UNIVERSIDAD POLITÉCNICA DE VALENCIA
Departamento de Tecnología de Alimentos

APROXIMACIONES A UNA ESTRATEGIA INTEGRADA PARA EL CONTROL NO CONTAMINANTE DE LAS PODREDUMBRES VERDE Y AZUL EN POSCOSECHA DE CÍTRICOS

TESIS DOCTORAL

Presentada por:
Pedro Antonio Moscoso Ramírez

Director:
Dr. Lluís Palou Vall

Tutora:
Dra. María Dolores Ortolá Ortolá

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D. Lluís Palou Vall, Investigador del Centro de Tecnología Poscosecha del Instituto Valenciano de Investigaciones Agrarias

CERTIFICA: Que la memoria titulada "Aproximaciones a una Estrategia Integrada para el Control no Contaminante de las Podredumbres Verde y Azul en Poscosecha de Cítricos", que, para aspirar al grado de Doctor en Ciencia, Tecnología y Gestión Alimentaria presenta D. Pedro Antonio Moscoso Ramírez, realizada bajo mi dirección en el Centro de Tecnología Poscosecha del Instituto Valenciano de Investigaciones Agrarias, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que

AUTORIZO: Al interesado a su presentación en el Departamento de Tecnología de Alimentos de la Universidad Politécnica de Valencia.

Y para que conste a los efectos oportunos, presento la referida memoria, firmando el presente certificado en Valencia a 5 de Noviembre de 2013.

Fdo. D. Lluís Palou Vall

Yo solo sé que no sé nada

Sócrates.

Dedicado a mi esposa Bolivia y mis hijos Estefanía Guadalupe, Iván Alejandro y David Antonio, a quienes los amo y me han brindado su apoyo y confianza para terminar esta encomienda.

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RESUMEN

Las podredumbres verde y azul, cuyos agentes causales son los hongos fitopatógenos *Penicillium digitatum* (Pers.: Fr.) Sacc. y *Penicillium italicum* Wehmer, respectivamente, son las responsables de grandes pérdidas de importancia económica en poscosecha de cítricos a nivel mundial. En España, las pudriciones de la fruta en poscosecha pueden llegar al 10% del producto cosechado en una estación típica. Sin embargo, bajo condiciones favorables a la enfermedad, las pérdidas pueden alcanzar el 50%. Actualmente, el sector aún depende del uso de fungicidas químicos sintéticos para controlar las enfermedades de poscosecha de fruta fresca en general y de cítricos en particular. Pero el uso de estos fungicidas conlleva el problema de la presencia de residuos químicos en la fruta, el desarrollo de cepas de *P. digitatum* y *P. italicum* resistentes a estos tratamientos y la contaminación del medio ambiente por vertido inadecuado de caldos fungicidas.

Un control adecuado de las enfermedades de poscosecha sin la utilización de fungicidas convencionales no puede enfocarse en una sola estrategia de control, sino que debe considerar todos los factores que influyen en la incidencia. En este sentido, el control integrado no contaminante de enfermedades de poscosecha (CINCEP) es una estrategia global, que toma en cuenta la epidemiología de la enfermedad y todos los factores de precosecha, cosecha y poscosecha para incidir sobre ellos en forma combinada para reducir las pérdidas económicas. En base a la problemática anterior y en un contexto de CINCEP, la meta general de la presente tesis doctoral fue profundizar en el conocimiento de algunos factores de incidencia de enfermedad y buscar estrategias alternativas no contaminantes para el control en poscosecha de las podredumbres verde y azul de los cítricos.

Primeramente se determinó el efecto de la desverdización comercial con etileno, en las condiciones españolas, en la susceptibilidad del fruto y el desarrollo de las podredumbres verde y azul en mandarinas ‘Clemenpons’, Clemenules’ y ‘Nova’, y naranjas ‘Navelina’ de recolección temprana, así como también el efecto en los atributos de calidad de estos cultivares de alta importancia económica (capítulo 1). La desverdización comercial a 2 μL L^{-1} de

etileno a 21ºC y 95-100% HR durante 3 días, no tuvo efecto en la susceptibilidad de frutos a las podredumbres verde y azul en los cultivares de cítricos desverdizados, inoculados unas 2 h después con *P. digitatum* o *P. italicum* e incubados a 20°C y 90% HR durante 7 días; y tampoco tuvo efecto en la incidencia de las podredumbres cuando los frutos se desverdizaron unas 2 h después de la inoculación fúngica y se almacenaron a 20°C y 90% HR durante 7 días o a 5°C y 90% HR durante 14 días. En cambio, incrementó la severidad de las podredumbres, pero sólo en frutos con un índice de color inicial de la piel relativamente alto. Por otro lado, con la excepción del color, la desverdización no afectó de manera general a los atributos de calidad externa e interna de los frutos.

Como una alternativa al control químico convencional, se evaluaron tratamientos de poscosecha en aplicaciones preventivas y curativas con inductores químicos seleccionados por su capacidad general para inducir resistencia en plantas (capítulo 2). Se evaluaron en pruebas primarias de selección *in vivo* distintas concentraciones de silicato de sodio (SSi), ácido 2,6-dicloroisonicotínico (INA), ácido β-aminobutírico (BABA), benzotiadiazol (BTH), ácido salicílico (SA), ácido acetil salicílico (ASA) y la proteína Harpin. Solamente los cuatro primeros de estos siete compuestos a las concentraciones respectivas de 1000, 0,03, 0,3, y 0,9 mM, redujeron significativamente las podredumbres verde y azul en naranjas 'Valencia' o 'Lanelate' inoculadas unas 2 h después del tratamiento e incubadas a 20°C durante 7 días. SSi a 1000 mM fue el tratamiento más eficaz para prevenir las podredumbres, pero fue descartado por la presencia de residuos en la piel del fruto y los consiguientes riesgos de producción de fitotoxicidad. Los inductores químicos carecieron de actividad curativa contra las podredumbres cuando los patógenos se inocularon 24 h antes del tratamiento.

Se evaluó también la actividad preventiva y curativa de tratamientos de poscosecha con silicato de potasio (PSi) (capítulo 3). En pruebas primarias de selección *in vivo*, tratamientos preventivos con PSi a 90 mM, redujeron la incidencia de las podredumbres verde y azul hasta un 52% en naranjas almacenadas a 20°C durante 6 días. Un tiempo de 2 h entre este tratamiento y la inoculación de *P. digitatum* redujo significativamente la incidencia y la severidad de la

podredumbre verde, mientras que con tiempos de 24, 48, 72 y 96 h la reducción no fue significativa. En ensayos de influencia espacial, este tratamiento no tuvo la capacidad para inducir resistencia sistémica en la piel del fruto contra *P. digitatum* porque no hubo control en heridas inoculadas localizadas a 10, 20 o 30 mm de distancia del punto de aplicación del tratamiento. Las mejores condiciones de tratamiento con PSi a 90 mM en soluciones acuosas fueron una temperatura de 20°C y un tiempo de inmersión de 60 s, que redujeron la incidencia y la severidad de las podredumbres hasta un 50% en naranjas 'Lanelate' almacenadas a 20°C durante 7 días. Una temperatura de 50°C y un tiempo de 150 s no mejoraron significativamente la efectividad del tratamiento. Los baños seleccionados también redujeron, aunque en menor cuantía, las podredumbres en naranjas almacenadas a 5°C durante 6 semanas.

Finalmente, se evaluó la capacidad de control de tratamientos de poscosecha con sales sódicas de parabenos, sustancias catalogadas como GRAS (Generally Regarded as Safe), incluyendo el metil parabeno sódico (SMP) (capítulo 4), el etil parabeno sódico (SEP) (capítulo 5) y el propil parabeno sódico (SPP) (capítulo 6), en especies y cultivares de cítricos de importancia comercial. SMP a 200 mM, SEP a 80 mM y SPP a 100 mM se seleccionaron en ensayos primarios *in vivo* como las concentraciones más efectivas (reducción de incidencia de hasta el 100%) contra las podredumbres verde y azul en frutos inoculados unas 24 h antes del tratamiento. Las mejores condiciones de baño para estos tratamientos fueron una temperatura de 20°C y un tiempo de inmersión de 60 s. Los tratamientos calientes a 50°C no mejoraron la efectividad de los baños respecto a la temperatura ambiente. Estos tratamientos de baño fueron compatibles y sinérgicos con dosis bajas de imazalil a 25 $\mu L\ L^{-1}$ (IMZ 25), independientemente de los cultivares y de las condiciones de almacenamiento ensayados. Los tratamientos combinados de SMP, SEP y SPP con IMZ 25 redujeron en más del 90% la incidencia de las podredumbres verde y azul en naranjas 'Valencia' incubadas durante 7 días a 20°C. El control también fue satisfactorio en naranjas 'Valencia' almacenadas a 5°C durante 8 semanas.

RESUM

Les podridures verda i blava, els agents causals de les quals són els fongs fitopatogens *Penicillium digitatum* (Pers.: Fr.) Sacc. i *Penicillium italicum* Wehmer, respectivament, són les responsables de grans pèrdues d'importància econòmica en postcollita de cítrics a nivell mundial. A Espanya, en una estació típica, les podridures dels cítrics en postcollita poden arribar al 10% del producte collit. No obstant això, si les condicions són favorables a les malalties, les pèrdues poden assolir el 50%. Actualment, el sector encara depén de l'ús de fungicides químics sintètics per controlar les malalties de postcollita de fruita fresca en general i de cítrics en particular. Però l'ús d'estos fungicides comporta problemes com la presència de residus químics a la fruita, el desenvolupament de soques de *P. digitatum* i *P. italicum* resistents a estos tractaments i la contaminació del medi ambient per abocament inadequat de caldos fungicides.

Un control adequat de les malalties de postcollita sense la utilització de fungicides convencionals no pot assolir-se amb una sola estratègia de control, sinó que cal considerar tots els factors que influïxen en la incidència. En este sentit, el control integrat no contaminant de malalties de postcollita (CINCEP) és una estratègia global, que té en compte l'epidemiologia de la malaltia i tots els factors de precollita, collita i postcollita per a incidir sobre ells de forma combinada per a reduir les pèrdues econòmiques. Basant-se en la problemàtica anterior i en un context de CINCEP, l'objectiu general de la present tesi doctoral va ser aprofundir en el coneixement d'alguns factors d'incidència de malaltia i buscar estratègies alternatives no contaminants per al control en postcollita de les podridures verda i blava dels cítrics.

Primerament es va determinar l'efecte de la desverdització comercial amb etilé, en les condicions espanyoles, en la susceptibilitat del fruit i el desenvolupament de les podridures verda i blava en mandarines 'Clemenpons', 'Clemenules' i 'Nova', i taronges 'Navelina' de recol.lecció primerenca, així com també l'efecte en els atributs de qualitat d'estos cultivars d'alta importància econòmica (capítol 1). La desverdització comercial amb 2 mL L^{-1} d'etilé a 21ºC i 95-100% HR durant 3 dies, no va tindre efecte en la susceptibilitat

dels fruits a les podridures verda i blava en els cultivars de cítrics desverditzats, inoculats unes 2 h després amb *P. digitatum* o *P. italicum* i incubats a 20°C i 90% HR durant 7 dies; i tampoc va tindre efecte en la incidència de les podridures quan els fruits es van desverditzar unes 2 h després de la inoculació fúngica i es van emmagatzemar a 20°C i 90% HR durant 7 dies o a 5°C i 90% HR durant 14 dies. En canvi, va incrementar la severitat de les podridures, però només en fruits amb un índex de color inicial de la pell relativament alt. D'altra banda, amb l'excepció del color, la desverdització no va afectar de manera general als atributs de qualitat externa i interna dels fruits.

