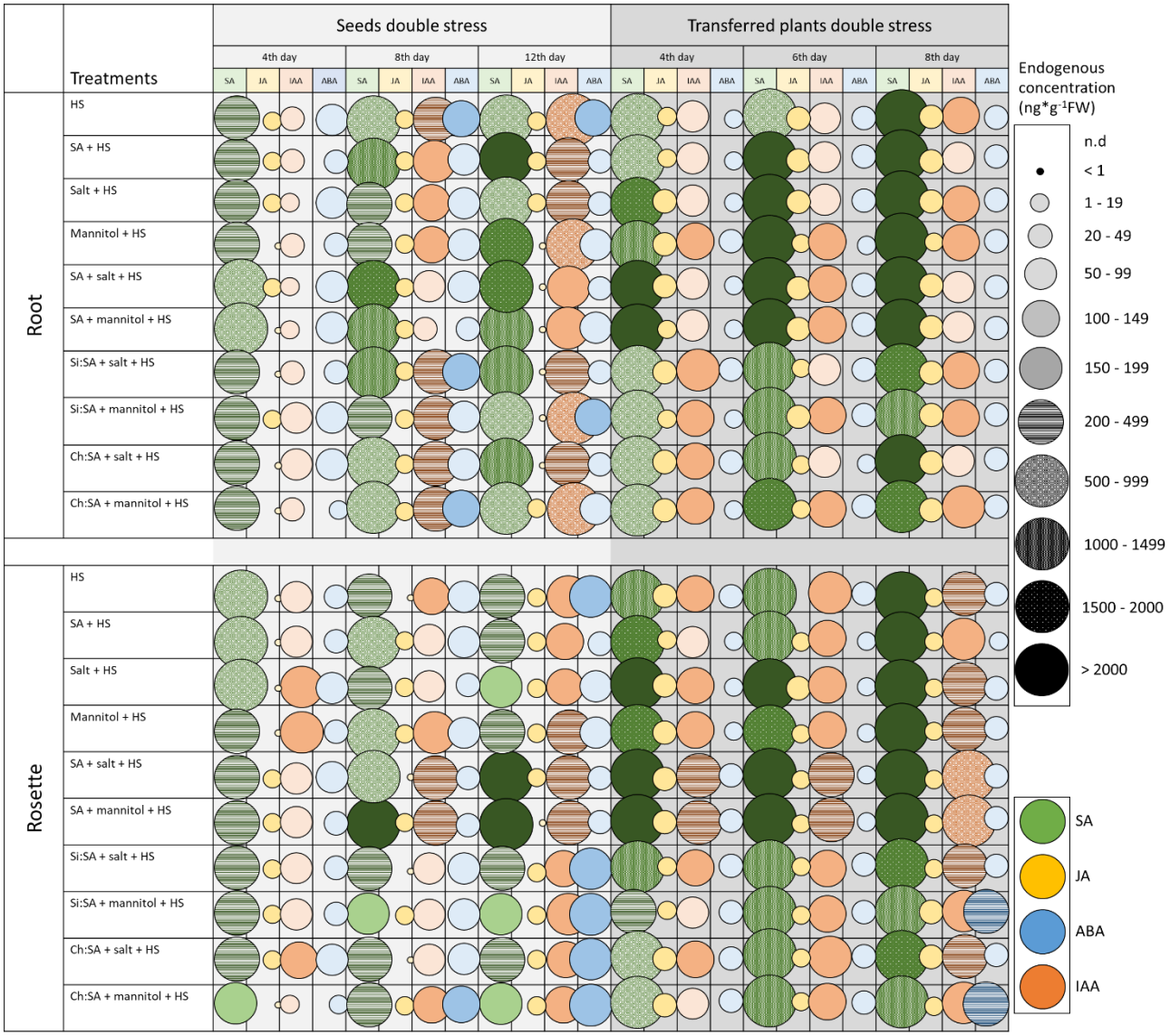

Figure 7. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants under salt and mannitol stress combined with heat (30°C) (double stress conditions). Analyses were performed on the two plant cultivation systems: seeds and 4-day-old transferred plants.

3.4. Encapsulated SA preserves root IAA levels in plants

To facilitate result analysis, focus was placed on treatments where the plants exhibited the worst morphological characteristics by stressful conditions, and the subsequent reversal of these effects by encapsulated SA. Thus, attention was directed to transferred plants under simple (mannitol) and double stress (mannitol and high temperature) conditions. Additionally, the study was restricted to a singular encapsulated material, Si:SA. To assess the impact of SA treatments on IAA root distribution, the

"DR5::GFP" reporter line of *Arabidopsis thaliana* was employed [71]. IAA is the primary auxin responsible for regulating root elongation and various developmental processes in plants, including tissue differentiation, cell division, and responses to diverse pathogens [72,73]. The DR5 system enables the monitoring of auxin distribution, particularly in the root tip, where significant accumulation of IAA occurs in response to various stimuli [74].

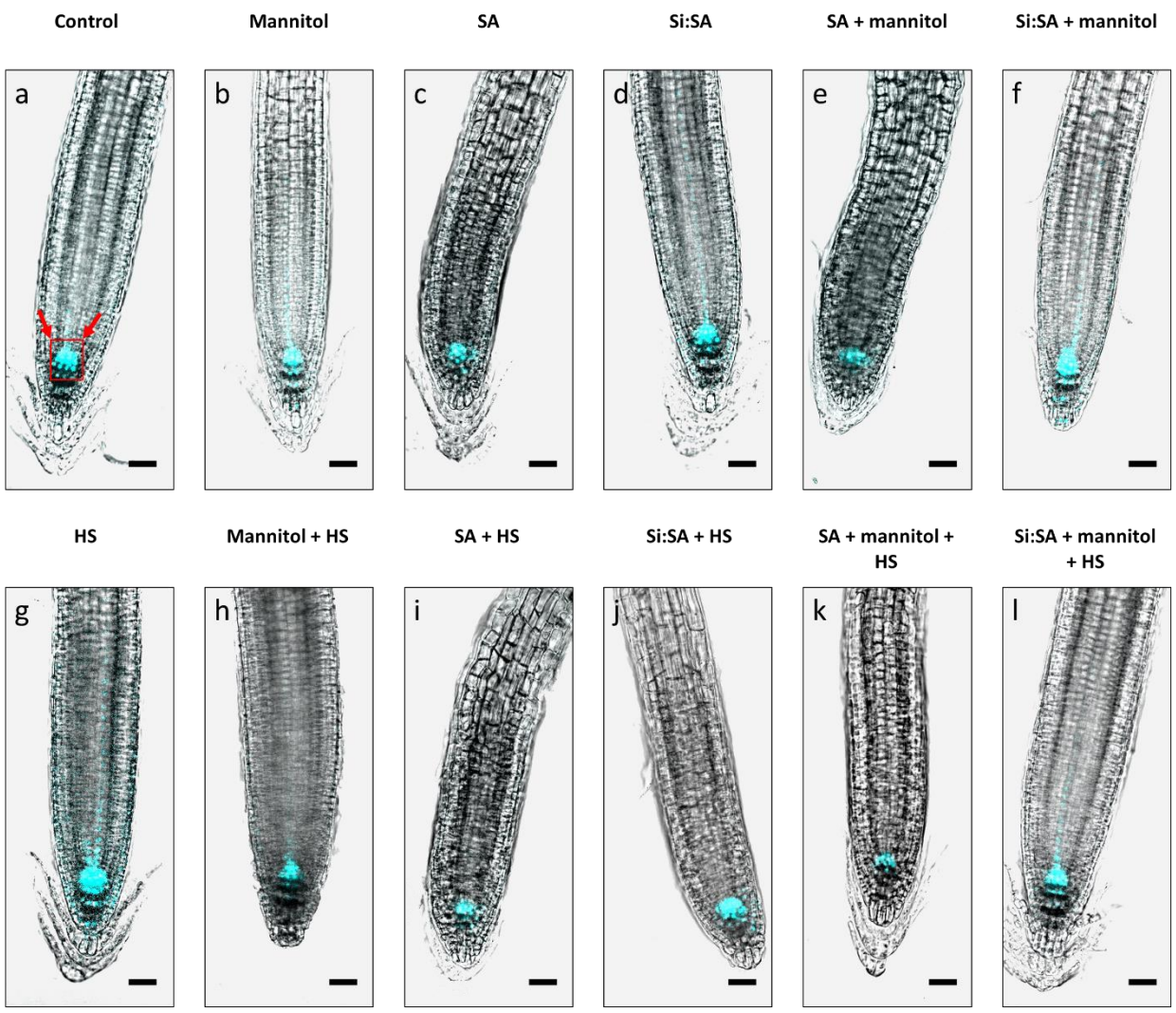

Figure 8. Effect of free SA and Si:SA on root fluorescence in *Arabidopsis thaliana* DR5::GFP plants, under mannitol stress at regular temperature (23°C) (simple stress conditions) and combined with heat (30°C) (double stress conditions). Four-day-old plants were exposed to media with the different treatments, and confocal images were taken after four days. Red square shows the IAA sensor activity. The scale represents 100 µm.

As expected, the activity of DR5 in the roots compares with control plants (Figure 8a) showed that the plants treated with Si:SA and Si:SA + Mannitol exhibited similarity in DR5 signalling patterns due to the controlled release of SA (Figure 8d-f, respectively). Nevertheless, plants treated with mannitol exhibited a slight decrease in DR5 activity (Figure 8b and Supplementary Figure 9). Mannitol is involved in the reduction of both root meristem length and cell number in the primary root [51]. Free SA and free SA + mannitol treatments produced the greatest reduction in DR5 activity (Figure 8c-e, respectively, and Supplementary Figure 9), likely due to the progressive accumulation of SA in the roots, which subsequently led to a decrease of IAA levels. Despite low-dose free SA treatment, plants exhibited a significant decrease in the DR5 signal, similar to results obtained by Pasternak, et al. (2019) [75].

Heat stress disrupts fundamental physiological processes, including hormone signalling pathways like those mediated by auxins in *Arabidopsis thaliana* [76]. The results revealed that control plants subjected to heat stress exhibited the highest DR5 activity (Figure 8g and Supplementary Figure 9). However, plants treated with free SA and/or mannitol in combination with heat stress (mannitol + HS, free SA + HS, and free SA + mannitol + HS), showed a reduced DR5 activity signal compared to the control plants (Figure 8h-i-k, respectively, and Supplementary Figure 9), consistent with the findings observed under simple stress conditions. The observed decrease in DR5 activity and accumulation of auxins in the rosette (sections 3.2.1 and 3.2.2) strongly suggest that treatments involving simple and double stress, as well as free SA treatment, affect auxin transport [65], rather than its biosynthesis [77]. Furthermore, the results indicate that plants treated with Si:SA + HS or Si:SA + mannitol + HS exhibited similar DR5 levels (Figure 8j-l, respectively, and Supplementary Figure 9) compared to control plants not subjected to heat stress (Figure 8a). This suggests that SA released in a controlled manner can enhance plant tolerance to simple or complex stresses due to the maintaining of endogenous IAA levels (Figure 9).

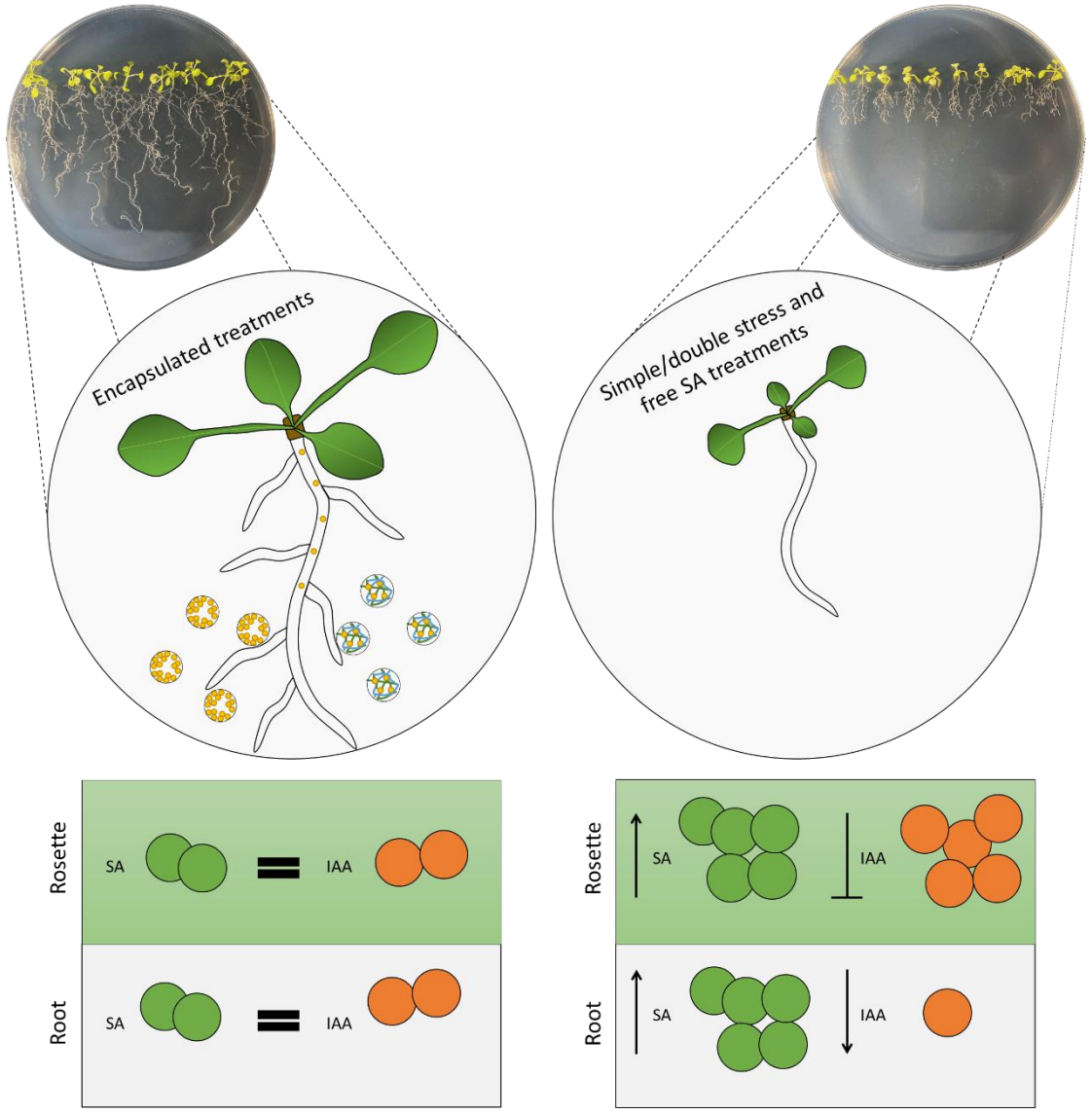

Figure 9. Schematic representation of the effects of encapsulated SA, simple/double stress, and free SA treatments on the endogenous levels of SA and IAA in the rosette and root of the treated plants.

3.5. Encapsulated SA treatment maintains key gene expression, preventing SA accumulation

Under stress conditions, SA triggers a signalling cascade in plants, essential for regulating the expression of defense-related genes [78]. The accumulation of SA in plants is required to promote local and systemic acquired resistance [79]. Furthermore, exogenous SA treatments can induce transcriptional reprogramming, diverting resources towards defense mechanisms at the expense of growth [80]. In general, SA biosynthesis begins (Figure 10b) with the suppression of ENHANCED DISEASE SUCEPTIBILITY 1 (EDS1) to promote the chorismate transformation into isochorismate by ISOCHORISMATE SYNTHASE 1 (ICS1) or phenylalanine by PHENILALANINE AMMONIA-LYASE 1 (PAL1), depending on the chosen synthetic pathway [81]. Elevated expression of ICS1 under

stress conditions suggests a preference for this pathway in SA synthesis [82]. Subsequently, the enzyme avrPphB SUSCEPTIBLE 3 (PBS3) converts isochorismate into SA [83]. Finally, SA is perceived by NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1), triggering the expression of defense genes [84].

To determine the effect of different treatments on SA synthesis, the expression of EDS1, ICS1, PAL1, PBS3 and NPR1, key genes in the SA biosynthesis pathway, was analyzed (Figure 10b). Once again, the transferred plant cultivate system under simple and double stress was selected for analysis. Gene expression was separately analyzed in both rosette and root tissues. Figure 10a revealed distinct gene expression patterns between roots and rosettes. Notably, EDS1, PAL1, and NPR1 genes were downregulated in roots compared to rosettes. This observation might be linked to SA uptake and translocation from roots to rosettes [45], subsequently promoting SA synthesis and accumulation there. Interestingly, free SA and free SA + mannitol treatments upregulate NPR1 in the root (Supplementary Figure 10). Conversely, plants treated with Si:SA and Si:SA + mannitol exhibited downregulation of EDS1, ICS1, and NPR1 genes. This suggest that SA uptake from the media is insufficient to induce changes in SA biosynthesis, preventing its accumulation. Moreover, plants treated with Si:SA + mannitol + HS exhibited upregulated ICS1 and PBS3 expression in the root compared to free SA + mannitol + HS. The co-expression of ICS1 and PBS3 might contribute to control SA accumulation, then preventing negative effects on the plants. This finding aligns with research suggesting that changes in specific SA biosynthesis genes can alter the regulation of downstream genes in the pathway, promoting SA accumulation [85,86].

Regarding the rosette, expression levels of PAL1, PBS3, and NPR1 in plants treated with Si:SA + mannitol were similar than in control plants (Supplementary Figure 11). However, these genes were upregulated in the rosette of plants treated with Si:SA + mannitol + HS, although did not reach the high levels observed in plants treated with SA + mannitol + HS. This further suggests that controlled SA release maintains endogenous SA levels within a physiological range, thereby preventing alterations in rosette size unlike treatments with free SA. These results likely stem from the slow release of SA, which maintains growth while enhancing stress tolerance in treated plants.

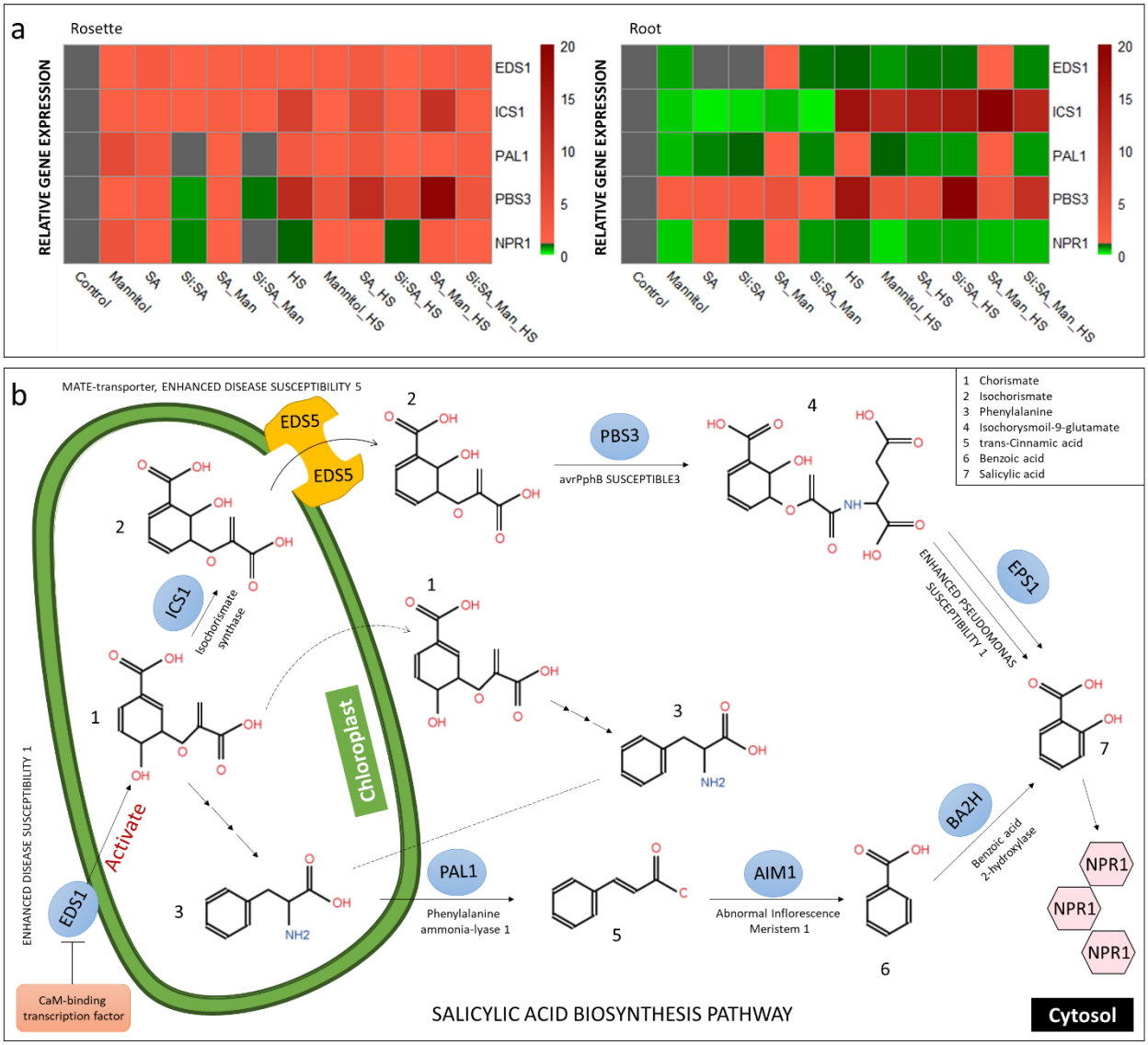

Figure 10. Effect of free SA and Si:SA on the relative expression of SA-related genes in roots and rosettes of 4-day-old transferred *Arabidopsis thaliana* plants under mannitol stress at regular temperature (23°C) (simple stress conditions) and combined with heat (30°C) (double stress conditions). a) Heat map represents differential expression of SA biosynthesis genes in both root and rosette at 8th evaluation day. b) Schematic representation of SA biosynthesis pathway.

4. Conclusions

Exogenous treatments with encapsulated SA effectively protects plants against various stress conditions, including salt or mannitol stress, and their combination with high temperature (30°C). This effectiveness was observed in the two different cultivation systems assayed: seeds germinated directly in media containing the different stressors and seedlings transferred to the media after four days of

growth. Plants treated with free SA displayed lower tolerance to stress conditions, likely because the applied SA exceeded physiological levels. In contrast, encapsulated treatments maintain or even enhance the positive morphological characteristics of treated plants compared to control plants. This includes maintaining or improving of root length, secondary root density, and rosette area. These effects could be related to the controlled release of SA, allowing plants to take up and utilize SA more efficiently, thus achieving a balance between plant defense and growth mechanisms. Plants under stress or treated with free SA accumulated important amounts of SA in both roots and rosettes, which explains their poorer morphological characteristics compared to control plants. This accumulation might lead to the observed alteration in IAA levels: a decreased in roots concomitant with an increase in rosettes. The auxin accumulation in rosettes could be attributed to the inhibition of its transport. The *Arabidopsis thaliana* DR5::GFP line supports this hypothesis, indicating IAA transport failing and decreased signalling in the quiescent center, probably due to increased endogenous SA levels. Analysis of SA-related genes in plants treated with free SA revealed upregulation of genes involved in SA biosynthesis pathway. This suggests that free SA treatment induces a positive feedback, increasing endogenous SA production, which added to the SA taken from media, leads to its uncontrolled accumulation (as observed by phytohormone quantification). Plants treated with encapsulated SA under stress conditions maintained expression levels of several SA biosynthesis genes, including EDS1, PAL1, and NPR1, similar to control plants, in both roots and rosettes. This managed regulation of gene expression prevented uncontrolled SA synthesis, ensuring only the necessary accumulation of SA for defense responses without compromising plant development and growth. This implies that encapsulated SA can prevent the harmful effects of abiotic stress in *Arabidopsis thaliana* by modulating endogenous phytohormone levels and regulating the expression of SA-related genes. Understanding the physiological and molecular mechanisms underlying plant tolerance to abiotic stresses, particularly the role of encapsulated SA as revealed in this study, will be valuable for future investigations on plant responses to stress, particularly for developing strategies to promote crop resilience to stress.