Com una alternativa al control químic convencional, es van avaluar tractaments de postcollita en aplicacions preventives i curatives amb inductors químics seleccionats per la seua capacitat general per induir resistència en plantes (capítol 2). Es van assajar en proves primàries de selecció *in vivo* distintes concentracions de silicat de sodi (SSi), àcid 2,6-diclorisonicotínic (INA), àcid β-aminobutíric (BABA), benzotiadiazol (BTH), àcid salicílic (SA), àcid acetil salicílic (ANSA) i la proteïna Harpin. Només els quatre primers d'estos set compostos a les concentracions respectives de 1000, 0,03, 0,3, i 0,9 mm, van reduir significativament les podridures verda i blava en taronges 'València' o 'Lanelate' inoculades unes 2 h després del tractament i incubades a 20°C durant 7 dies. SSi a 1000 mm va ser el tractament més eficaç per previndre les podridures, però va ser descartat per la presència de residus en la pell del fruit i els consegüents riscos de producció de fitotoxicitat. Els inductors químics no van tindre activitat curativa contra les podridures quan els patògens es van inocular 24 h abans del tractament.

Es va avaluar també l'activitat preventiva i curativa de tractaments de postcollita amb silicat de potassi (PSi) (capítol 3). En proves primàries de selecció *in vivo*, tractaments preventius amb PSi a 90 mM, van reduir la incidència de les podridures verda i blava fins a un 52% en taronges emmagatzemades a 20°C durant 6 dies. Un temps de 2 h entre este tractament i la inoculació de *P. digitatum* va reduir significativament la incidència i la severitat de la podridura verda, mentres que amb temps de 24, 48, 72 i 96 h la reducció no va ser significativa. En assajos d'influència espacial, este tractament no va

tindre la capacitat per a induir resistència sistèmica en la pell del fruit contra *P. digitatum* perquè no va haver-hi control en ferides inoculades localitzades a 10, 20 o 30 mm de distància del punt d'aplicació del tractament. La millors condicions de tractament amb PSi a 90 mM en solucions aquoses van ser una temperatura de 20°C i un temps d'immersió de 60 s, que van reduir la incidència i la severitat de les podridures fins a un 50% en taronges 'Lanelate' emmagatzemades a 20°C durant 7 dies. Una temperatura de 50°C i un temps de 150 s no van millorar significativament l'efectivitat del tractament. Els banys seleccionats també van reduir, encara que en menor quantia, les podridures en taronges emmagatzemades a 5°C durant 6 setmanes.

Finalment, es va avaluar la capacitat de control de tractaments de postcollita amb sals sòdiques de parabens, substàncies catalogades com GRAS (Generally Regarded as Safe), incloent el metil paraben sòdic (SMP) (capítol 4), l'etil paraben sòdic (SEP) (capítol 5) i el propil paraben sòdic (SPP) (capítol 6), en espècies i cultivars de cítrics d'importància comercial. SMP a 200 mm, SEP a 80 mm i SPP a 100 mm es van seleccionar en assajos primaris *in vivo* com les concentracions més efectives (reducció d'incidència de fins al 100%) contra les podridures verda i blava en fruits inoculats unes 24 h abans del tractament. Les millors condicions de bany per a estos tractaments van ser una temperatura de 20°C i un temps d'immersió de 60 s. Els tractaments calents a 50°C no van millorar l'efectivitat dels banys respecte a la temperatura ambient. Estos tractaments de bany van ser compatibles i sinèrgics amb dosis baixes d'imazalil a 25 $\mu L\ L^{-1}$ (IMZ 25), independentment dels cultivars i de les condicions d'emmagatzemament assajats. Els tractaments combinats de SMP, SEP i SPP amb IMZ 25 van reduir en més del 90% la incidència de les podridures verda i blava en taronges 'València' incubades durant 7 dies a 20°C. El control també va ser satisfactori en taronges 'València' conservades a 5°C durant 8 setmanes.

SUMMARY

Postharvest green mold, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., and postharvest blue mold, caused by *Penicillium italicum* Wehmer, are the most economically important postharvest diseases of citrus worldwide. In Spain, fruit postharvest losses due to molds may reach up to 10% under normal environmental conditions. However, under climatic conditions favorable to mold development, these losses might exceed 50%. Currently, these diseases are primarily controlled by application of synthetic fungicides. However, the use of these fungicides can lead to important problems such as chemical residues accumulation on/in fruit, the development of *P. digitatum* and *P. italicum* strains resistant to these fungicides and environmental pollution because of inadequate management of fungicide disposal.

An adequate control of postharvest diseases without the use of conventional fungicides does not rely on only one control strategy and should consider all factors that are influencing decay incidence. Therefore, the non-contaminant integrated management of postharvest diseases is a global strategy, which takes into account disease epidemiology, and all preharvest, harvest and postharvest factors determining disease to take action on each of them at the right moment in order to reduce economic losses. In this context, the general goal of this doctoral thesis was to increase the knowledge on some of these factors and search for non-polluting alternative strategies to control citrus postharvest green and blue molds.

The effect of commercial degreening with ethylene gas under Spanish conditions on fruit susceptibility and quality and on the development of postharvest green and blue molds on early-season 'Clemenpons', 'Clemenules' and 'Nova' mandarins and 'Navelina' oranges was determined (chapter 1). Commercial degreening with 2 μL L^{-1} ethylene at 21ºC and 95-100% RH for 3 days had no significant effect on fruit susceptibility to both green and blue molds on citrus cultivars degreened, inoculated 2 h later with *P. digitatum* or *P. italicum* and incubated at 20°C and 90% RH for 7 days. Also, no significant effect was observed on disease incidence on citrus cultivars inoculated 2 h before degreening and stored at either 20°C for 7 days or 5°C for 14 days. In contrast, commercial degreening significantly

increased the severity of the molds on fruit with higher initial rind color index (CI). On the other hand, besides rind color, commercial degreening did not significantly affect external and internal quality attributes of citrus cultivars.

Postharvest preventive and curative treatments with chemical inducers selected for their general capability to induce disease resistance in plants were evaluated as alternatives to conventional chemical control (chapter 2). In *in vivo* primary tests, different concentrations of sodium silicate (SSi), 2,6-dichloroisonicotinic acid (INA), *β*-aminobutyric acid (BABA), benzothiadiazole (BTH), salicylic acid (SA), acetylsalicylic acid (ASA) and harpin protein were assayed. Among these seven compounds, only the first four at respective concentrations of 1000, 0.03, 0.3, and 0.9 mM significantly reduced the incidence of green and blue molds on 'Valencia' or 'Lanelate' oranges inoculated 2 h after treatment and incubated at 20°C for 7 days. SSi at 1000 mM was the best treatment to reduce both molds, but it was discarded because of potential phytotoxicity. Chemical elicitors did not show any curative activity when the fruit were inoculated with the pathogens about 24 h before their application.

Preventive and curative antifungal activities of postharvest treatments with potassium silicate (PSi) against green and blue molds were evaluated on oranges (chapter 3). In *in vivo* primary tests, preventive treatments with PSi at 90 mM significantly reduced the incidence of green and blue molds up to 52% on oranges stored at 20°C for 6 days. PSi applied about 2 h before inoculation with *P. digitatum* showed higher preventive activity than applied before 24, 48 or 96 h. In preventive tests, no systemic activity was observed because no disease reduction was noticed when the distance between treatment and inoculation sites was 10, 20 or 30 mm. Dips with PSi at 90 mM at 20°C for 60 s were selected and subsequently applied on inoculated 'Valencia' oranges incubated at 20°C and 90% RH. These dips significantly reduced the incidence and severity of green and blue molds up to 50%. A temperature of 50°C and a dip time of 150 s did not improve the effectiveness of this dip treatment. Selected dips also reduced significantly the molds on oranges stored at 5°C for 6 weeks.

Finally, the control ability of postharvest treatments with sodium salts of parabens, which are classified as GRAS compounds, and included sodium methylparaben (SMP) (chapter 4), sodium ethylparaben (SEP) (chapter 5) and sodium propylparaben (SPP) (chapter 6) were evaluated in citrus species and cultivars of commercial significance. SMP at 200 mM, SEP at 80 mM and SPP at 100 mM were selected in *in vivo* primary screenings as the most effective concentrations (reduction of incidence up to 100%) against green and blue molds on fruits inoculated 24 h before treatment. A temperature of 20°C and an immersion time of 60 s were selected as the best dip treatment conditions. Dip treatments at 50°C did not improve the effectiveness of treatments at 20°C. These dip treatments were compatible with imazalil applied at doses as low as 25 μL L^{-1} (IMZ 25) and consistently improved its performance, irrespective of citrus cultivars and storage conditions. The combination of SMP, SEP, or SPP with IMZ 25 reduced up to 90% the incidence of green and blue molds in 'Valencia' oranges incubated for 7 days at 20°C. Also, these combined treatments were effective to control the molds on 'Valencia' oranges stored at 5°C during 8 weeks.

ABREVIATURAS / ABBREVIATIONS

AAC	1-aminociclopropano-1-ácido carboxílico
ACS	1-aminociclopropano-1-carboxilato sintasa
ANOVA	analysis of variance
APX	ascorbate peroxidase
ASA	acetylsalicylic acid / ácido acetil salicílico
BABA	*β*-aminobutyric acid / ácido *β*-aminobutírico
BM	blue mold
BTH	benzothiadiazole / benzotiadiazol
CI	color index / índice de color
DRBC	dichloran rose-bengal chloramphenicol agar
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
GLU	*β*-1,3-glucanase
GM	green mold
GRAS	generally regarded as safe
HR	humedad relativa
IDA	ingesta diaria aceptable
IMZ	imazalil
IMZ 25	imazalil a 25 μL L^{-1} / 25 μL L^{-1} IMZ
IMZ 50	imazalil de 50 μL L^{-1} / 25 μL L^{-1} IMZ
INA	2,6-dichloroisonicotinic acid / ácido 2,6-dicloroisonicotínico
ISR	induced systemic resistance / resistencia sistémica inducida
LAR	local acquired resistance / resistencia adquirida local
LMR	límites máximos de residuos
LSD	least significant difference

M	metionina
MCP	methylcyclopropene
MI	maturity index / índice de madurez
MTA	5´-metiltioadenosina
PAL	phenylalanine ammonia-lyase / fenilalanina amonia liasa
PD	*Penicillium digitatum*
PDA	potato dextrose agar / papa dextrosa agar
PI	*Penicillium italicum*
POD	peroxidase / peroxidasa
PPO	polyphenoloxidase / polifenol oxidasa
PR	pathogenesis-related
PRs	proteínas relacionadas con la patogénesis
PSi	potassium silicate / silicato de potasio
RH	relative humidity
SA	salicylic acid / ácido salicílico
SAM	S-adenosil metionina
SAMS	S-adenosil metionina sintasa
SAR	systemic acquired resistance / resistencia adquirida sistémica
SEP	sodium ethylparaben / etil parabeno sódico
SMP	sodium methylparaben / metil parabeno sódico
SPP	sodium propylparaben / propil parabeno sódico
SSi	sodium silicate / silicato de sodio
SSC	soluble solids concentration / contenido de sólidos solubles
TA	titratable acidity / acidez titulable
TMV	tobbaco mosaic virus / virus del mosaico del tabaco
UE	Unión Europea

INDICE

INTRODUCCIÓN GENERAL

Dentro de las frutas, los cítricos ocupan el primer lugar en volumen de producción a nivel mundial. Se cultivan en más de 100 países distribuidos en seis continentes, tanto en climas tropicales como en subtropicales (Saunt, 1999). La superficie mundial cosechada de cítricos fue aproximadamente de 8,7 millones de hectáreas con una producción de 131,2 millones de toneladas en el año 2011. España es el quinto productor de cítricos con 5,7 millones de toneladas, después de China, Brasil, USA y México y es el primer exportador de fruta fresca, con 3,1 millones de toneladas (más de la mitad de su producción) (FAO, 2011). En España, la distribución de las especies de cítricos en la producción es: 48% de naranja dulce, 38% de mandarina, 12% de limonero y 1% de pomelo (MAGRAMA, 2010).

Las podredumbres verde y azul causadas por los hongos patógenos *Penicillium digitatum* (Pers.: Fr.) Sacc y *Penicillium italicum* Wehmer, respectivamente, son las responsables de grandes pérdidas económicas en poscosecha de cítricos a nivel mundial (Droby et al., 1998). En España, las pudriciones de la fruta en poscosecha fluctúan del 3 al 10% durante una estación típica (Tuset, 1987). Sin embargo, bajo condiciones favorables a la enfermedad, las pérdidas pueden alcanzar el 50% (Fischer et al., 2009). Las podredumbres verde y azul son las enfermedades de poscosecha más importantes de los cítricos en España (Tuset, 1987), California y todas las áreas de producción caracterizadas por lluvias escasas en verano (Ecker y Eaks, 1989).

Han transcurrido más de 30 años de investigación con un enfoque alternativo al uso de fungicidas convencionales para controlar enfermedades de poscosecha de los cultivos y se han logrado avances significativos en esta área tales como la comercialización de algunos productos de biocontrol (Biosave 100®, Aspire™, etc.). También se han logrado avances importantes en estudios de resistencia adquirida sistémica (SAR) y resistencia sistémica inducida (ISR) tanto natural como artificial, así como en la evaluación de sustancias conocidas como GRAS (Generally Regarded as Safe) (Stanojevic et al., 2009; Valencia-Chamorro et al., 2009), o en el uso de tratamientos físicos como el calor o la luz ultravioleta (Palou et al., 2008). Todos estos enfoques constituyen estrategias de control alternativo no contaminante. Sin embargo, actualmente seguimos dependiendo del uso de fungicidas químicos sintéticos para controlar las enfermedades

de poscosecha de fruta fresca en general, y de cítricos en particular (D'Aquino et al., 2006). Asimismo, existe un incremento de la demanda en los mercados internacionales de frutas y hortalizas frescas de productos de buena calidad pero libres de residuos químicos. Además, en el caso de los cítricos, el uso de fungicidas convencionales conlleva el problema del desarrollo de resistencias en cepas de *P. digitatum* y *P. italicum* (Kinay et al., 2007; Lesar, 2008). Por otro lado, los resultados en efectividad de los métodos de control no contaminantes no han sido equivalentes a los obtenidos con métodos de control químico convencionales (Palou, 2011). Por todo ello, sigue siendo un reto la búsqueda e implementación comercial de estrategias no contaminantes efectivas para el control de las podredumbres verde y azul de los cítricos.

1. Principales enfermedades de poscosecha de los cítricos

Las pérdidas de frutos cítricos debidas a las enfermedades de poscosecha son muy variables y dependen del área de producción, de las variedades, de la edad y condición del árbol, de las condiciones ambientales durante el crecimiento y la cosecha, de daños físicos a la fruta durante la cosecha y del manejo en poscosecha. (Iqbal et al., 2012). Las más importantes en las condiciones españolas, y objeto de esta tesis doctoral, son las podredumbres verde y azul. Otras enfermedades que pueden causar pérdidas económicas en zonas productoras y momentos concretos de la campaña son la podredumbre amarga, la podredumbre negra, la podredumbre gris, la antracnosis y las podredumbres pedunculares, causadas respectivamente por los hongos patógenos *Geotrichum citri-aurantii, Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, y *Lasiodiplodia theobromae* o *Phomopsis citri*.

1.1. Podredumbre verde (*Penicillium digitatum* (Pers.:Fr.) Sacc.)

Es la enfermedad de poscosecha económicamente más importante de todas las regiones del mundo con escasas lluvias durante el verano (Smilanick et al., 2006). El agente causal de la podredumbre es el hongo *P. digitatum*. *P. digitatum* produce cadenas de conidios (6-7 x

6-8 μm) (Brown y Eckert, 2000). Los conidios de *P. digitatum* germinan fácilmente en papa dextrosa agar (PDA), pero también pueden germinar en agua conteniendo glucosa, ácido ascórbico y mezcla de ácidos orgánicos y azúcares simples. Las esporas de *P. digitatum* germinan poco o no germinan en agua-agar (Pelser y Eckert, 1977a). Se demostró que compuestos volátiles y no volátiles de la piel de los cítricos estimulan la germinación y el crecimiento de *P. digitatum* y *P. italicum* (Droby et al., 2008).

1.1.1. Síntomas

El síntoma inicial de la podredumbre verde se manifiesta como una mancha acuosa suave, que es visible en unas 48 h después de la infección (Droby et al., 2008) y, a medida que la lesión aumenta de tamaño aparece un micelio de color blanco que invade la mayor parte de la piel del fruto en sólo 7 días, y en seguida aparece una coloración verde-oliva debida a la esporulación del hongo (Ismail y Zhang, 2004). Una área amplia de micelio blanco rodea al área de esporulación, mientras que el resto del área de la lesión presenta un ablandamiento de la piel del fruto (Brown, 2003). Este ablandamiento es causado por la acción de enzimas pectolíticas que degradan sustancias pécticas de la lámina media de la pared celular (Barmore y Brown, 1980).

1.1.2. Ciclo de la enfermedad

Las esporas de *P. digitatum* se producen en grandes cantidades sobre la superficie de fruta infectada y se diseminan muy fácil por el aire. Estas esporas penetran en el albedo del fruto en presencia de heridas debidas mayoritariamente a daños mecánicos durante la cosecha y manejo, donde germinan y en presencia de agua libre y nutrientes finalmente se establece una infección irreversible dentro de 48 h a 20-25°C (Smoot y Melvin, 1967; Pelser y Eckert, 1977b). Las esporas de *P. digitatum* son dispersadas por el aire hacia los equipos de centrales citrícolas, almacenes, contenedores en tránsito y mercados al pormenor. Además, esporas liberadas de la superficie de fruta infectada pueden acumularse en el agua utilizada en los

drenchers y tanques de agua durante el proceso de lavado de la fruta a su llegada a los almacenes. El ciclo de infección y esporulación pueden ser repetido varias veces en las centrales citrícolas durante almacenamientos prolongados (Brown, 2003). El ciclo de la enfermedad tarda de 5 a 7 días a temperaturas ambientales comunes (20-25°C), y una sola fruta infectada puede producir de 1 a 2 billones de conidios (Holmes y Eckert, 1995).

1.2. Podredumbre azul (*Penicillium italicum* Wehmer)

P. italicum produce cadenas de conidios de forma oblonga a elipsoides y son algo más pequeños que los de *P. digitatum* (2-3 x 3-5 μm) (Brown y Eckert, 2000). Los primeros síntomas de la podredumbre azul son similares a los de la podredumbre verde. Pero a medida que crece la lesión aparece un micelio de color blanco que no ocupa una franja tan amplia como la de *P. digitatum.* Al esporular el hongo, la lesión adopta un color azul característico que da nombre a la enfermedad. La podredumbre azul es particularmente importante en frutos cítricos bajo condiciones comunes de almacenamiento en frío de los cítricos (3-5°C), puesto que *P. italicum* crece más rápido que *P. digitatum* en estas condiciones (Brown y Eckert, 2000; Plaza et al., 2003) y a diferencia de la podredumbre verde, puede diseminarse por contacto de fruta enferma a fruta sana durante el almacenamiento (Barmore y Brown, 1982). En cambio, *P. italicum* crece más lento que *P. digitatum* a temperatura ambiente. Se sabe también que la germinación de conidios de *P. italicum* es estimulado por compuestos volátiles como citral, nonanal o citrela (French et al., 1978). El ciclo de la podredumbre azul es similar al de podredumbre verde.

2. Control de las podredumbres verde y azul

2.1. Control químico convencional con fungicidas de síntesis

El control químico de las podredumbres verde y azul se realiza principalmente a base de fungicidas sintéticos, tales como imazalil, tiabendazol u o-fenilfenato, los cuales fueron registrados desde hace varios años para uso en poscosecha de cítricos en la Unión Europea

(UE). Por otro lado, el pirimetanil y el miclobutanil se incluyeron mucho más recientemente como parte de una lista de fungicidas autorizados (Palou, 2011). Actualmente, otros fungicidas sintéticos, tales como fludioxonil, azoxystrobin y trifloxystrobin son ingredientes activos nuevos, clasificados por la agencia de protección del medio ambiente en los EE UU como fungicidas de bajo riesgo (Kanetis et al., 2007) y se ensayaron y mostraron actividad como tratamientos de poscosecha en la UE (Schirra et al., 2010). No obstante a la fecha no hay formulados registrados disponibles para su uso en España (Palou, 2011). Las ventajas que tienen estos fungicidas sintéticos es que son relativamente económicos, fáciles de aplicar, persistentes y con acción curativa y preventiva. Hay estimaciones que indican que las pérdidas aplicando estos fungicidas sintéticos son del orden del 2-4%, mientras que sin estos tratamientos pueden fluctuar del 15 al 30% (Smilanick et al., 2006). El uso de fungicidas es especialmente importante en partidas refrigeradas por periodos largos o enviadas a mercados lejanos de exportación.

2.1.1. Problemática del uso de fungicidas de síntesis

El uso masivo e indiscriminado de los fungicidas sintéticos ha generado una serie de problemáticas, dentro de las cuales se encuentra el desarrollo de cepas de los hongos patógenos resistentes a los fungicidas, el incremento de residuos en la fruta a niveles no permitidos, y la contaminación del medio ambiente (Palou et al., 2008).

Aunque en el caso de los cítricos la piel del fruto fresco no es una parte comestible, la presencia de residuos de fungicidas químicos en ella sigue representando un problema para los consumidores, ya que pueden ser transferidos de forma sistémica a los gajos comestibles y al zumo. En el caso de aplicaciones de agroquímicos en campo, estos riesgos aumentan cuando no se respetan los intervalos de tiempo recomendados entre las aplicaciones de fungicidas y la cosecha. En el caso de fungicidas de poscosecha, los niveles de residuos no deben rebasar los Límites Máximos de Residuos (LMR) establecidos por la legislación de cada país. No obstante, en la práctica es más común que los LMR sean emitidos de forma particular por los grandes

importadores y cadenas de distribución europeas, que demandan niveles más bajos que los de la legislación para así diferenciarse de su competencia dando una imagen de mayor interés por la salud del consumidor. En el caso de España, los LMR son de 5 ppm para imazalil, tiabendazol y o-fenilfenol (Reglamento CE 149/2008). Estudios realizados en los últimos años reportan un aumento en la cantidad de residuos de imazalil en la fruta, cuando los tratamientos de baños con fungicida son calentados (50°C) con respecto a tratamientos controles a temperatura ambiente (D´Aquino et al., 2006).

En centrales citrícolas españolas, se encontraron cepas tanto de *P. digitatum* como de *P. italicum* resistentes al tiabendazol y al imazalil (Palou, 2002). Además, se observaron reducciones en la efectividad del imazalil contra la podredumbre verde en centrales citrícolas de California debido a la proliferación de biotipos de *P. digitatum* resistentes a esta materia activa (Eckert et al., 1994; Holmes y Eckert, 1999). Asimismo, aislamientos de *P. digitatum* en huertas comerciales de cítricos mostraron un aumento en resistencia a los fungicidas imazalil, tiabendazol y o-fenilfenol de 1988 a 1994. Casos de resistencia más recientes se reportaron en aislamientos de *P. digitatum* resistentes a imazalil en centrales citrícolas de California (Kinay et al., 2007) y a los fungicidas imazalil, o-fenilfenato y procloraz en la industria citrícola de Sudáfrica (Lesar, 2008; Erasmus et al., 2013).