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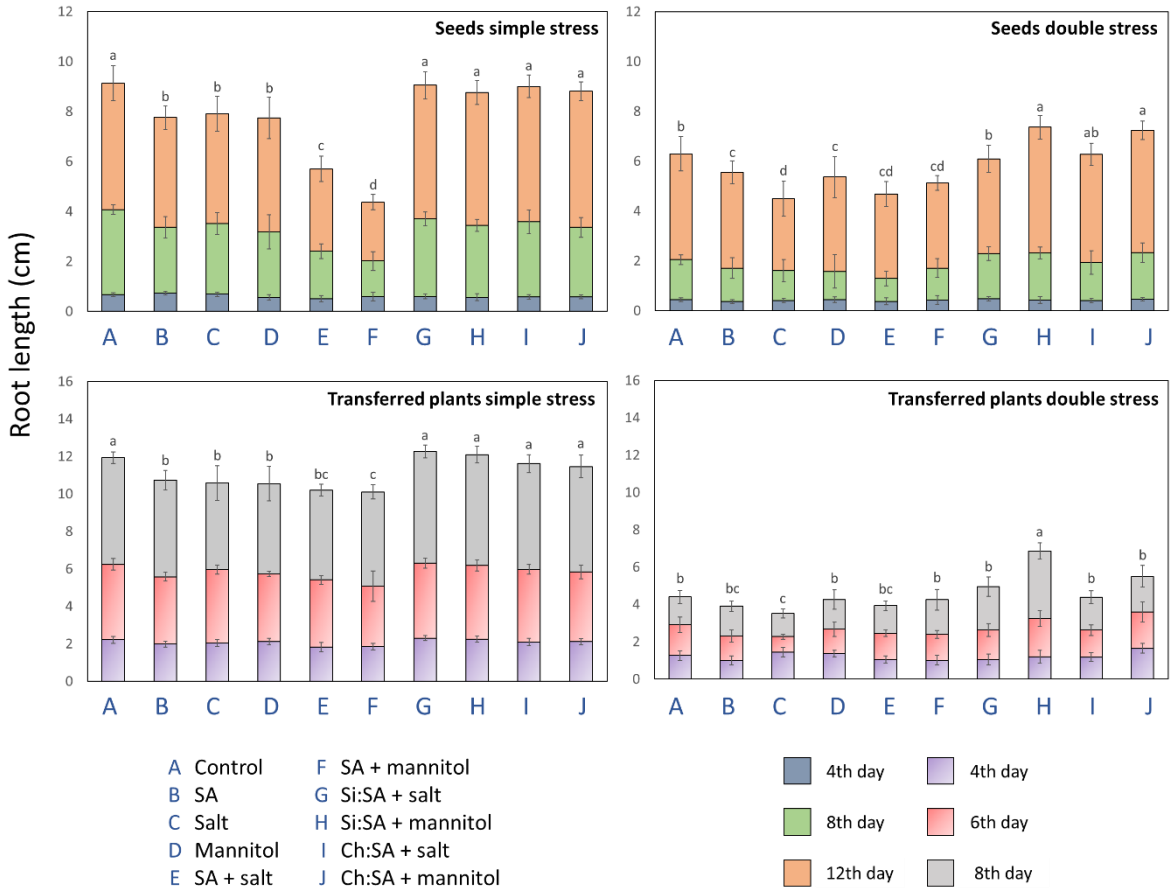
Supplementary material

Supplementary Table 1. Treatment conditions chosen to ensure no observed effects in *Arabidopsis thaliana* plants.

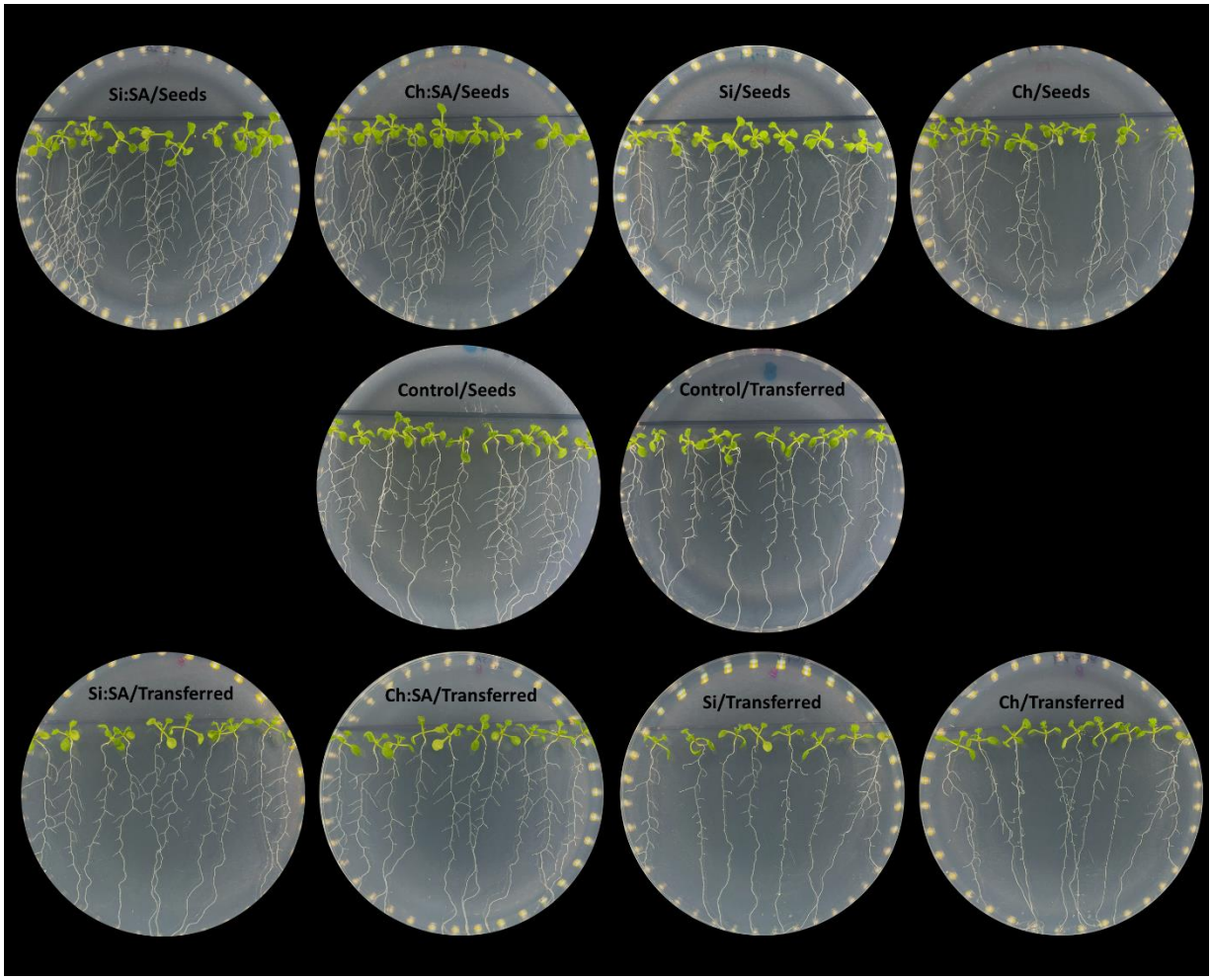
Treatment	Description	Concentration
Control	Murashige & Skoog (MS)	-
Si	Silica capsule	1 µM
Ch	Chitosan capsule	1 µM
Si:SA	Encapsulated SA in silica	1 µM
Ch:SA	Encapsulated SA in chitosan	1 µM

Supplementary Table 2. Primers used for gene expression analysis.

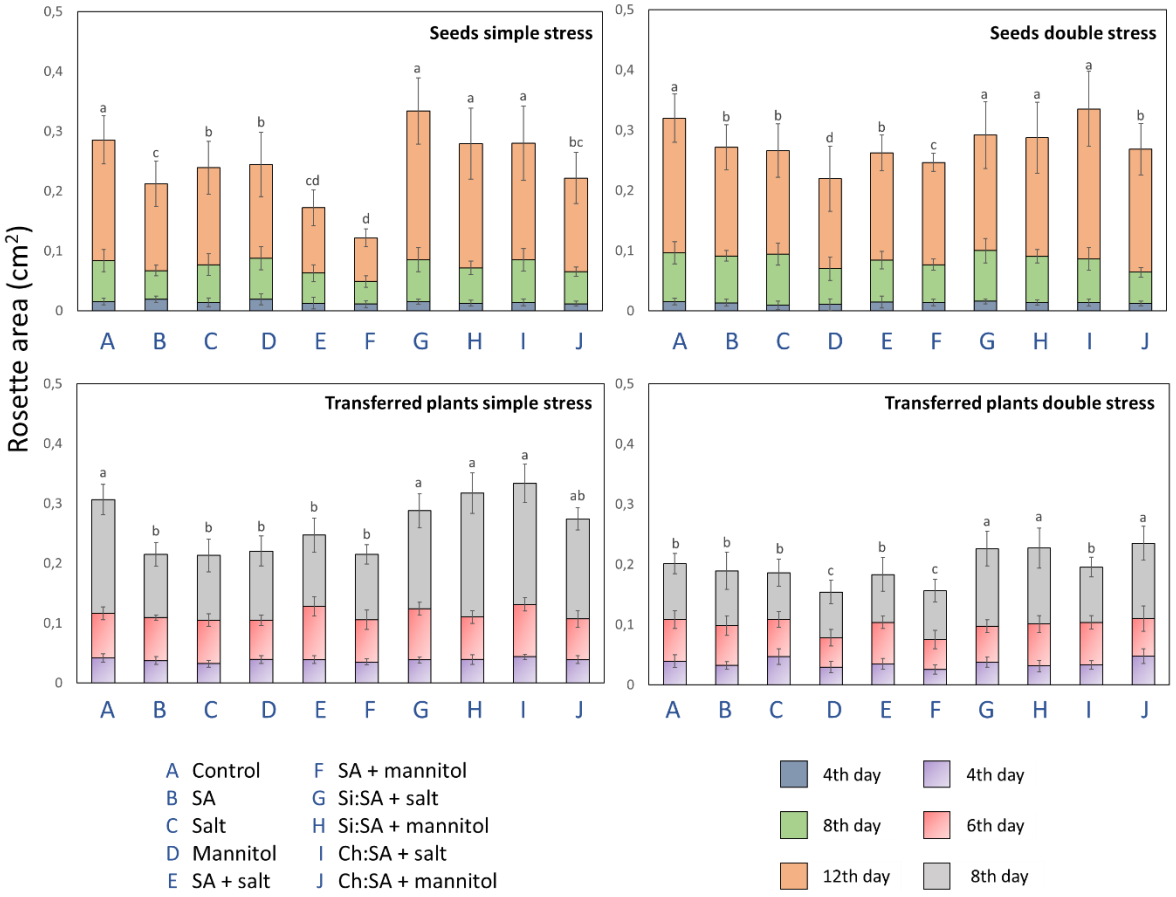
Gene	Accession	Primer sequence (5′→3′)		Amplicon size (bp)
		Forward	Reverse	
ACT2	At3g18780	GCCATCCAAGCTGTTCTCTC	CAGTAAGGTCACGTCCAGCA	157
EDS1	At3g48090	CCTCGTTGTGTGACATTTGG	AATTGGGCAAGAACATGAGG	182
ICS1	At1g74710	GAACTCAAATCTCAACCTCC	ACTGCGACGAGAGAAGAAAC	138
PAL1	At2g37040	GCGATTCACGGTGGTAACTT	CCAAACTTGGATTCCTCGAA	175
PBS3	At5g13320	TACTGCCATTTGCTCTGTGG	CCCAAGTCTGTGACCCAGTT	167
NPR1	At1g64280	GATGGATTCGCCGATTCTTA	AACAAGCTTAGCGTCGCTGT	195