2.2. Control integrado no contaminante de enfermedades de poscosecha (CINCEP)

Los resultados de numerosas investigaciones realizadas hasta la fecha indican que la efectividad, persistencia y espectro de acción de los tratamientos alternativos no contaminantes, solos o combinados, son inferiores a los de los fungicidas químicos convencionales; por tanto y de forma general, no pueden implementarse solos como sustitutos a nivel comercial. En este contexto, un control adecuado de las podredumbres no puede reducirse a la aplicación en poscosecha de tratamientos antifúngicos, sino que el control debe enfocarse a una estrategia más amplia de manejo, que coadyuve actuaciones sobre todos los factores para minimizar las pérdidas económicas. Bajo este esquema, el control integrado no contaminante de enfermedades de

poscosecha (CINCEP) constituye una estrategia global de control sin la utilización de fungicidas químicos convencionales, y se basa en un conocimiento profundo de la epidemiología de los patógenos, de los factores que determinan la incidencia en precosecha, cosecha y poscosecha, para incidir de forma holística y particular sobre cada uno de estos factores en el momento adecuado, con el objetivo de minimizar las pérdidas económicas causadas por las enfermedades (Palou, 2011). La posibilidad de usar una estrategia de CINCEP requiere dedicar esfuerzos a múltiples frentes de investigación, muchos de sus resultados podrían ser de aplicación en sistemas de producción ecológica y para la obtención de marcas o sellos de calidad 'Residuo Cero'. En los siguientes apartados se describen actuaciones de aplicación en estrategias de CINCEP.

2.2.1. Reducir la contaminación fúngica en precosecha

Incluye principalmente prácticas culturales, tales como eliminar frutos podridos y material de plantas infectadas, hacer podas de saneamiento y evitar recolectar cuando el tejido superficial de la fruta acumule agua (Johson, 1947) o se presenten condiciones ambientales favorables para el desarrollo de las enfermedades (Bonnardeaux y Robinson, 1994). Además, puede incluir tratamientos con insecticidas para evitar heridas en el fruto producidas por insectos, las cuales pueden ser puerta de entrada para los patógenos de poscosecha. Por otro lado, pueden realizarse aplicaciones de precosecha con materiales antifúngicos tales como fungicidas químicos, entre los que destaca el tiofanato de metilo (Ritenour et al., 2004) sustancias GRAS (Youssef et al., 2012) o agentes de biocontrol como la bacteria *Pantoea agglomerans* (Cañamas et al., 2008). Se ha observado que estos tratamientos pueden ser eficaces para reducir la contaminación en campo de especies de *Penicillium*.

2.2.2. Reducir las poblaciones de los patógenos en las centrales citrícolas

Tanto la fruta como los envases llevan esporas, micelio o restos de hongos a las centrales citrícolas, por tanto se establecen distintos focos

de infección dentro del área de la central citrícola. La higienización tiene como objetivo la limpieza y desinfección de la infraestructura y equipo de las centrales citrícolas, incluyendo el área de recepción de la fruta, las líneas de confección, las cámaras frigoríficas, así como también los embalajes; de esta manera se minimizan posibles reinfecciones en la fruta por los patógenos de poscosecha (Smilanick y Mansour, 2007). La limpieza se realiza con agua a presión y jabones alcalinos, eliminando suciedades como grasas, aceites, resinas, restos microbiológicos y restos orgánicos de hojas y frutos. La limpieza puede ser manual o con máquinas lavadoras. La desinfección es el proceso capaz de disminuir los niveles de inóculo de los hongos a valores no peligrosos, y se pueden usar sales de amonio cuaternarios, formaldehído, hipocloritos, peróxido de hidrógeno, hidróxido sódico o ácido peracético. En algunas centrales se usa también el fungicida o-fenilfenol como desinfectante de superficie, además de cómo medio de control de infecciones ya establecidas (Smilanick et al., 2006; Cerioni et al., 2009). Recientemente se ha encontrado que tratamientos desinfectantes secuenciales con hipoclorito de sodio, peróxido de hidrógeno y una sal cúprica mostraron actividad antifúngica contra *P. digitatum* aplicados solos o en combinación con bicarbonato de sodio (Cerioni et al., 2012).

2.2.3. Efecto de operaciones de manejo en poscosecha en la incidencia de las podredumbres verde y azul

Estudios recientes mostraron que nuevas tecnologías basadas en la adquisición de imágenes hiper-espectrales y redes neurales artificiales pueden clasificar mandarinas afectadas por las podredumbres verde y azul de forma no visible a simple vista, con una precisión de hasta un 93% (Gómez-Sanchis et al., 2013). Asimismo, ensayos de detección temprana de infecciones causadas por *P. digitatum* en naranjas 'Navelate' usando imágenes *backscattering* mostraron una precisión de hasta un 96% (Lorente et al., 2013). La implementación comercial de estas tecnologías podría contribuir decisivamente en la reducción de la incidencia final de podredumbres en los almacenes citrícolas. El reto está en la integración de estos sistemas en las líneas de confección de una forma coste-efectiva.

La desverdización con etileno exógeno es una tecnología de poscosecha muy usada en la citricultura española con variedades tempranas de mandarinas y naranjas. Sin embargo, su efecto sobre la incidencia y el desarrollo de las podredumbres verde y azul no está clara y existen en la literatura estudios que indican que el etileno puede inducir resistencia, susceptibilidad o no tener efectos, dependiendo de las características de la interacción entre el gas, el patógeno y el fruto huésped (El-kazzaz et al., 1983; Van Loon y Pennings, 1993). Por ello uno de los capítulos de esta tesis se ha dedicado a establecer los efectos de la desverdización comercial con etileno en las condiciones españolas sobre las podredumbres verde y azul.

Teniendo en cuenta que la temperatura es el factor más importante en la vida poscosecha de los productos hortofrutícolas, la frigoconservación, más o menos prolongada en función del mercado de destino es la tecnología de poscosecha más extendida en los frutos cítricos. El objetivo es prolongar el periodo de comercialización del fruto y mantener su calidad durante el transporte a mercados distantes de las zonas productoras puesto que reduce la actividad metabólica de los frutos y su senescencia, pero paralelamente, se utiliza como un método para inhibir el desarrollo de las podredumbres y reducir su incidencia (Kader, 2002).

2.2.4. Tratamientos antifúngicos en poscosecha

Los tratamientos antifúngicos en poscosecha alternativos a los fungicidas químicos convencionales que pueden usarse en estrategias de CINCEP para el control de las podredumbres verde y azul de los cítricos pueden ser físicos, biológicos y químicos.

Los tratamientos físicos tienen la ventaja de no dejar residuos en los frutos y tienen un impacto mínimo en el medio ambiente. Sin embargo, tienen las desventajas de que su efectividad puede ser limitada y variable, y normalmente carecen de actividad preventiva y persistencia. Dentro de los tratamientos físicos más estudiados (Montesinos-Herrero y Palou, 2010) se encuentran, el calor (curado, agua caliente y vapor caliente) y la radiación ultravioleta (Palou et al.,

2007, 2008; Fatemi y Borji, 2011). Por ejemplo, los tratamientos con agua caliente o curado pueden tener un efecto directo sobre la elongación del tubo germinativo e inactivar esporas en germinación y también un efecto indirecto de inducción de resistencia en el fruto huésped (Schirra et al., 2000; Plaza et al., 2003b). Tratamientos como el curado a 34-35°C durante 48-72 h inducen la producción de lignina y compuestos fenólicos en las heridas del fruto, y pueden contribuir a la resistencia de los frutos (Ben-Yehoshua et al., 1989). Tratamientos térmicos con agua caliente pueden inducir la formación de sustancias de resistencia en frutos, como la escoparona y escopoletina (Ferguson et al., 2000), asi como también proteínas relacionadas con la patogénesis, tales como, quitinasa y ß-1,3-glucanasa, o inhibir la síntesis de enzimas hidrolíticas de la pared celular (poligalacturonasa) (Schirra et al., 2000).

El control biológico de las podredumbres verde y azul está basado en el uso de antagonistas microbiales, cuyo modo de acción puede ser la competencia con el patógeno por los nutrientes y el espacio, la secreción de antibióticos, o la inducción de mecanismos de defensa. Los casos más exitosos de control biológico en el control de podredumbres de poscosecha de cítricos se registraron a principios de la década de los 2000, con el registro y la aparición en el mercado de los productos de biocontrol Aspire^{TM} (*Candida oleophila*) y Biosave^{TM} (*Pseudomona syringae*) (Zheng et al., 2005; Palou et al., 2008). Reportes recientes han mostrado el potencial de nuevos antagonistas microbiales en el control de las podredumbres verde y azul de los cítricos (Hao et al., 2011; Nashwa Salam et al., 2012).
Los productos químicos alternativos al uso de los fungicidas convencionales deben ser naturales o sintéticos, de toxicidad baja para mamíferos y el medio ambiente. Estos químicos incluyen sustancias naturales como aceites esenciales o extractos de plantas, el quitosano o algunos peptidos y proteínas. También incluyen a los inductores químicos naturales o sintéticos y a los compuestos clasificados como aditivos alimentarios o sustancias GRAS (Palou et al., 2008). La caracterización de tratamientos poscosecha con algunos de estos componentes para el control de las podredumbres verde y azul en frutos cítricos constituye uno de los objetivos primordiales de la presente tesis doctoral.

3. Desverdización

3.1. Generalidades

La desverdización con etileno exógeno es un proceso artificial que acelera el cambio del color verde natural al color naranja de la piel de los frutos (Poole y Gray, 2002), y es una práctica comercial común en muchas partes del mundo (Smilanick et al., 2006). En el caso particular de la citricultura española, se usa para adelantar la campaña de cultivares de naranjas y mandarinas de estación precoz, cuando éstas alcanzan su madurez interna, y posteriormente, la fruta fresca puede ser colocada comercialmente en los mercados locales y de exportación, lo cual repercute en mejores ganancias económicas. La efectividad de la desverdización depende en primera instancia de las condiciones del proceso, tales como la concentración de etileno, temperatura y humedad así como también del cultivar y del índice de color externo de la fruta (CI) que se desverdiza. La desverdización a concentraciones de 1-5 $\mu L\ L^{-1}$ de etileno causan un cambio del color de la fruta sin afectar la calidad de la fruta (Cuquerella et al., 2005). Las condiciones óptimas para la desverdización de cítricos españoles son: temperaturas de 20 a 22°C y HR del 90% o mayor (Martínez-Jávega et al., 2008). A nivel celular y molecular, la desverdización comercial con etileno exógeno degrada los pigmentos de la clorofila (Purvis y Barmore, 1981; Peng et al., 2013) e incrementa la biosíntesis de carotenoides (Zhou et al., 2010).

3.2. Etileno

El etileno, (C_2H_4), es un compuesto gaseoso producido naturalmente por las plantas (Salveit, 1999). Es esencial para el crecimiento, desarrollo, regulación, reproducción y supervivencia de las plantas (Bleecker y Kender, 2000; Wang et al., 2002). Además, el etileno también está involucrado en procesos de senescencia (Kader, 1985), madurez y desórdenes fisiológicos en fruta fresca (Sdiri et al., 2013). Asimismo, tiene varios efectos sobre el crecimiento y desarrollo de muchos cultivos de frutas, vegetales y ornamentales, siendo efectivo a concentraciones muy pequeñas (del orden de ppm, $\mu L\ L^{-1}$) (Salveit, 1999). El efecto del etileno en las plantas puede ser

benéfico o perjudicial según los casos. Por ejemplo, mientras que la inducción floral en piña es un efecto benéfico, el aborto de flores en varias plantas es un efecto detrimental. Asimismo, las plantas producen etileno, pero sólo los frutos climatéricos en la madurez y los tejidos dañados o enfermos lo producen en suficiente cantidad como para afectar tejidos adyacentes (Alonso y Stepanova, 2004; Broekaert et al., 2006).