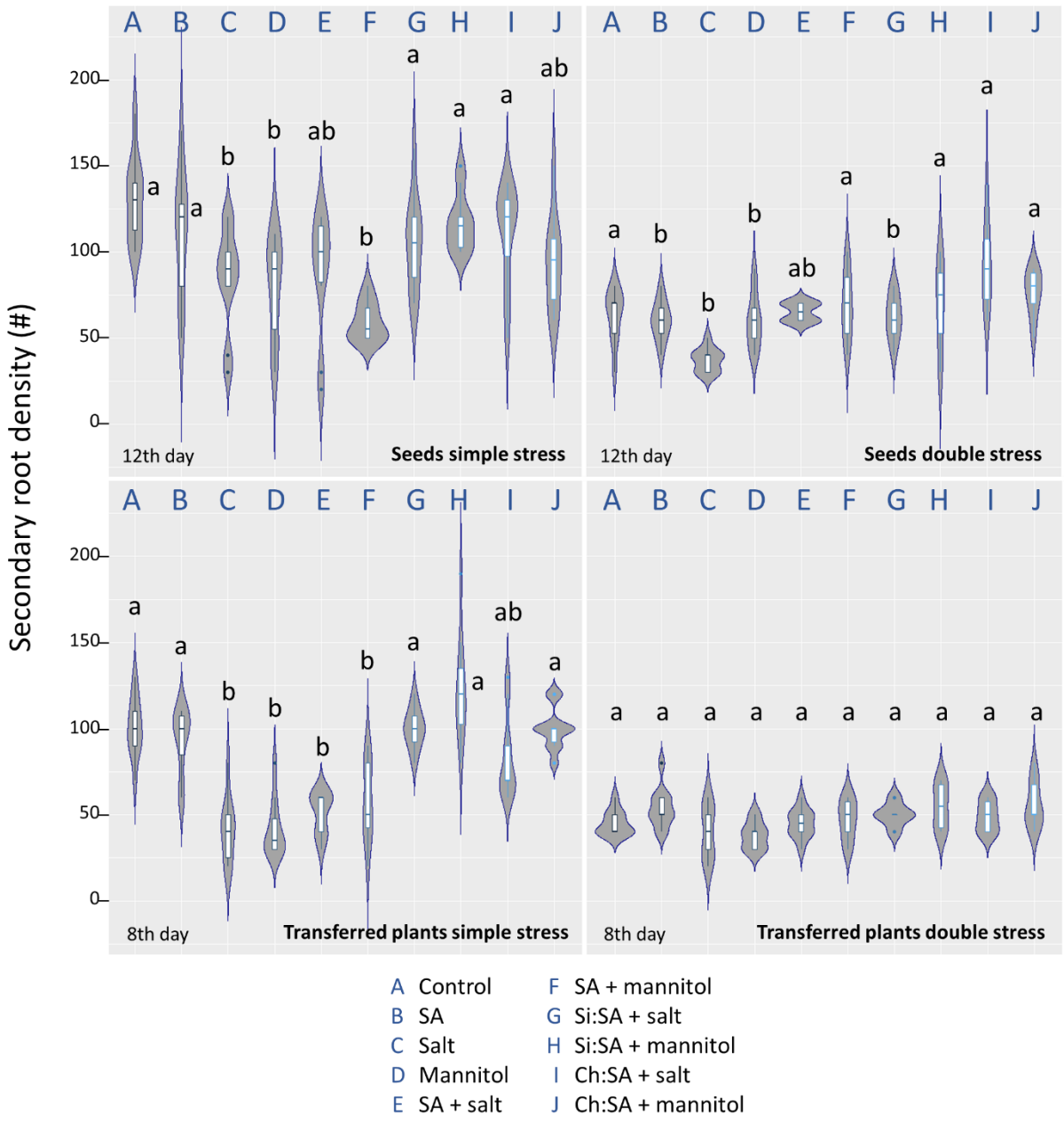
Supplementary Figure 1. Effect of free SA, Si:SA, and Ch:SA on root length in *Arabidopsis thaliana* plants grown from seeds and 4-day-old transferred plants, under salt and mannitol stress at regular temperature (23°C) and with heat (30°C) (simple and double stress conditions, respectively).



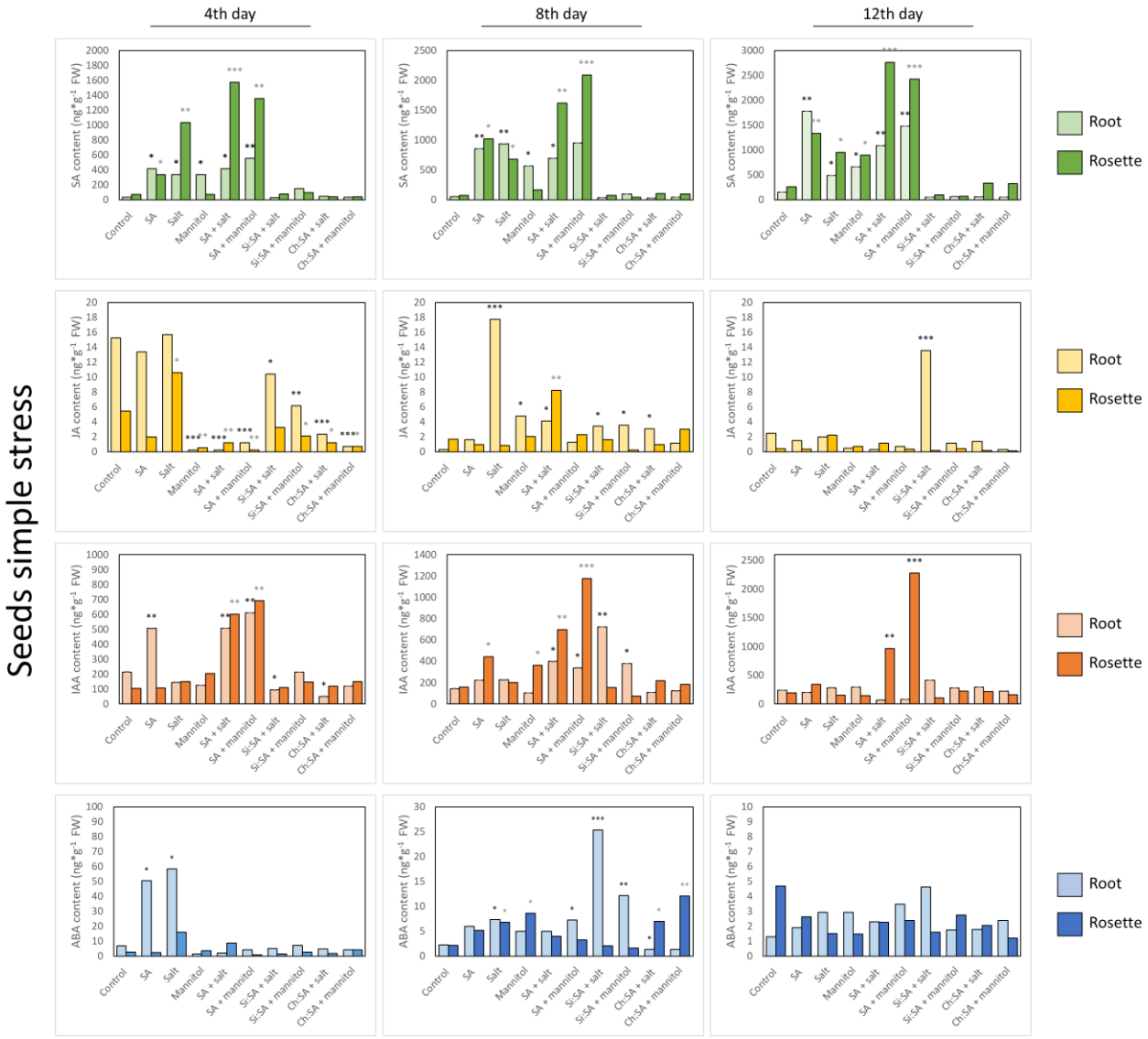
Supplementary Figure 2. Effect of Si:SA, Ch:SA, Si and Ch on *Arabidopsis thaliana* seeds and 4-day-old transferred plants. Photographs were taken on the 12th day after sowing, and on the 8th day after transferred.



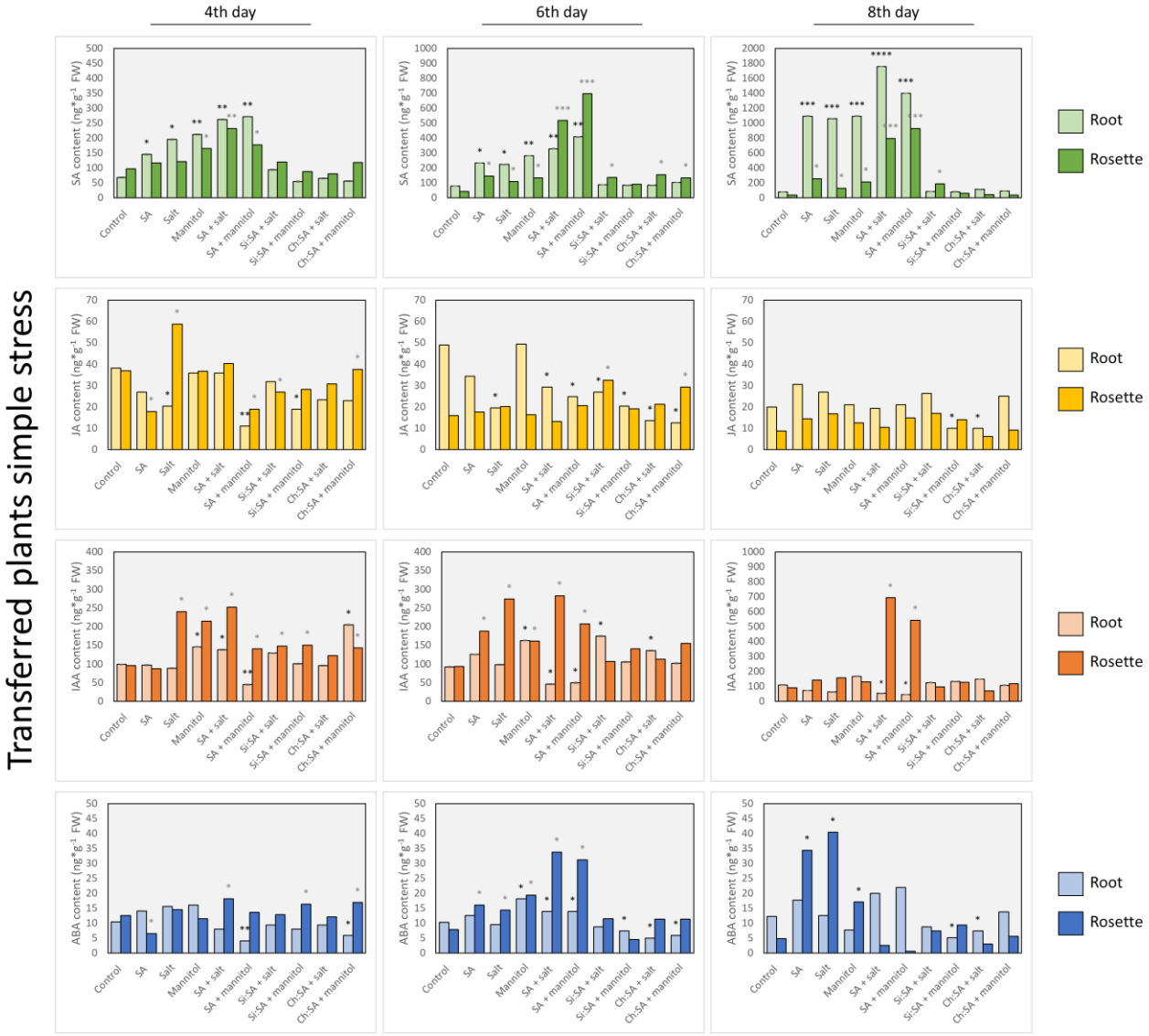
Supplementary Figure 3. Effect of free SA, Si:SA, and Ch:SA on rosette area in *Arabidopsis thaliana* plants grown from seeds and 4-day-old transferred plants, under salt and mannitol stress at regular temperature (23°C) and with heat (30°C) (simple and double stress conditions, respectively).