Todos los frutos cítricos producen niveles bajos de etileno durante su desarrollo y no exhiben un climaterio en su madurez (McCollum y Maul, 2007). Pero se ha observado que los frutos cítricos producen cantidades elevadas de etileno en respuesta a una variedad de estreses tales como heridas en la piel (Hyodo, 1977; Evenson et al., 1981; Riov y Yang, 1982), exposición a bajas temperaturas (McCollum y McDonald, 1991) o infecciones por patógenos (Achilea et al., 1984; Mullins et al., 2000; Broekaert et al., 2006).

El etileno se sintetiza a partir del aminoácido metionina (M), el cual es convertido a S-adenosil metionina (SAM) por la enzima S-adenosil metionina sintasa (SAMS). La SAM está involucrada en las reacciones de metilación de lípidos, proteínas y ácidos nucleicos y es convertida por la enzima 1-aminociclopropano-1-carboxilato sintasa (ACS) a 5´-metiltioadenosina (MTA), la cual es convertida nuevamente a M vía ciclo de Yang y a 1-aminociclopropano-1-ácido carboxílico (AAC), siendo este compuesto el precursor del etileno. Finalmente, el AAC es oxidado por la enzima 1-aminociclopropano-1-carboxilato oxidasa para formar etileno, cianuro y dióxido de carbono (Broekaert et al., 2006; Figura 1).

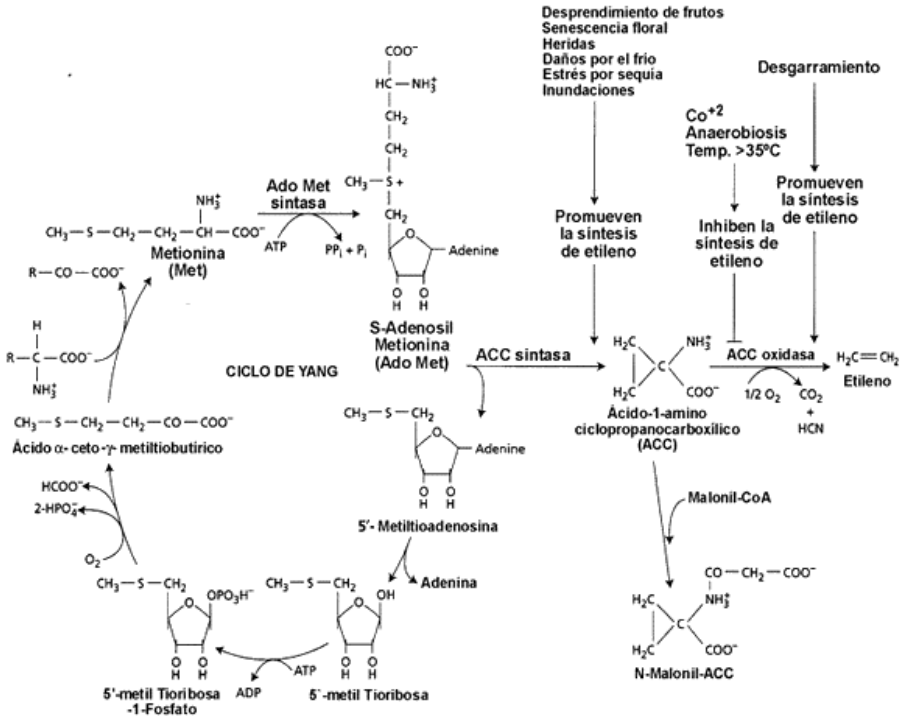

Figura 1. Biosíntesis de etileno

3.3. Efecto de la desverdización en las podredumbres verde y azul de los cítricos

El etileno puede activar procesos relacionados con los mecanismos de defensa de las plantas a enfermedades, incluyendo la producción de fitoalexinas (Díaz et al., 2002) y proteínas relacionadas con la patogénesis (PRs), tales como la β-1,3-glucanasa o la quitinasa; la inducción de la ruta de los fenilpropanoides y el reforzamiento de la pared celular mediante mecanismos tales como la lignificación y la acumulación de proteínas en la pared celular ricas en hidroxiprolina (Boller, 1990). En general, la aplicación de etileno exógeno puede inducir resistencia, susceptibilidad o no tener efectos, dependiendo de la interacción planta-patógeno (El-kazzaz et al., 1983; Van Loon y Pennings, 1993). Las escasas investigaciones desarrolladas para clarificar las relaciones que existen, entre la aplicación de etileno exógeno y el desarrollo de enfermedades de poscosecha causadas por hongos fitopatógenos, especialmente en frutos cítricos han dejado resultados controvertidos en función de factores relacionados con la concentración de etileno, las condiciones ambientales o de tratamiento y la interacción hospedero-planta (McCornack, 1971; Brown, 1973, 1975, 1986; Barmore y Brown, 1985). Varios trabajos indican que la desverdización con etileno exógeno en frutos cítricos redujo la incidencia o la severidad de la podredumbre verde o la podredumbre azul (Brown, 1973; El-kazzaz et al., 1983; Porat et al., 1999). Por contra, la desverdización aumentó la incidencia de la podredumbre peduncular (*L. theobromae*) (McCornack, 1971; Barmore y Brown, 1985; Brown, 1986; Zhang, 2004) y la antracnosis (*C. gloeosporioides*) (Brown, 1975), cuando la fruta se desverdizó a 30°C, debido a que es una temperatura óptima para el desarrollo del hongo (Barmore y Brown, 1985). Se sabe que *C. gloeosporioides* forma estructuras de dormancia, llamados apresorios, los cuales se encuentran sobre la superficie del fruto y forman una hifa infectiva durante la desverdización, que penetra la piel intacta del fruto, siendo más susceptible cuando el tiempo de desverdización sobrepasa las 36 h (Brown, 1975). Asimismo, otras investigaciones sugieren que la desverdización con etileno exógeno no tiene efecto sobre la incidencia de las podredumbres verde o azul de frutos cítricos (Chalutz, 1979; Mullins et al., 2000; Plaza et al., 2004). Una investigación a nivel de biología molecular sugiere que el etileno promueve respuestas de

defensa en frutos cítricos contra el hongo *P. digitatum* mediante evidencias de acumulación de ARNm relacionado con mecanismos de defensa (Marcos et al., 2005). Asimismo, en un estudio sobre cambios transcriptómicos en el tejido de la piel de frutos de cítricos se señalan efectos de los genes de la biosíntesis de etileno en la resistencia inducida a *P. digitatum* (Ballester et al., 2011).

3.4. Efecto de la desverdización en la calidad

La calidad de la fruta puede ser definida como un problema de preferencia del consumidor, y es la suma de aquellos atributos (tamaño, forma, color, apariencia etc.) que atraen al consumidor. La calidad es una preocupación comercial y de investigación en cualquier parte de la cadena productiva, desde el productor hasta el consumidor, y nunca es un tratamiento per se en una investigación; es una respuesta medida, a menudo, una de muchas en un experimento (Castle, 1995). La firmeza de los frutos cítricos esta relacionada con la deformación de la piel del fruto por la aplicación de una fuerza, y los cambios en firmeza son debidos a cambios en los contenido en agua o turgor o en la química de los componentes de la pared celular, pectinas y hemicelulosa, los cuales ocurren durante el crecimiento, desarrollo y almacenamiento de los frutos (Sams, 1999). Varios estudios demostraron que el etileno no afectó la pérdida de peso, firmeza, o contenidos de sólidos solubles totales en naranjas 'Shamouti' (Porat et al., 1999), mandarinas 'Oronules' (Carvalho et al., 2006) o mandarinas 'Mioro' (Abad et al., 2003). Sin embargo, un estudio demostró que la desverdización a 20°C durante 24 horas incrementó la firmeza del fruto con respecto al tratamiento control, pero no tuvo efecto sobre contenidos de sólidos solubles totales y acidez titulable del zumo en mandarinas 'Clemenules' (Plaza et al., 2004).

Actualmente, la calidad nutricional y las cantidades de compuestos activos tales como vitamina C, compuestos fenoles, flavonoides y flavonas están tomando importancia como consecuencia de la competencia en el mercado global y el interés público por los beneficios nutricionales de los productos hortofrutícolas (Patil et al., 2009). Estudios recientes reportaron que la desverdización con etileno

no afectó los contenidos de vitamina C, fenoles totales y flavonoides en el zumo de cítricos (Nishikawa et al., 2002; Mayuoni et al., 2011) y, en general, tampoco afectó a los atributos de calidad externa y a los componentes bioactivos de distintos cultivares de frutos cítricos (Sdiri et al., 2012).

4. Sistemas químicos de control alternativos

El control de las enfermedades de poscosecha por medio de químicos alternativos a los fungicidas convencionales deben realizarse con sustancias naturales o sintéticas de toxicidad baja sobre mamíferos y medio ambiente. En este tipo de sustancias se incluyen los aditivos alimentarios, compuestos llamados GRAS (Generally Recognized as Safe) clasificados por la FDA (Food and Drug Administration) de los EE UU, inductores químicos sintéticos de resistencia, así como también compuestos naturales extraídos de plantas, animales o microorganismos, incluyendo compuestos fenólicos, extractos de plantas, antibióticos, alcaloides, propóleos y quitosano (Troncoso-Rojas y Tiznado-Hernández, 2007).

4.1. Sustancias inductoras de resistencia

Las plantas han desarrollado un número de mecanismos de defensa inducibles contra ataque de patógenos (Durran y Dong, 2004). La resistencia inducida puede ser definida como un incremento de expresión de mecanismos de defensa natural de las plantas contra varios tipos de patógenos que pueden ser inducidos por factores físicos, biológicos y químicos (van Loon et al., 1998; Edreva, 2004; Iqbal et al., 2012). La resistencia en las plantas inducida por patógenos fue reconocida primero en 1901 (Beauverie, 1901; Ray, 1901) y 30 años más tarde estas investigaciones fueron confirmadas (Chester, 1933). Sin embargo, evidencias convincentes de la resistencia inducida no fueron obtenidas hasta los años 60, cuando Ross demostró que las plantas de tabaco infectadas con el virus del mosaico del tabaco (TMV), subsecuentemente desarrollaban un incremento de resistencia a infecciones secundarias en el tejido distal. Esta propagación de resistencia a través de los tejidos de la planta, se llamó

Resistencia Adquirida Sistémica (SAR por sus siglas en inglés) (Ross, 1961). Desde el punto de vista molecular, la SAR se caracteriza por un incremento en la expresión de un gran número de genes relacionados con la patogénesis, tanto a nivel local como sistémico. Las PRs fueron descritas por van Loon en 1970, quien observó una acumulación de éstas en tejido infectado del TMV (van Loon y van Kammen, 1970; van Loon y van Strien, 1999). Más tarde, los trabajos de Malamy et al. (1990) y Métraux et al. (1990), mostraron la evidencia de que el ácido salicílico (SA) era una señal de la inducción de SAR, cuando observaron un aumento en la concentración de SA en tejidos locales y sistémicos de plantas después de la infección de tabaco con el TMV y una correlación con las PRs. Existen definidas seis tipos de resistencia: específica-parásito, específica-cultivar, no hospedera, específica-órgano, relacionada a la edad e inducida (Kuc, 2001). Algunos agentes químicos que inducen resistencia sistémica inducida (ISR por sus siglas en inglés) son más efectivos contra algunas enfermedades que contra otras, lo cual puede ser explicado por efectos diferentes sobre componentes distintos de la respuesta de resistencia (Kuc, 2001). Diferentes compuestos y rutas pueden mediar diferentes resistencias bioquímicas. Estos mediadores incluyen al ácido salicílico, el ácido jasmónico, el ácido abscísico, el etileno y el óxido nítrico (Kuc, 2001).

Las sustancias inductoras de resistencia, llamados “elicitors” en inglés son sustancias que inducen respuestas protectivas en plantas. La primera sustancia de este tipo fue descubierta en 1968. La naturaleza química de los inductores es diversa: polisacáridos, proteínas, polipéptidos, glicoproteínas, compuestos conteniendo lípidos, u otras sustancias. Las plantas tratadas con inductores desarrollan una resistencia general (no específica), parecida a la resistencia horizontal (poligénica) con la única diferencia que esta última es una característica genética, mientras que la resistencia inducida es una característica fenotípica (Ozeretskovskaya y Vasyukova, 2002). Los criterios aplicados a los inductores de SAR como agentes de protección de plantas son: 1) ni el inductor ni su metabolito deben tener una actividad antimicrobial directa *in vitro* o *in vivo*; 2) el agente modifica la interacción planta-patógeno, la cual incluye mecanismos relacionados a defensas, inducidos antes o después de la infección; 3) el agente debe proteger una planta contra patógenos (Sticher, 1997).

4.1.1. Inductores químicos de resistencia

El uso de productos químicos como inductores de resistencia es un área en crecimiento que tiene como propósito desarrollar nuevos compuestos para el control de enfermedades. Los inductores químicos ideales poseen las siguientes características: carecen de toxicidad directa contra el patógeno, no son tóxicos a plantas y animales, no tienen efectos negativos sobre el crecimiento y desarrollo de las plantas, poseen un amplio espectro de defensa en cantidades bajas, proporcionan una protección duradera, y tienen un bajo costo económico. Estas características lo diferencian de los fungicidas y pesticidas, que tienen efecto tóxico directo contra los patógenos, suelen ser más o menos venenosos para el medio ambiente, y poseen un estrecho espectro de defensa (Kuc, 2001). Los inductores químicos sintéticos no tienen actividad antimicrobial, sin embargo, la actividad antimicrobial ha sido mostrada en algunos trabajos documentados y se asociaron a concentraciones muy altas (Tosi y Zazzerini, 2000; Rohilla et al., 2002). La SAR puede ser activada en plantas pretratadas con inductores químicos, la mayoría de los cuales parecen actuar como análogos funcionales del SA (Gozzo, 2003). Aunque la mayoría de las investigaciones se han enfocado a enfermedades de precosecha (Joyce y Johnson, 1999), algunos inductores químicos aplicados en pre o en poscosecha mostraron inducción de respuestas de defensa en enfermedades de poscosecha en distintos cultivos hortofrutícolas.

4.1.1.1. Ácido salicílico (SA)

El SA se sintetiza por la ruta de los fenilpropanoides (Sticher et al., 1997). La fenilalanina es convertida a ácido cinámico, el cual es transformado tanto a ácido o-fumárico como a ácido benzoico, dependiendo de la especie, tejido o condiciones de la planta (Lee et al., 1995; Schneider et al., 1996; Figura 2). La translocación del SA del sitio primario de infección a otras partes de la planta en pepinos, sugirió que el SA puede contribuir a la inducción de SAR (Meuwly et al., 1995). El metil salicilato es un metabolito del SA que se ha localizado en muchas plantas y que se encontró en grandes cantidades en hojas de tabaco infectadas, pero no en hojas con heridas (Shulaev et al., 1997).

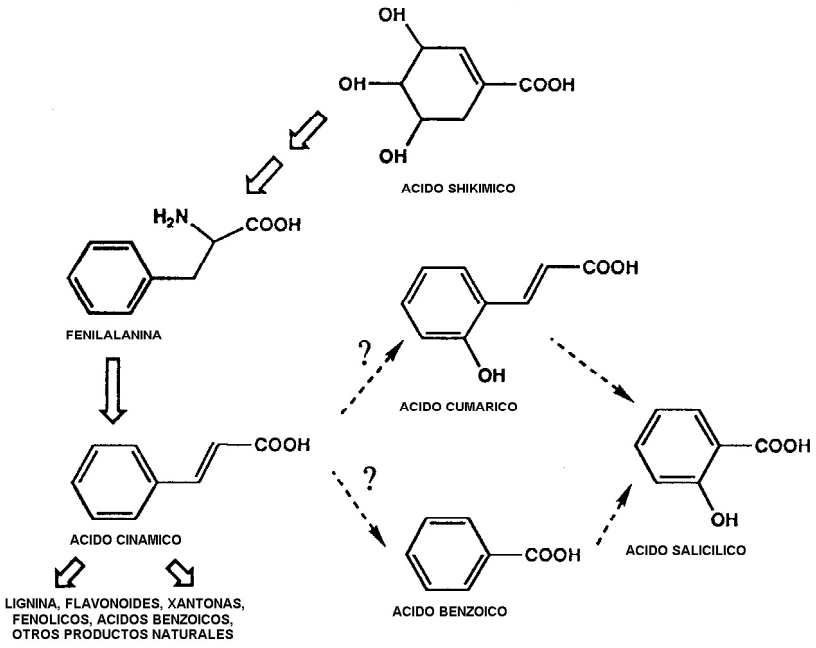

Figura 2. Biosíntesis del ácido salicílico

El SA a altas concentraciones (1 mM) puede inhibir varias enzimas tales como la catalasa, la ascorbato peroxidasa, o la aconitasa (Durner y Klessig, 1995; Ruffer et al., 1995). Niveles elevados de peroxido de hidrogeno (H_2O_2) inducen la acumulación de SA, sugiriendo que el H_2O_2 puede posiblemente inducir la acumulación de PRs a través de la inducción del SA (Leon et al., 1995; Summermatter et al., 1995). El H_2O_2 se produce por la actividad de NADPH oxidasas y peroxidasas, y puede ser directamente tóxico a los patógenos. Se sabe que el H_2O_2 puede contribuir al refuerzo estructural de las paredes celulares de las plantas por oxidación y entrecruzamiento de glicoproteínas ricas en prolina e hidroxiprolina. Además, el H_2O_2 actúa en la síntesis de compuestos fenólicos como ligninas y suberinas por la acción de la peroxidasa. El H_2O_2 es eliminado de las células por conversión en H_2O y O_2, debido a la acción de las enzimas catalasa, ascorbato peroxidasa y glutato peroxidasa. Además, el SA puede

inhibir un paso de la biosíntesis del ácido jasmónico, el cual es un compuesto involucrado en la expresión de genes inducidos por heridas (Farmer y Ryan, 1992). El SA exógeno puede ser fitotóxico, pero si se aplica a concentraciones óptimas puede inducir resistencia de forma muy efectiva.

Tratamientos en precosecha de SA a 8 mM redujeron los diámetros de lesión, la pudrición de frutos y la esporulación de la podredumbre verde en naranja 'Lanelate'. Sin embargo, el control de las podredumbres verde y azul no fue satisfactorio con tratamientos aplicados en poscosecha (Iqbal et al., 2012). Resultados similares se obtuvieron en mandarina 'Ponkan' con tratamientos poscosecha de SA (Zheng y Zhang, 2004). Por otro lado, Bokshi et al. (2003) reportaron que el SA controló enfermedades de tubérculo de papas en aplicaciones de poscosecha. El SA tiene actividad antifungica contra algunos patógenos de mango (Cao et al., 2006), cítricos (Shaat y Galal, 2004) y pera (Zainuri et al., 2001).

4.1.1.2. Benzotiadiazol (BTH)

Es un análogo sintético del SA y fue desarrollado como un potente activador de SAR de plantas, capaz de inducir resistencia a enfermedades fúngicas, bacterianas y víricas en un gran número de especies de plantas monocotiledóneas y dicotiledóneas (Friedrich et al., 1996; Gozzo, 2003). Originalmente, el BTH salió al mercado para controlar el mildiu polvoriento del trigo y la cebada en la UE (Gorlach et al., 1996). El BTH inhibe la enzima catalasa y la ascorbato peroxidasa. Las primeras evidencias exitosas de control de hongos con tratamientos precosecha de BTH se reportaron en cultivos tales como gramíneas (Morris et al., 1998; Stadnik y Buchenauer, 1999) y solanáceas bajo condiciones de campo (Csinos et al., 2001; Abbasi et al., 2002; Buonaurio et al., 2002; Matheron y Porchas, 2002; Pérez et al., 2003). Además, el BTH mostró resultados prometedores para el control de enfermedades de campo en cítricos, como son la roña (*Elsinoe fawcettii),* la melanosis (*Diaporthe citri*) o la mancha negra por Alternaria (*Alternaria alternata*) (Agostini et al., 2003). El BTH aplicado en precosecha mostró, además, capacidad de control de enfermedades de poscosecha en frutos de melón y fresa (Huang et al.,

2000; Terry y Joyce, 2000). Trabajos más recientes han demostrado que tratamientos en precosecha con BTH pueden controlar la podredumbre azul (*Penicillium expansum*) y la podredumbre negra (*A. alternata*) sobre frutos de pera (Cao y Jiang, 2006). Asimismo, otros investigadores revelaron que tratamientos poscosecha de BTH indujeron resistencia a frutos de durazno contra *P. expansum* (Liu et al., 2005). Por otro lado, el BTH aplicado al suelo fue reportado para el control del cáncer en árboles jóvenes de cítricos (Graham y Myers, 2012).

4.1.1.3. Ácido β-aminobutírico (BABA)

El BABA es un aminoácido no proteico que se ha llegado a considerar un tratamiento alternativo prometedor debido a su carácter natural (Zimmerli, 2000), su inducción de respuestas de defensa en tejidos de distintas plantas (Jakab et al., 2001) y su actividad antifúngica (Tavalalli et al., 2008). El BABA puede inducir resistencia a varios organismos, incluyendo hongos, bacterias, virus y nemátodos (Cohen, 2001; Jakab et al., 2001; Cohen, 2002). Varios reportes señalan que el modo de acción del BABA puede ser la acumulación de PRs en pimiento (Cohen, 1994), tomate (Cohen y Grisi, 1994) o tabaco (Hwang et al., 1997). En otros casos se ha observado una respuesta hipersensitiva, deposición de callosa o acumulación de lignina (Cohen et al., 1999; Zimmerli et al., 2000; Ton y Mauch-Mani, 2004). Algunas investigaciones demostraron que aplicaciones de BABA a la raíz o a la parte vegetativa de la planta inducen resistencia sistémica a patógenos (Cohen, 1994; Hong et al., 1999). Se ha observado que este inductor de resistencia sistémica puede tener efecto curativo en condiciones *in vitro* e inducir resistencia en la piel del fruto (Cohen, 1994, Porat et al., 2003; Tavalalli et al., 2008).

Trabajos más recientes con BABA, mostraron una reducción de la incidencia o de los diámetros de lesión de la podredumbre azul o la podredumbre verde en fruta almacenada a 5°C de naranja dulce (Tavalalli et al., 2008) y de pomelo (Porat et al., 2003). Esta misma investigación, además, reportó una inhibición de la germinación de esporas y elongación del tubo germinativo de *P. italicum in vitro*. BABA indujo un incremento significativo de las actividades de la

quitinasa, la ß-1,3-glucanasa y la peroxidasa en frutos de manzana (Zhang et al., 2011).

4.1.1.4. Ácido 2,6-dicloroisonicotínico (INA)

Tiene un modo de acción similar al SA y protege contra un rango amplio de patógenos en varias plantas mono o dicotiledóneas (Sticher et al., 1997). El INA induce genes de expresión de SAR, algunas veces antes de la inoculación, y otras veces después del ataque del patógeno únicamente (Gozzo, 2003). Aplicaciones foliares de INA demostraron efectividad para controlar varias enfermedades en plantas de algodón infectadas de forma natural (Colson-Hanks et al., 2012).