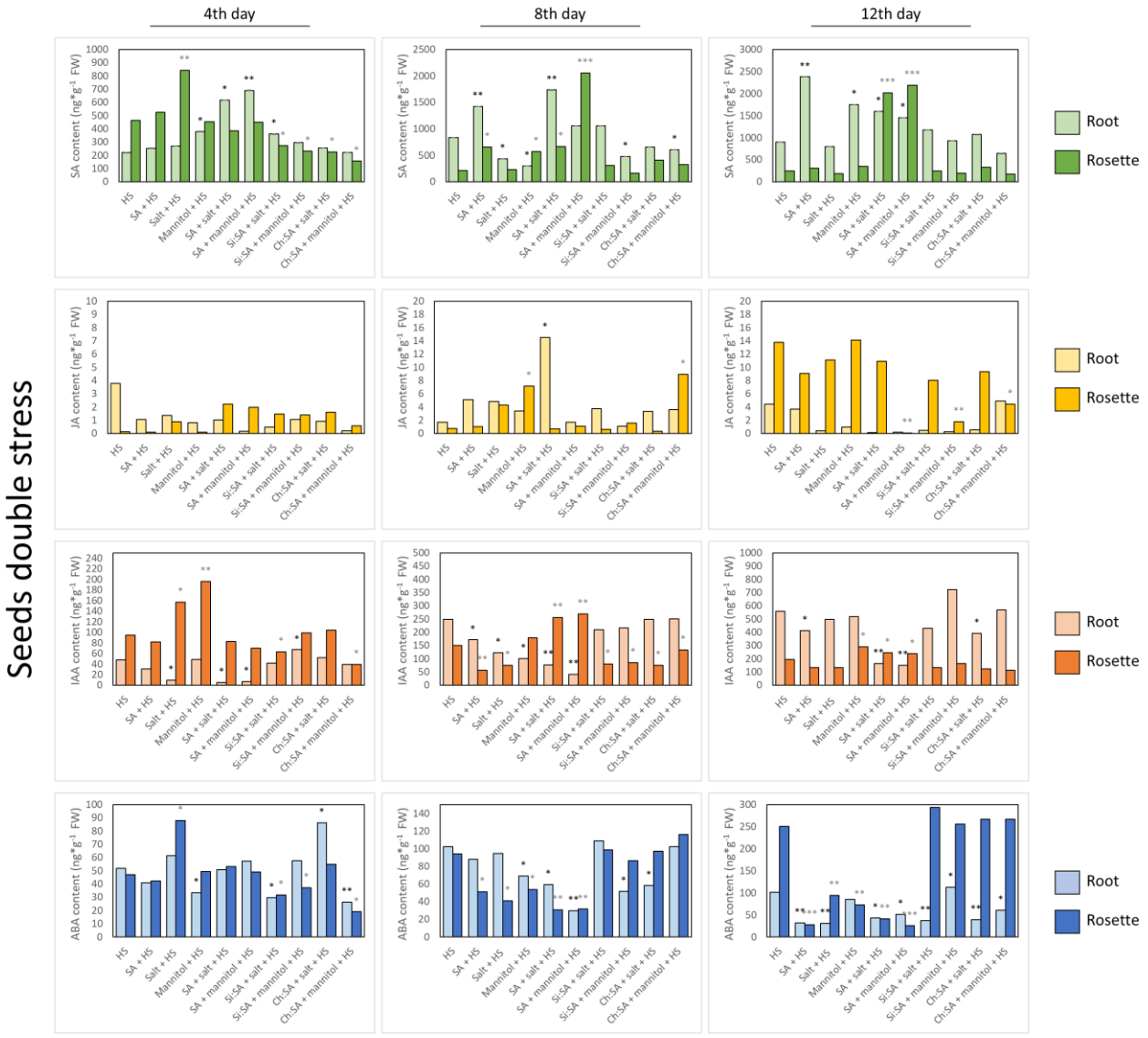
Supplementary Figure 4. Effect of free SA, Si:SA, and Ch:SA on secondary root density in *Arabidopsis thaliana* plants grown from seeds and 4-day-old transferred plants, under salt and mannitol stress at regular temperature (23°C) and with heat (30°C) (simple and double stress conditions, respectively).



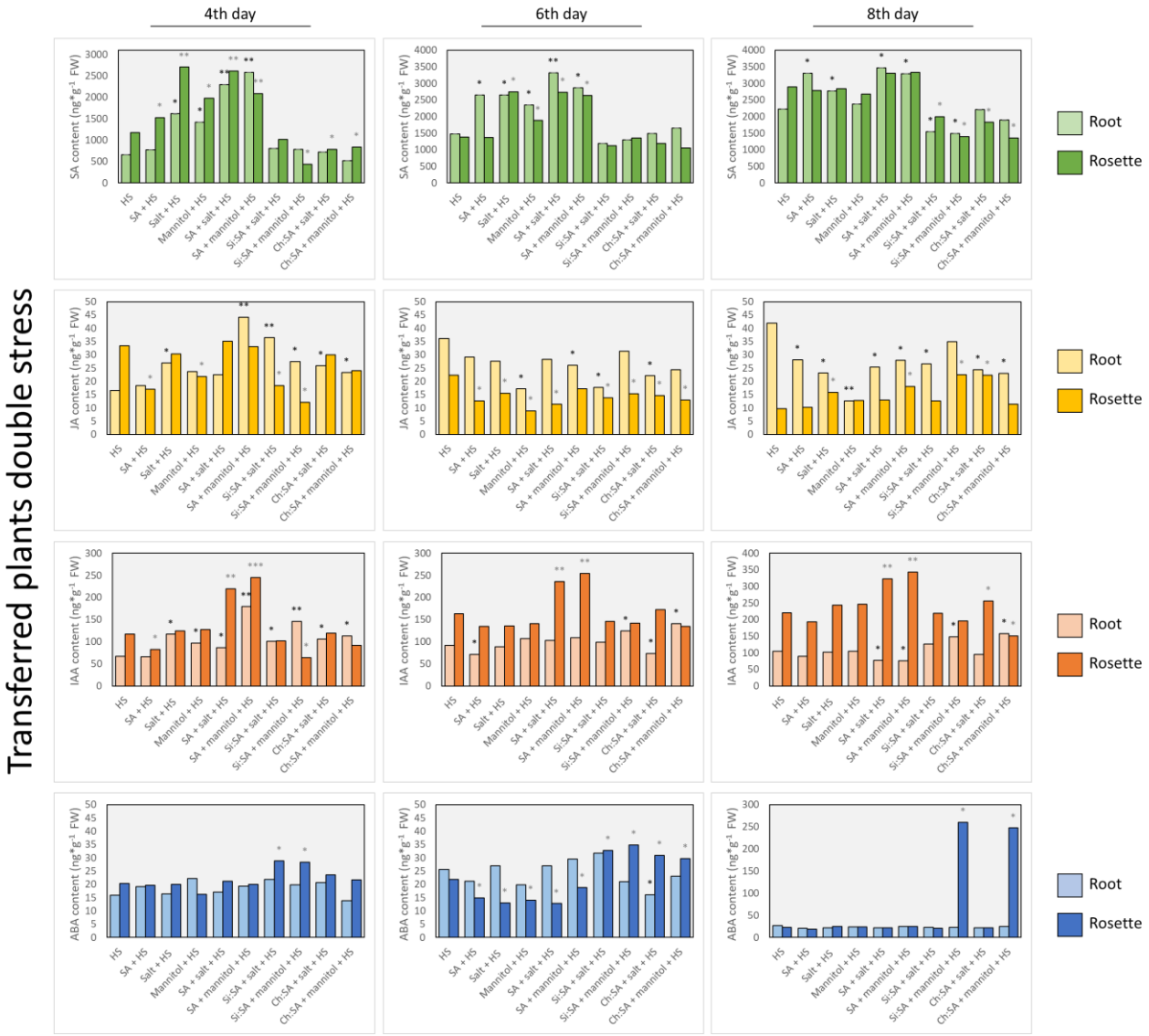
Supplementary Figure 5. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants grown from seeds, under salt and mannitol stress at regular temperature (23°C) (simple stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.



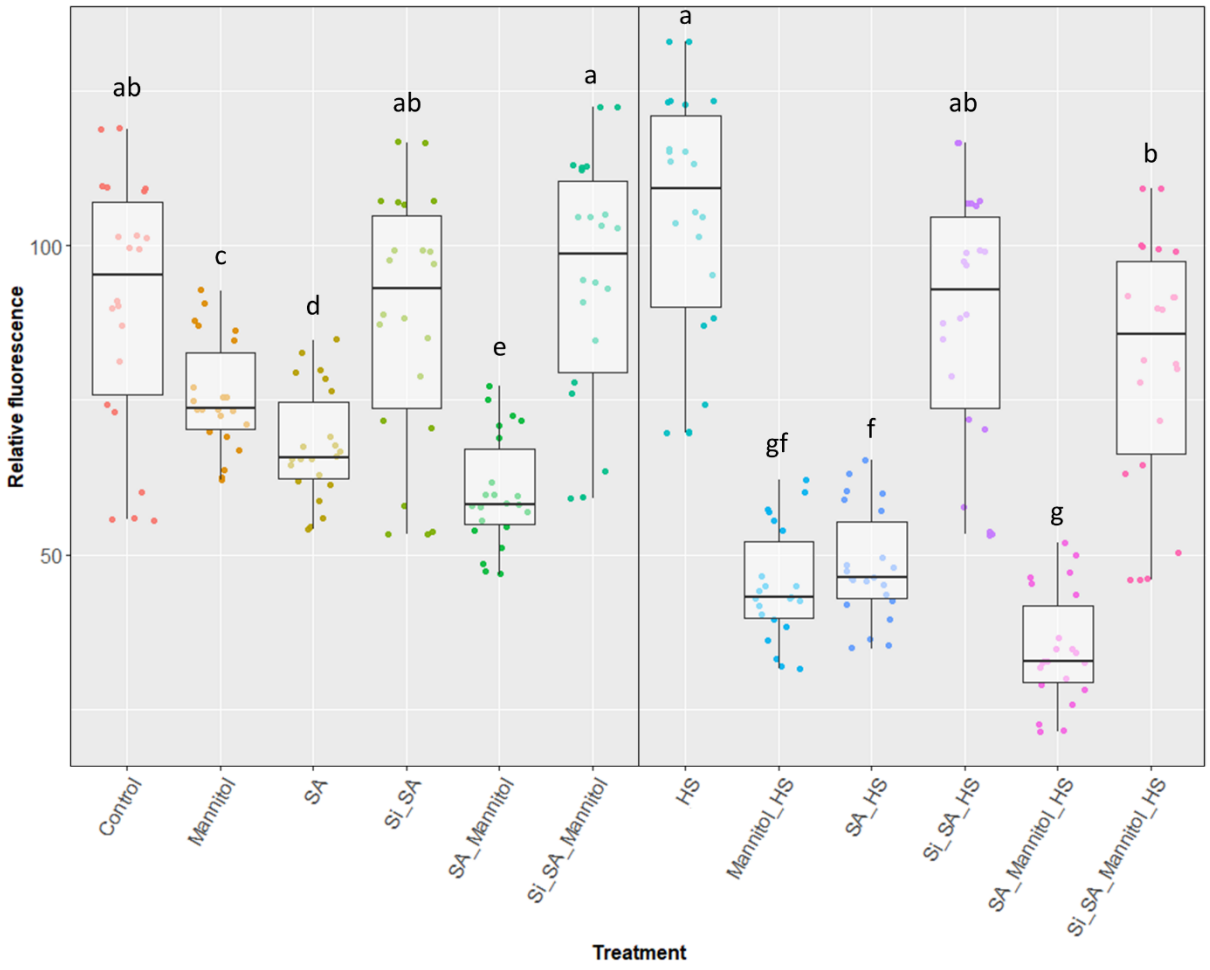
Supplementary Figure 6. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants grown from 4-day-old transferred plants, under salt and mannitol stress at regular temperature (23°C) (simple stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.



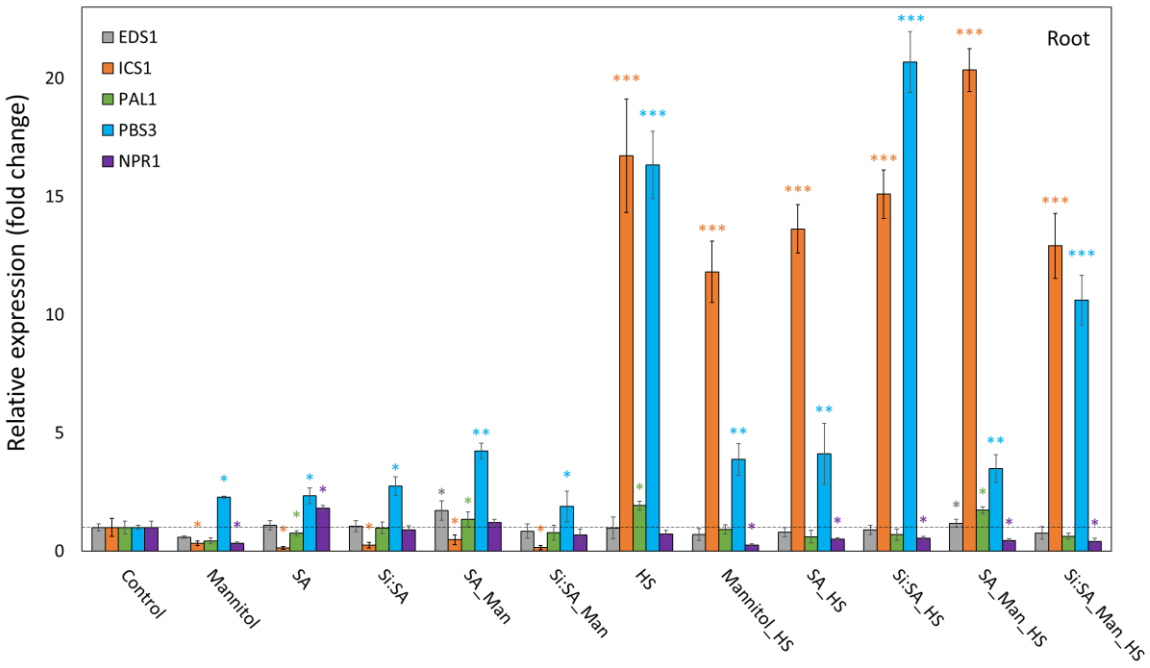
Supplementary Figure 7. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants grown from seeds, under salt and mannitol stress combined with heat (30°C) (double stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.



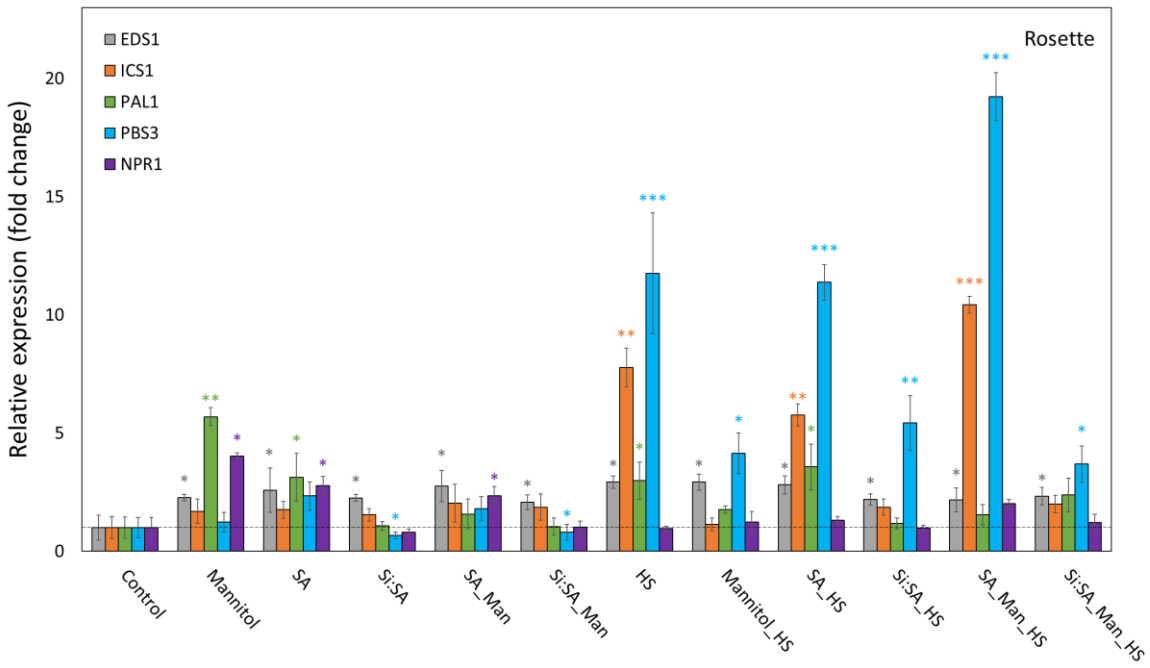
Supplementary Figure 8. Effect of free SA, Si:SA and Ch:SA on endogenous phytohormone levels in roots and rosettes of *Arabidopsis thaliana* plants grown from 4-day-old transferred plants, under salt and mannitol stress combined with heat (30°C) (double stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.



Supplementary Figure 9. Effect of free SA and Si:SA on fluorescence intensity in the principal root of *Arabidopsis thaliana* DR5:GFP plants under mannitol stress at regular temperature (23°C) (simple stress conditions) and combined with heat (30°C) (double stress conditions). The relative fluorescence quantification was performed in the quiescent center zones of treated plants.

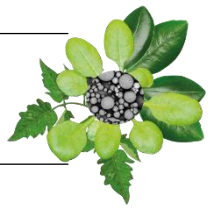


Supplementary Figure 10. Effect of free SA and Si:SA on the relative expression of SA-related genes in the roots of 4-day-old transferred *Arabidopsis thaliana* plants under mannitol stress at regular temperature (23°C) (simple stress conditions) and combined with heat (30°C) (double stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.



Supplementary Figure 11. Effect of free SA and Si:SA on the relative expression of SA-related genes in the rosettes of 4-day-old transferred *Arabidopsis thaliana* plants under mannitol stress at regular temperature (23°C) (simple stress conditions) and combined with heat (30°C) (double stress conditions). Values with *, **, ***, are statistically different at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

GENERAL DISCUSSION



In recent years, human activities have contributed to altering climate, leading to extreme weather events that cause desertification, flooded soils, extreme temperatures, and pathogen emergence. These harsh conditions directly affect agriculture by decreasing crop productivity and quality [1]. Plants have developed an arsenal of defences, including modifying gene expression, adjusting growth rates, altering metabolism, and producing secondary metabolites [2]. These secondary metabolites include various acids, flavonoids, carotenoids, unsaturated fatty acids, and phytohormones (key regulators of plant development and growth [3]).

Among phytohormones salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and indole acetic acid (IAA) play important roles in plant responses to adverse conditions [4,5]. They stimulate specific plant responses and trigger crosstalk among them, enabling plants to adapt to challenging environments. Several studies have investigated the exogenous application of phytohormones on plants under adverse conditions, demonstrating enhanced resilience against environmental stress [6]. SA is particularly versatile, as it boosts plant defences against various stresses while also promoting their development in turn [7].

In general, SA is applied into plants by two ways: either mixed into a nutrient solution for root absorption or sprayed directly onto the leaves and stems [8]. While SA has shown beneficial effects in promoting plant tolerance, there are several concerns regarding its use as an exogenous treatment: i) determining the right dose, and ii) its degradation by environmental factors such as pH or temperature [9]. Encapsulation offers a potential solution by enabling more precise management of both application and dosage of SA. Encapsulation allows SA to be packaged in a carrier, creating capsules with enhanced biological characteristics while promoting its controlled release [10].

Therefore, this thesis focuses on the study of the physico-chemical, and biological characteristics of encapsulated SA. The research began with formulating encapsulated SA using two shell materials: silica and chitosan. This was followed by optimizing the encapsulation process by analysing its key variables. Next, the effects of free SA versus encapsulated SA at different concentrations were investigated in *Arabidopsis thaliana* plants. Finally, the research explored the mechanism by which encapsulated SA mitigates abiotic stress on stressed Arabidopsis plants.

The first chapter of this thesis focuses on obtain particles of SA encapsulated in chitosan (Ch) or silica (Si) at several ratios. The suspension formulation of the capsule active compound (Si/Ch:SA) was initially produced by homogenizing their respective amounts using a planetary mill. The rheological behaviour was evaluated to test the suspension for spraying. Viscosity analysis showed well-dispersed particles on Si:SA suspensions which is remarkable considering their nanometric size. This also evidences both low shear thinning behaviour and low pseudoplasticity, attributed to their symmetrical spherical geometry even between tested ratios [11]. Ch:SA suspensions showed a mild shear thinning behaviour and increased pseudoplasticity related with the tested ratio. This behaviour could be attributed to the low solid content in Ch:SA suspensions and the irregular geometry of the Ch, which causes particle alignment. In contrast, the spherical geometry of Si particles prevents this alignment phenome.

Despite the differences, the suspension of both Si:SA and Ch:SA were suitable for spray drying, obtained dry powder of each tested ratio. For each dry powder, several physico-chemical parameters were analyzed to determine the effectiveness of encapsulation process and its potential biological effect. The capsule size distribution was obtained through image analysis, determining the equivalent diameters (D_{10}, D_{50} and D_{90}) from the accumulated frequency vs diameter curve. The size of encapsulated samples can influence toxicological effects in plants, when they are applied exogenously [12,13]. Interestingly, the capsule size distribution for both samples at the three ratios exhibited a remarkable similarity.