4.1.1.5. Harpin

Harpin es una proteína acídica, estable al calor, rica en glicina, producida por la bacteria *Erwinia amylovora*. Harpin es el primer producto de orígen bacteriano capaz de inducir la reacción de hipersensibilidad en plantas (Baker et al., 1993; Mullin et al., 1998; Dong et al., 1999). También induce SAR en tabaco y arabidopsis (Dong et al., 1999). Tratamientos poscosecha de harpin a diferentes concentraciones aplicados en frutos de manzana 'Red Delicious' redujeron el progreso de la podredumbre azul de poscosecha (*P. expansum*) (de Capdeville et al., 2002, 2008). De forma similar, tratamientos poscosecha de harpin inhibieron significativamente la mancha negra (*Guignardia citricarpa*) en naranjas 'Valencia' en Brasil (Lucon et al., 2010).

4.1.1.6. Silicio

El silicio es considerado un nutriente funcional de las plantas y juega un papel importante como constituyente de la pared celular (Laing et al., 2006). De acuerdo a resultados de investigación, se sugirió que el silicio puede tener varios modos de acción, incluyendo un efecto directo sobre el patógeno (Liu et al., 2010) y un efecto indirecto sobre la fruta hospedera. Este último incluye la formación de

barreras físicas y mecánicas a la penetración del patógeno a nivel de la pared celular (Buonaurio et al., 2009) y la inducción bioquímica de defensas como la acumulación de lignina, compuestos fenólicos y PRs (Epstein, 1999).

Los primeros trabajos realizados sobre el control de enfermedades con silicio fueron reportados en los años 1920 y 1930 en cultivos de cereal. Wagner (1940) fue el primero en encontrar una interacción entre fertilización de silicio y el control del mildiu polvoriento en pepino. El uso de silicio soluble aplicado en precosecha como solución de nutrientes llegó a ser importante en invernaderos. Por ejemplo, casos exitosos de control se reportaron en pepino contra mildiu polvoriento (Chérif y Bélanger, 1992) y la pudrición de raíz por *Pythium* (Menzies et al., 1991), en el anublo del arroz (Seebold et al., 2001) y *Micosphaerella pinodes* en haba (Dann y Muir, 2002).

Estudios más recientes indicaron que tratamientos poscosecha de silicio en la forma de silicato de sodio mostraron control contra *A. alternata*, *Fusarium semitectum* y *Trichothecium roseum* en melones (Bi et al., 2006; Guo et al., 2007). De forma parecida, el silicato de sodio controló las podredumbres verde y azul en mandarinas clementinas (Ligorio et al., 2007) y fue capaz de dañar la membrana plasmática de esporas de *P. digitatum* e inhibir la germinación de esporas, la elongación del tubo germinativo y el crecimiento micelial en condiciones *in vitro*, además de reducir significativamente la podredumbre verde en frutos cítricos (Liu et al., 2010). En otro trabajo reciente, silicato de sodio tuvo una concentración mínima inhibitoria del 0.25% para *P. digitatum* y *P. italicum* en condiciones *in vitro*, y además, tratamientos poscosecha con este mismo inductor, redujeron significativamente las podredumbres verde y azul en frutos cítricos (Youssef et al., 2012).

4.2. Sustancias generalmente reconocidas como seguras (GRAS) Parabenos

4.2.1. Descripción y propiedades

En su forma pura, los parabenos son cristales incoloros pequeños o polvos cristalinos, sin olor, insípidos e higroscópicos, y son preparados por esterificación del ácido *p*-hydroxibenzoico con el alcohol correspondiente en presencia de un catalizador ácido.

Los parabenos son un grupo homólogo del ácido hidroxibenzoico esterificado en la posición C4 e incluyen al metil, etil, propil,butil, heptil y bencil parabenos y se usan solos o combinados para ejercer un efecto antimicrobiano. Los parabenos tienen un efecto inhibitorio sobre el transporte de membranas y la función mitocondrial, y se usaron como agentes antimicrobianos en productos farmacéuticos en la década de 1920 (Sabalitschka, 1930). Los parabenos tienen un amplio espectro de actividad, son seguros y estables en un rango amplio de pH y son suficientemente solubles en agua como para producir la concentración efectiva en la fase acuosa. No obstante, a menudo se usan sus sales porque son más solubles en agua (Suhr y Nielsen, 2004). En esta tesis doctoral se han evaluado las sales sódicas del metil, etil y propil parabeno.

Los parabenos se usan desde hace más de 50 años en alimentos tales como jugo de frutas, extractos de café, salsas, refrescos, pasteles, cremas, helados o pastas a concentraciones que varían de 0,03 a 0,1% (Daniel, 1986).

Los parabenos tales como el metil y el etil parabeno y sus sales sódicas, con los números E 218, 214, 219 y 215 respectivamente, están autorizados para uso en alimentos por las normativas de la UE y son generalmente reconocidas como sustancias seguras (GRAS) por la legislación de los EE UU (Milss et al., 2004). La excepción la constituye el propil parabeno y su sal sódica que, con los antiguos números E 216 y 217, fueron recientemente excluidos de la lista de aditivos autorizados. No obstante, puesto que la piel de los cítricos no

se utiliza para el consumo humano, sigue teniendo sentido evaluar su efectividad como tratamiento antifúngico alternativo.

4.2.2. Actividad antimicrobiana

Los parabenos y sus sales sódicas son más efectivos contra hongos que contra bacterias a bajas concentraciones, y más activos contra bacterias Gram positivas que contra Gram negativas (Soni et al., 2005). Generalmente, los parabenos son efectivos en soluciones con pH de rango 4-8 (Aalto et al., 1953; Thompson, 1994). Sin embargo, los parabenos a un pH superior a 8 pueden hidrolizarse y reducir su eficiencia preservativa (Aalto et al., 1953; Rosen y Berke, 1973). Los parabenos actúan tanto en la fase germinativa como en la fase vegetativa del desarrollo microbiano en general y son más efectivos sobre la germinación de esporas que sobre el crecimiento micelial de hongos (Bomar, 1962; Watanabe y Takesue, 1976). Los parabenos, especialmente el propil parabeno inhibieron completamente el crecimiento micelial de varias especies de hongos de los géneros *Aspergillus*, *Fusarium* y *Penicillium* a concentraciones de 1-2 mM en condiciones *in vitro* (Thompson, 1994). La actividad antimicrobiana de los parabenos incrementa a medida que aumenta la cadena del radical alquilo o grupo éster.

Los parabenos y sus sales tienen actividad inhibitoria en el transporte de membranas y actividad mitocondrial; y se comportan como un ácido fenólico débil. Cuando un ácido débil se disuelve en agua, ocurre un equilibrio entre moléculas ácidas no disociadas e iones cargados. La proporción de ácido no disociado se incrementa a un pH bajo y pasa por difusión hacia la membrana del plasma, la cual tiene un pH más alto en su interior y, como consecuencia, el ácido se disocia causando una acumulación de protones y aniones que producen una inhibición del metabolismo celular (Brul y Coote, 1999). Por otro lado, se ha observado que de manera general combinaciones de metil parabeno con propil parabeno resultan en un efecto sinergístico o aditivo en formulaciones acuosas (Gilliland et al., 1972; Berenbaum, 1977).

Estudios toxicológicos en ratas indican que los parabenos y sus sales son no tóxicos para mamíferos. Los parabenos se absorben, metabolizan y excretan de forma rápida. Además, la estructura de los parabenos no es un indicativo de un potencial carcinogénico, como lo han demostrado estudios experimentales. Sin embargo, la detección de parabenos en una muestra pequeña de tumores de pecho y efectos adversos en la reproducción ocasionaron controversia en el uso de estas sustancias (Soni et al., 2005).

4.2.3. Uso de parabenos en el control de las podredumbres verde y azul

Varios tratamientos poscosecha con aditivos alimentarios o compuestos clasificados como GRAS, han mostrado capacidad significativa de control contra las podredumbres verde y azul en cítricos. Dentro de ellos destacan las sales de carbonatos (Smilanick et al., 1999; Palou et al., 2001, 2002b; Larrigaudiére et al., 2002; Youssef et al., 2012), las sales de acetatos y molibdatos (Palou et al., 2002c), y el sorbato de potasio (Smilanick et al., 2008; Montesinos et al., 2009; Cerioni et al., 2013).

Las sales de sodio de parabenos tales como el metil parabeno sódico (SMP), el etil parabeno sódico (SEP) y el propil parabeno sódico (SPP) han demostrado potencial para el control de enfermedades de poscosecha de cítricos y fresa (Yildirm y Yapici, 2007; Valencia-Chamorro et al., 2008, 2009). Por ejemplo, Yildirm y Yapici (2007) reportaron una concentración mínima de inhibición de 1000 μg mL^{-1} de metil y propil parabeno sódico contra *B. cinerea* en condiciones *in vitro*. Sin embargo, en estos estudios los parabenos fueron aplicados en condiciones *in vitro* y/o como ingredientes de recubrimientos comestibles. Asimismo, se encontró una inhibición de crecimiento de *P. digitatum* y *P. italicum* con metil, etil y propil parabeno sódico a concentraciones de 1-1,5% en condiciones *in vitro* (Valencia-Chamorro et al., 2008). Además, el SMP incorporado a un recubrimiento comestible sobre la piel del fruto de cítricos 24 h después de la inoculación con *P. digitatum* y *P. italicum* redujo la incidencia de las podredumbres verde y azul de hasta un 50% y menos del 20% en naranjas 'Valencia' y mandarinas 'Clemenules',

respectivamente, después de 7 días de incubación a 20°C y 90% HR (Valencia-Chamorro et al., 2009). En la presente tesis doctoral se contempla la evaluación de tratamientos poscosecha con soluciones acuosas de estas sustancias.

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OBJETIVOS

OBJETIVO GENERAL

Evaluar aproximaciones a una estrategia integrada no contaminante para el control en poscosecha de las podredumbres verde y azul de los frutos cítricos.

Objetivos específicos

1. Determinar el efecto de la desverdización comercial con etileno exógeno sobre la susceptibilidad y la calidad del fruto y sobre el desarrollo de las podredumbres verde y azul en frutos de mandarinas y naranjas de estación temprana.

2. Evaluar la actividad preventiva y curativa de tratamientos de poscosecha con inductores químicos de resistencia contra las podredumbres verde y azul de los cítricos.

3. Evaluar tratamientos de poscosecha preventivos y curativos con soluciones acuosas de silicato de potasio para controlar las podredumbres verde y azul en frutos de naranja.

4. Caracterizar la actividad antifúngica de tratamientos de poscosecha con soluciones acuosas de metil parabeno sódico contra las podredumbres verde y azul de cítricos. Evaluar la compatibilidad con dosis bajas de imazalil y la capacidad de control en cultivares comerciales y en frutos conservados en frío.

5. Caracterizar la actividad antifúngica de tratamientos de poscosecha con soluciones acuosas de etil parabeno sódico contra las podredumbres verde y azul de cítricos. Evaluar la compatibilidad con dosis bajas de imazalil y la capacidad de control en cultivares comerciales y en frutos conservados en frío.

6. Caracterizar la actividad antifúngica curativa de tratamientos de poscosecha con propil parabeno sódico contra las podredumbres verde y azul de cítricos. Evaluar la

compatibilidad con dosis bajas de imazalil y la capacidad de control en cultivares comerciales y en frutos conservados en frío.