Another important parameter to consider is the thermal stability of encapsulated samples. Simultaneous Differential Thermal and Thermogravimetric Analysis (DTA-TGA) of pure raw materials showed that Si has high thermal stability with minimal mass loss (around 5%). In contrast, SA decomposes completely at 266°C, while chitosan undergoes a two-stage thermal decomposition: moisture evaporation (90°C) and dehydration/decomposition (313°C). In the case of Si:SA, encapsulation decreased the SA decomposition rate, while for Ch:SA, the two-stage decomposition showed new peaks at 200°C and 400°C, respectively, suggesting that encapsulated SA was decomposed at higher temperature than free SA. This observation confirms the successful encapsulation process for all three ratios studied within each capsule material [14,15].

Likewise, the Energy Dispersive X-ray Spectroscopy coupled with Scanning Electron Microscopy (SEM-EDS) allowed confirmation of the successful encapsulation of SA. EDS analysis of Si:SA revealed three main elements: carbon, oxygen, and silicon. When SA is encapsulated, carbon content increases while silicon decreases, probably due to a gradual distribution of SA within the silica matrix. In the EDS analysis of Ch:SA, five elements were identified: carbon and oxygen from Ch and SA, nitrogen from Ch, and phosphorus and sodium from the TPP-Na crosslinker. Unlike Si:SA, Ch:SA has

not a unique element for easy differentiation. However, microanalysis of Ch:SA revealed higher levels of nitrogen, phosphorus, and sodium, consistent with the presence of Ch and TPP-Na [14,16]. This suggests SA entrapment in Ch by TPP-Na crosslinking, confirming the SA encapsulation.

Once confirmed that SA was correctly encapsulated in both Si and Ch, encapsulation efficiency (EE%) and kinetic release parameters were analysed. The EE% results showed mean values of 61.9% and 46.7% for Si:SA and Ch:SA sample, respectively. These differences may be originated from disparities in the encapsulation process, where SA seems to saturate the Si capsule from the exterior towards the interior in Si:SA samples and become trapped by Ch as the polymeric chains crosslink with TPP-NA in Ch:SA samples. Additionally, specific surface area of Si (117 m^2/g) might contribute to higher SA loading. To study the release mechanisms of SA, in vitro kinetic assays were accessed. Results showed that SA was released in the first 6 hours of evaluation in a single stage for both samples. The Korsmeyer-Peppas model allowed to determine the kind of release mechanisms (Fickian or non-Fickian diffusion) [17], as well as the speed of this release. Diffusion analysis (n) revealed non-Fickian release (case II transport) for both Si:SA and Ch:SA, suggesting that SA is released through the dissolution or relaxation of the capsules [18,19]. With respect of the speed of release (k), higher ratios samples showed a faster release kinetic, correlating with higher EE%. Encapsulated SA samples with the highest ratio could experience capsule saturation due to the higher amount of SA, diminishing the effective encapsulation capacity [20].

The biological effect of SA on pathogenic fungi was evaluated at different concentrations through analysis of mycelial growth inhibition. The lowest ratio samples: Si:SA (1:0.25) and Ch:SA (1:0.5) at 1000 µM exhibited significantly growth inhibition against *Alternaria alternata* (62.5%) and *Botrytis cinerea* (80.1%) compared to free SA (45.8% and 37.3%, respectively). This stronger inhibition suggests that SA has an important antifungal activity which is enhanced by its encapsulation. Finally, the effect of Si:SA (1:0.25) and Ch:SA (1:0.5) at 100 µM on *Arabidopsis thaliana* growth was investigated. While free SA treatment inhibited root and shoot growth compared to control plants, encapsulated SA treatments mitigated this inhibition. This suggests encapsulation diminishes SA toxicity. The lowest ratios promote controlled SA release, reducing plant growth inhibition [21].

After characterizing the physico-chemical and biological properties of encapsulated SA samples, we investigated several questions regarding the encapsulation process. The encapsulation process involves formulating a suspension, followed by spray drying. Spray drying requires elevated temperatures (higher than 100°C) to fully evaporate the solvent within the suspension. A concern existed regarding the potential impact of this spray temperature on the structure and properties of the encapsulated SA. In this sense, the second chapter of this thesis consisted of formulating encapsulated SA samples in Si and Ch, using an organic solvent (acetone) to lower the spray temperature. To formulate the encapsulated

SA samples, a water/acetone mixture was tested, and preliminary experiments were conducted to optimize the grinding speed in the Ch:SA samples (with lower solid content) and minimize the wear of the grinding medium during the process, which could interfere with the encapsulation process.

The Ch:SA (1:1) sample was selected for optimizing the rotational speed of the suspension preparation due to its lower solid content, exacerbating wear issues. To determine the optimal speed, parameters such us the suspension density, the solid waste generated (not incorporated into the suspension), and grinding wear were parameterized. Results showed that speeds below 150 rpm resulted in decreased density and increased solid waste, likely due to poor dispersion of the raw materials [22]. The best results of density (0.963 g/mL) and grinding wear (30%) with no solid waste was achieved at 160 rpm. Once the milling conditions for Ch:SA were established, suspensions with both capsules, Si:SA and Ch:SA, were formulated in acetone and spray-dried at 100°C. Several parameters specific to suspensions and dry powders were analysed, highlighting the differences between ace-Si:SA and ace-Ch:SA in terms of performance. The ace-Si:SA samples exhibited higher performance values (41.6%–59.8%) than those of ace-Ch:SA samples (17.8%–24.4%). This difference could be attributed to the partial solubility of chitosan during the grinding process or its lower solid content in the formulation.

Similar trends were observed with the other analyzed parameters: viscosity, particle size distribution (D_{50}), elemental chemical composition, and thermal stability, when compared to samples formulated in water. This trend suggests effective encapsulation in both water and acetone. The kinetic and antifungal experiments confirmed that there were no differences attributable to the solvent used (drying process temperature). Kinetic analysis revealed the same release mechanism (case II transport) for samples formulated in both water and acetone, consistent with the results on the study of water-formulated samples[11]. Antifungal experiments revealed that both water-formulated and acetone-formulated samples displayed inhibitory effects on the micelial growth of *Alternaria alternata* and *Penicillium digitatum*. Both Si:SA and ace-Si:SA, as well as Ch:SA and ace-Ch:SA, exhibited similar inhibition rates at the lowest ratio tested of 65% and 50%, for *Alternaria alternata* and *Penicillium digitatum*, respectively. Acetone does not seem to alter the encapsulation process or the biological activity of SA; thus, there is no justification for using it as a solvent to lower the spray temperature. Encapsulated samples formulated in water and acetone exhibited no significant differences, suggesting that spray temperature (within the studied range) did not affect the structure of SA.

Once the possibility of SA degradation due to atomization temperature was ruled out, the next part of this chapter focused on optimizing the encapsulation process conditions. This involved a thorough examination of the main variables, each assigned two values—one high and one low: solid content (6.4 and 4.0%), milling speed (220 and 160 rpm), milling time (80 and 40 min), spray dryer temperature (160 and 130°C), spray dryer feed rate (12 mL/min and 5 mL/min) and spray dryer airflow (90% and

70%). A fractional factorial experimental design was employed to evaluate various combinations of these variables [23,24], which allowed reducing the number of experiments to 16. The density and viscosity measurements across the 16 suspensions remained unchanged. After the suspensions were sprayed, SEM images analysis of the powders showed that all experiments exhibited a consistent doughnut shape, as well as a similar range of mean capsule size distribution values (17–26 μm).

Among the 16 experiments, the experiments 7 and 15 stood out due to their high performance values. A closer analysis revealed that a high performance could related with a high grinding wear. These two experiments exhibited lower mass loss compared to the others, possibly because alumina was encapsulated instead of SA. Furthermore, they showed decreased moisture content, which could be related to a reduction in specific surface area (S_{BET}) caused by grinding wear, then preventing water retention [25]. However, despite their high performance values, the low EE% suggests that alumina might be competing with SA for being encapsulated. Additionally, the low antifungal activity of experiments 7 and 15 against pathogenic fungi indicates that the actual amount of encapsulated SA might be lower, with alumina potentially occupying the majority of the capsule. On contrary, experiments 2 and 10 showed the lowest grinding wear values. As expected, lower grinding wear resulted in moderate performance values, but conversely led to increased encapsulation efficiency (EE%). Additionally, decreased mass loss, coupled with low antifungal activity, suggests insufficient SA encapsulation.

The most favourable outcomes were observed in experiment 8, characterized by the highest values of EE% and inhibition rate, as well as a controlled release of SA. Additionally, average values for performance, grinding wear, moisture and S_{BET} were noted, indicating an optimal balance between SA content and the grinding wear during formulation. The highest EE% directly promote a controlled release of SA which enhance the effect on fungi micelial growth. This experiments analysis revealed that the solid content, milling speed, and milling time are the most influential variables during encapsulation process. These three variables impact on the correct material homogenization, and control the grinding wear [26]. A lower solid content in combination with the higher speed and extended time, can lead to alumina detachment, which impacts the performance, moisture, S_{BET}, and EE% values. In addition to these three variables, it is important to keep a low feed rate with a low airflow to promote proper powder drying.

The third chapter focuses on studying the physiological effects of free SA and encapsulated SA treatments on Arabidopsis thaliana plants at various concentrations. During stress conditions, plants modulate their endogenous phytohormone levels to activate defense responses [27]. SA plays an important role controlling the plant defense while maintaining its growth and development [28]. However, when endogenous SA levels exceed concentrations of 100 μM, germination and plant growth become compromised [29]. This negative effect can be supressed by encapsulation, promoting

controlled SA release, as demonstrated in the first chapter with the lowest ratios of Si:SA and Ch:SA. To study this, several doses (1, 10, 50, 100 and 500 µM) of free SA, Si:SA (1:0.25) and Ch:SA (1:0.5), were applied on Arabidopsis plants to determine their impact on primary root development. After five days, plants treated with free SA showed a severely decrease on the length of primary root at all doses. Plants treated with encapsulated SA maintained root length similar to controls at 1 and 10 µM, while at higher concentrations (50, 100, 500 µM) a moderate decrease was observed.

Examination of rosette size revealed a dose-dependent decrease in plants treated with free SA. Rosette area became progressively smaller with increasing SA concentration. In contrast, plants treated with encapsulated SA exhibited rosette size similar to control plants at 1 and 10 µM concentrations. Remarkably, even the lowest doses of free SA treatment resulted in a potential decrease in size. These findings suggest that encapsulation reduces the availability of free SA in the growth medium [30], thereby controlling the amount absorbed by the plant. Gravitropism response of principal root was also investigated. In a similar trend, plants treated with free SA maintain normal gravitropism at low doses but it was disrupted at doses higher than 50 µM, resulting in progressively impaired root reorientation. These findings suggest that free SA disturbs normal gravitropism, possibly through altered auxin signalling. In contrast, plants treated with encapsulated SA exhibited reduced agravitropic phenotypes even at the highest doses. Once again, the controlled release of SA achieved through encapsulation significantly mitigated agravitropism.

The observed changes in the morphology of plants treated with free SA could be related to increased SA endogenous levels, potentially affecting IAA endogenous levels [31,32]. To corroborate this assumption, we analyzed the endogenous phytohormone profiles (SA, JA, ABA, and IAA) in both roots and rosettes of plants treated with free and encapsulated SA at all doses, after 28 days of treatment. Plants treated with free SA exhibited significantly increased endogenous SA levels in both rosettes and roots. In addition, these plants exhibited a decrease on IAA levels in roots, being undetectable at 500 µM. This suggests a disrupted balance between SA and IAA endogenous levels. Encapsulation significantly reduced the accumulation of SA in both roots and rosettes on treated plants, diminishing the dramatic impact on IAA levels.

To validate the decreased IAA levels on treated plants, the *Arabidopsis thaliana* DR5::GFP reporter line sensor was used to monitor auxin distribution [33]. An increased of relative fluorescence of the reporter line was exhibited in the root tip, on DR5::GFP plants treated with free SA at 1, 10 and 50 µM doses. However, these plants displayed a decreased and complete absence of fluorescence signal at 100 µM and 500 µM, respectively. Conversely, DR5::GFP plants treated with encapsulated SA maintained this increase even until 100 µM, and showed a slight decrease at the highest dose of 500 µM. Remarkably, encapsulated SA prevented IAA decreased at higher doses, allowing for continued root

and rosette growth on treated plants, unlike plants treated with free SA. The controlled release of SA by encapsulation prevents an early SA uptake by roots, reducing translocation to the rosette. Consequently, this regulation of endogenous SA and IAA levels minimizes pleiotropic effects in plants.

PCA revealed a correlation between negative effects showed in root (short length and agravitropism) with SA content [34]. This supports that free SA treatment, increase endogenous SA while alter IAA endogenous levels, reducing root development. In the Individuals-PCA graphic, free SA treatments cluster together, with higher doses causing an important variability in plant responses. Conversely, Si:SA at lowest doses and Ch:SA treatments group near to control plants, suggesting minimal developmental impact.

The third chapter demonstrates that encapsulated SA can reverse the negative effects caused on treated plants by exogenous free SA. Therefore, the final chapter investigates the mechanism by which encapsulated SA promotes a protective effect on plants under abiotic stresses. Experiments employed two *Arabidopsis thaliana* cultivation systems: seeds or 4-day-old transferred plants. These plants were subjected to salt stress, mannitol stress, and their combination with heat stress, with or without treatment by free or encapsulated SA [Si:SA (1:0.25) or Ch:SA (1:0.5)]. Plants subjected to salt or mannitol stress, and combined or not with heat stress (HS), showed a decreasing on principal root length in comparison with control plants. In addition, plants under stress conditions treated with free SA exhibited a more severe decrease in root length, probably because free SA enhances the defense response but compromises root development [35]. In the case of plants under stress conditions but treated with Si:SA or Ch:SA, root lengths were similar to those of control plants. This suggests a gradual uptake of released SA, which activates the defense response while maintaining normal root growth and development.

Analysis of the rosette in treated plants showed that stress conditions and free SA treatment resulted in a smaller rosette size compared to control plants. Conversely, plants under stress conditions but treated with encapsulated SA maintained rosette size similar to control plants. However, 4-day-old transferred plants exhibited a greater decrease in rosette size compared to seeds. This could be because these plants first require the induction of acquired tolerance to resist the stressful conditions [36], compromising their normal development. However, 4-day-old transferred plants under the same conditions did not show a difference between treatments. This suggests that plants prioritize primary root growth at the expense of secondary roots during the initial stress response. Surprising, secondary root density declined in plants under salt and mannitol stress conditions, suggesting a disruption in ion balance. The free SA treatment did not improve the number of secondary roots, while encapsulated SA treatments reverted this negative effect. Seeds treated with encapsulated SA subjected to salt or mannitol stress and combined with HS, exhibited an increase in secondary root number compared to control plants,

while in 4-day-old plants under the same conditions did not show a difference between treatments. This suggests that plants prioritize principal root growth of secondary roots during stress conditions.

Phytohormone profiles were analyzed in treated plants to identify specific interactions. Plants treated with free SA under stress conditions exhibited elevated endogenous SA levels, while JA levels decreased. These plants also displayed a decrease in IAA levels, with a notably greater decrease observed in the roots compared to the rosette. This suggests that increased SA might affect IAA transporter through hyperphosphorylation [37], conditioning IAA transport from the rosette to the root. Additionally, ABA levels in all treated plants remained similar to those in the control plants. Conversely, plants treated with encapsulated SA displayed significantly lower SA levels compared to plants under stress conditions, those treated with free SA, or those with the combination of stress and free SA. These plants maintained endogenous IAA levels comparable to those in controls in both rosettes and roots, which can facilitate plant growth and adaptation to stress conditions. Under heat stress, phytohormone profiles of treated plants was similar to previous observations in plants under simple stress conditions. Similar trends in SA and IAA levels were observed, but with an important increase on their values due to HS. Again, plants treated with encapsulated SA showed SA and IAA levels similar to control plants, suggesting enhanced thermotolerance provided by the controlled release of SA. Plants under mannitol + HS, and treated with encapsulated SA, showed an increase in endogenous ABA levels. This suggest that SA levels were sufficient to protect plant against stress conditions without disrupting the phytohormone homeostasis.

Plants exhibiting the most substantial stress-induced morphological changes and their reversal by encapsulated SA were selected for auxin distribution analysis. Transferred plants system cultivation under mannitol and mannitol + HS, and treated or not with Si:SA, were selected for analysis. *Arabidopsis thaliana* DR5::GFP reporter line was used to determinate the influence of SA on IAA root distribution [38]. Stress conditions and free SA treatments derived in decreased DR5 activity in treated plants. In contrast, plants treated with Si:SA or Si:SA combined with mannitol showed similar DR5 activity, probably by the controlled release of SA. This reduction in DR5 activity could be explained by the progressive accumulation of SA in the roots and the subsequent decrease on IAA levels. Plants treated with stress conditions, free SA, and its combination with HS, had a significant reduction in DR5 activity compared to control plants. Interestingly, plants treated with encapsulated SA (Si:SA) in combination with HS, or with both HS and mannitol, maintained DR5 activity levels similar to control plants. The controlled release of SA helps maintain IAA levels, thereby enhancing plant tolerance to abiotic stresses.

Additionally, the effect of exogenous treatments on the SA biosynthesis pathway was analyzed. The relative expression of EDS1, ICS1, PAL1, PBS3, and NPR1 genes were analyzed in both rosette and

root tissues separately. Interesting, different gene expression patterns was observed between roots and rosettes. EDS1, PAL1, and NPR1 were downregulated in roots compared to rosettes. This suggest that SA uptake from root was translocated in direction to rosette, subsequently promoting SA synthesis and its accumulation [39]. The roots of plants treated with Si:SA and Si:SA + mannitol showed that EDS1, ICS1, and NPR1 genes were downregulated, suggesting that the SA uptake in these cases is not sufficient to activate the SA biosynthesis and subsequent accumulation. Furthermore, plants treated with Si:SA + mannitol + HS upregulated ICS1 and PBS3, which possible prevents SA accumulation in the root [40,41]. In the case of rosettes, plants treated with Si:SA + mannitol and Si:SA + mannitol + HS, exhibited relative expression values of PAL1, PBS3, and NPR1 similar to control plants. The slow release of SA from Si:SA did not induce SA accumulation, allowing normal growth and stress tolerance on treated plants.

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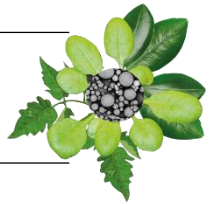
CONCLUSIONS



- Encapsulation emerges as an important tool, acting as nanocarrier for phytohormones. The lowest ratios of encapsulated SA samples [Si:SA (1:0.25) and Ch:SA (1:0.5)] provide a better controlled release of SA than the other ratios through efficient encapsulation without saturating the Si or Ch capsules.

- Encapsulated SA exhibit a stronger antifungal effect on *A. alternata, B. cinerea, F. oxysporum*, and *G. candidum*, because controlled release of SA prolonged its anti-pathogenic effect over time. In turn, the slow release of SA helps to revert the toxic effect on plant growth caused by free SA, reducing its over accumulation.

- Encapsulated SA formulated in water and acetone present similar physical-chemical, and biological characteristics. This comparison between encapsulated SA samples reveals that SA is not affected during spray drying at 150°C. The use of organic solvent is unnecessary, saving costs and mitigating a potential environmental damage.

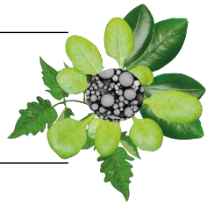
- The analysis of SA encapsulation optimization determines that solid content, milling speed, and milling time are more influential variables than spray temperature, feed rate and airflow. The fractional factorial experimental design offers a straightforward method to enhance the encapsulation process, making it suitable for scalable industrial manufacturing.

- Encapsulated SA reverts the negative effects of free SA on the root length, rosette size and gravitropism of treated plants by decreasing the SA available in the medium. Plants treated with free SA take up the maximum SA available, accumulating it in both the rosette and root. Additionally, encapsulated SA prevents the uncontrolled SA release even at high doses, maintaining a proper balance between SA and IAA, thus avoiding a decrease of IAA in the root tip.

- Encapsulated SA enhances plant tolerance to salt or mannitol stress, as well as their combination with HS. This effectiveness is attributed to maintaining a balance between SA and IAA. Plants under stress or treated with free SA exhibited IAA accumulation in the rosette likely due to increased endogenous SA levels, which affect IAA transport from the rosette to the root.

- Plants subjected to stress conditions and treated with encapsulated SA maintain expression of SA biosynthesis genes: EDS1, PAL1, and NPR1, in both roots and rosettes compared to control plants. This regulation of gene expression promotes the necessary SA synthesis for plant defense without overaccumulation. Plants are able to tolerate adverse conditions because encapsulated SA modulates endogenous phytohormone levels and regulates the expression of SA-related genes.

APPENDIX



Escola de Doctorat · ED

Yo **Vicente Vives Peris**, como coautor/ coautora doy mi **autorización** a Jimmy Andres Sampedro Guerrero para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2023). Efficient strategies for controlled release of nanoencapsulated phytohormones to improve plant stress tolerance. Plant Methods, 19(47). https://doi.org/10.1186/s13007-023-01025-x

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Improvement of salicylic acid biological effect through its encapsulation with silica or chitosan. International Journal of Biological Macromolecules, 199:108-120. https://doi.org/10.1016/j.ijbiomac.2021.12.124

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Encapsulation Reduces the Deleterious Effects of Salicylic Acid Treatments on Root Growth and Gravitropic Response. International Journal of Molecular Sciences, 23(22):14019. https://doi.org/10.3390/ijms232214019

Asimismo, **renuncio** a poder utilizar estas publicaciones como parte de otra tesis doctoral. Y para que conste firmo el presente documento,

Castellón, 10 de mayo del 2024

*Firmado por VICENTE VIVES PERIS - NIF:***8365** el día 10/05/2024 con un certificado emitido por ACCVCA-120*

Todo ello, atendiendo al artículo 28 del Reglamento de los estudios de doctorado de la Universitat Jaume I de Castelló, regulados por el RD 99/2011, en la Universitat Jaume I (Aprobado en la sesión nº 8/2020 del Consejo de Gobierno de 02 /10/2020):

"(...)

4. En el caso de publicaciones conjuntas, todas las personas coautoras deberán manifestar explícitamente su autorización para que la doctoranda o doctorando presente el trabajo como parte de su tesis y la renuncia expresa a presentar este mismo trabajo como parte de otra tesis doctoral. Esta autorización se adjuntará como documentación en el momento del inicio de evaluación de la tesis.

Escola de Doctorat · ED

Yo **Aurelio Gómez Cadenas**, como coautor/ coautora doy mi **autorización** a Jimmy Andres Sampedro Guerrero para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2023). Efficient strategies for controlled release of nanoencapsulated phytohormones to improve plant stress tolerance. Plant Methods, 19(47). https://doi.org/10.1186/s13007-023-01025-x

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Improvement of salicylic acid biological effect through its encapsulation with silica or chitosan. International Journal of Biological Macromolecules, 199:108-120. https://doi.org/10.1016/j.ijbiomac.2021.12.124

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2022). Encapsulation Reduces the Deleterious Effects of Salicylic Acid Treatments on Root Growth and Gravitropic Response. International Journal of Molecular Sciences, 23(22):14019. https://doi.org/10.3390/ijms232214019

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Relación de publicaciones:

- Sampedro-Guerrero, J., Vives-Peris, V., Gomez-Cadenas, A., Clausell-Terol, C. (2023). Efficient strategies for controlled release of nanoencapsulated phytohormones to improve plant stress tolerance. Plant Methods, 19(47). https://doi.org/10.1186/s13007-023-01025-x

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