RESULTADOS Y DISCUSIÓN

CAPÍTULO 1

Effect of citrus ethylene degreening on the development of postharvest penicillium molds and fruit susceptibility and quality

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

The effect of commercial degreening with ethylene gas on fruit susceptibility and quality and development of postharvest green (GM) and blue (BM) molds on early season citrus fruits was investigated. Each cultivar was harvested with different rind color indexes. Fruit were exposed for 3 d to 2 μL L^{-1} ethylene at 21ºC and 95-100% RH before or after artificial inoculation with *Penicillium digitatum* or *P. italicum*. Control fruit were kept at the same environmental conditions without ethylene. Fruit were stored at either 20ºC for 7 d or 5ºC for 14 d and disease incidence (%) and severity (lesion diameter) were assessed. No significant effect of commercial degreening was observed on fruit susceptibility to both GM and BM on citrus cultivars inoculated after degreening. Likewise, no significant effect was observed on disease incidence on citrus cultivars inoculated before degreening and stored at either 20°C for 7 d or 5°C for 14 d. In contrast, in cultivars like 'Clemenules' mandarins and 'Navelina' oranges, degreening significantly increased the severity on fruit with higher initial CI (-3.6 and 1.7, respectively). GM and BM severity on degreened and control 'Clemenules' mandarins incubated at 20°C for 7 d was 146 and 118 mm and 56 and 46 mm, respectively. In general, commercial degreening did not significantly affect external and internal quality attributes of citrus cultivars. Commercial degreening after inoculation of less green (more mature) fruit showed a trend to increase mold severity, presumably through an aging effect (acceleration of rind senescence).

Keywords: Orange, mandarin, postharvest disease, *Penicillium digitatum*, *P. italicum*, rind color index, fruit quality

1. INTRODUCTION

Postharvest green mold (GM), caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., and postharvest blue mold (BM), caused by *Penicillium italicum* Wehmer, are the most economically important postharvest diseases of citrus in Spain and all citrus production areas characterized by low summer rainfall (Eckert and Eaks, 1989; Palou et

al., 2007). Blue mold is especially important on citrus fruit kept under cold storage (Whiteside et al., 1993; Palou et al., 2002).

On the other hand, external color of citrus fruits is more often related to climatic conditions than to internal maturity (Terblanche, 1999). Color break is the natural process of degreening and it happens in a stage of maturity in which the green fruit turns to a yellow and orange color (Poole and Gray, 2002). This stage is characterized by the degeneration of thylakoids in the chloroplasts of the peel and it is accompanied by a decrease of the carotenogenesis, a degradation of the green chlorophyll pigments (Purvis and Barmore, 1981; Peng et al., 2013) and finally an increase in carotenoid biosynthesis (Zhou et al., 2010; Mayuoni et al., 2011a). Degreening with ethylene (C_2H_4) is employed to accelerate artificially the natural color break (Mayuoni et al., 2011b) in which fruit turns from green to orange/yellow. Citrus degreening with ethylene is a common commercial practice in many parts of the world (Smilanick et al., 2006). Particularly, ethylene degreening of Spanish citrus fruits is an important operation that is extensively implemented to market early season mandarins and oranges that have achieved internal maturity with little or no change in external appearance. It is typically conducted from September to December, and it is calculated that more than 1.2 million ton of early season mandarin fruit is degreened annually.

Since 1970's decade to date, several research works have been conducted to clarify the relationship between ethylene applications and postharvest decay by phytopathogenic fungi on citrus fruit, finding a non-well-determined trend (McCornack, 1971; Brown, 1973, 1975, 1986; Barmore and Brown, 1985). Discrepancies observed in research results seem to be related to different environmental conditions, the amount of ethylene applied and/or different host-pathogen interactions. On the one hand, resistance induction to mold fungi by fruit degreening has been reported. For instance, the incidence of green mold caused by *P. digitatum* was reduced by degreening naturally infected oranges at 30°C and relative humidity (RH) of 90-96% (Brown, 1973). In other work, 'Robinson' tangerines were extremely susceptible to anthracnose (caused by *Colletotrichum gloeosporioides*), especially when were degreened before artificial fungal inoculation (Brown, 1975). Moreover, oranges treated with

ethylene gas for 3 d at 20°C before inoculation with *P. italicum* became more resistant to decay and developed smaller lesions (El-kazzaz et al., 1983a). Another report revealed a decrease by 10% of the incidence of mold rots (caused by *P. digitatum* and *P. italicum*) in naturally infected and ethylene-treated 'Shamouti' oranges (Porat et al., 1999). On the other hand, other research results have suggested an increase in the incidence of postharvest diseases in citrus following ethylene exposure. For example, degreening after artificial fungal inoculation increased the incidence of stem-end rots, but not of green mold, in 'Hamlin' and 'Valencia' oranges (McCornack, 1971). In addition, concentrations of ethylene of 5-10 μL L^{-1}, required for optimum degreening of 'Valencia' oranges, significantly increased the incidence of stem-end rot caused by *Lasiodiplodia theobromae* (Brown, 1986). The incidence of stem-end rot on 'Valencia' oranges increased with exposure to increasing concentrations of ethylene from 0 to 50 μL L^{-1} (Barmore and Brown, 1985). In a later study, fruit treated with ethylene at 0, 5 and 50 μL L^{-1} for 60 h showed a stem-end rot incidence of 10.0, 33.3 and 73.3% in 'Valencia' oranges (Zhang, 2004). It was reported in another work that degreening in standard conditions after fungal inoculation had no effect on the incidence of both green and blue molds on 'Clemenules' mandarin fruit (Plaza et al., 2004).

In some of these reports ethylene degreening has been found to induce fruit resistance or susceptibility to disease, depending on the studied plant-pathogen interaction and the experimental conditions. Given that the effects of degreening on the incidence and development of citrus pathogenic fungi can vary depending on specific handling and environmental conditions, it is important to establish them for each particular situation. Standard commercial degreening practices for early season mandarins and oranges in Spain and other Mediterranean countries consist of fruit exposure for 2-4 d to 2-5 μL L^{-1} ethylene at 20-22ºC and RH > 90% (Sdiri et al., 2012a), while in other citrus producing areas like Florida or Brazil, commercial degreening is typically performed at temperatures surrounding 30ºC (Brown, 1973, 1975). Regarding the effects of ethylene exposure on the quality of harvested horticultural produce, the responses are numerous and varied, and they can be beneficial or detrimental

depending on each case (Saltveit, 1999). For example, promoting chlorophyll destruction would be detrimental in lettuce or in stored limes, but it would be beneficial for the commercialization of mandarins, oranges or lemons, or for tobacco curing. In general, ethylene can influence the postharvest life of both climacteric and non-climacteric fruit by affecting their quality attributes and the development of physiological disorders and postharvest diseases (Palou et al., 2003). In the case of citrus fruit, it is crucial to examine the effects of ethylene degreening on fruit quality attributes other than rind color, especially on fruit destined to prolonged storage or long-distance markets. For example, neither ethylene nor 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, influenced fruit weight loss, firmness, total soluble solids and acid contents in 'Shamouti' oranges (Porat et al., 1999). In a more recent study with 'Clemenules' mandarins, it was found that fruit degreened at 20°C for the first 24 h showed an increase in firmness in comparison with control fruit kept at 20°C without ethylene exposure. The same study reported that the degreening treatment had no effect on soluble solids concentration (SSC) and titratable acidity (TA) of the juice at the end of the degreening period (Plaza et al., 2004). Moreover, no significant differences were found in weight loss, firmness, SSC, TA and flavor among degreening treatments on 'Oronules' mandarin fruit (Carvalho et al., 2006). In addition, fruit degreened with exogenous ethylene at 5-7 $\mu L\ L^{-1}$ had no detrimental effects on SSC, TA and maturity index (MI) on 'Mioro' mandarin fruit (Abad et al., 2003).

Over the last few years, due to increased competition in global markets and increasing public interest of the nutritional benefits of horticultural produce; growers and consumers are attaching increasing importance to the nutritional quality and bioactive compounds of fruits and vegetables (Patil et al., 2009), and, for this reason, research is also being directed to establish the effects of ethylene degreening on these compounds. For instance, it has been reported that ethylene degreening had no significant effect on levels of health promoting compounds such as vitamin C, total phenols and flavonoids of citrus juice (Nishikawa et al., 2002; Mayuoni et al., 2011a). On the other hand, research has focused on the evaluation of internal fruit quality on a normal clementine cultivar and a late-ripening clementine mutant defective in ethylene perception; it was found that the mutation did

not affect internal ripening characteristics such as juice TA and SSC content (Distefano et al., 2009). Likewise, effects of ethylene on the transcriptome of 'Michal' mandarin flesh revealed that exposure to ethylene activated gene expression and stimulated various adaptation and metabolic processes , which might impact on fruit internal and nutritional quality (Mayuoni et al., 2011b).

Before the interest of the Spanish citrus industry to optimize degreening treatments and overall fruit handling in the packinghouses for the most representative commercial cultivars, it is important to determine the influence of ethylene degreening in our particular conditions on the development of green and blue molds, the most economically important cause of postharvest decay, and also on fruit susceptibility to these diseases. Therefore, the aims of this research were to: (i) determine the effect of commercial degreening with ethylene gas on the susceptibility of intact early season mandarins and oranges to green and blue molds, (ii) assess the effect of degreening on the incidence and development of green and blue molds on fruit previously inoculated with *P. digitatum* and *P. italicum* and incubated at 20ºC or stored at 5ºC, and (iii) study the effect of degreening at standard commercial conditions on internal and external fruit quality attributes. Preliminary results from this research have been recently published (Moscoso-Ramírez and Palou, 2012).

2. MATERIALS AND METHODS

2.1. Fruit

The trials were conducted with 'Clemenpons' (two experiments) and 'Clemenules' (three experiments) clementine mandarins (*Citrus reticulata* Blanco), 'Navelina' oranges (*Citrus sinensis* (L.) Osbeck) (three experiments), and 'Nova' [*C. reticulata* x (*Citrus reticulata* x (*Citrus reticulata* x *Citrus paradisi*)), synonym: 'Clemenvilla'] hybrid mandarins (two experiments) from 2008 to 2011. Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same d or stored up to 1 week at 5°C and 90% RH before use. Before each experiment, fruit were selected, randomized, washed, disinfected superficially by immersion for 2 min in a 0.5% sodium hypochlorite

solution, rinsed with tap water to eliminate residual chorine, and allowed to air dry at room temperature. Fruit of each cultivar were harvested at different season periods according to different rind color indexes (CI = -0.07, 0.9 for 'Clemenpons' mandarins; CI = -6.5, -3.6, 2.2 for 'Clemenules' mandarins; CI = -5.3, 1.1, 1.7 for 'Navelina' oranges; CI = 12.3 for 'Nova' hybrid mandarins).

2.2. Commercial degreening procedure

Fruit were transported to a 1,000 t commercial degreening room in a local citrus packinghouse (Fontestad S.A., Montcada, Valencia). Fruit were exposed to 2 μL L^{-1} ethylene (±0.5 μL L^{-1}) at a constant temperature of 21ºC (±0.5ºC) and RH>95% for 72 h, with the exception of fruit from one of the experiments with 'Clemenules' mandarins that needed 24 h of additional exposure to develop acceptable commercial color. At the same time, non-degreened (control) fruit were exposed to the same environmental conditions but without exposure to exogenous ethylene in a cold room located in the IVIA CTP pilot plant.

2.3. Fungal inoculation

Penicillium digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25ºC. Conidia of each fungus from 7-to 14-day-old were taken from the plate surface with a sterile glass rod and transferred to a sterile aqueous solution of 0.05% Tween 80® (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 1 x 10^5 spores mL^{-1} using a haemocytometer. Fruit were wounded and inoculated with the pathogens at the same time by immersing the tip of a stainless steel rod (2 mm length and 1 mm diameter) into the conidial suspension and making a puncture on the rind in the equatorial region of the fruit. Different lots of fruit were inoculated with each fungus.

2.4. Effect on fruit susceptibility to disease

To evaluate the effect of degreening on the fruit susceptibility to GM and BM, fruit were artificially inoculated with *P. digitatum* or *P. italicum* about 2 h after ethylene degreening. This set of experiments was performed with fruit of each cultivar harvested at the lowest CI (CI of -0.07, -6.5, -5.3 and 12.3 for 'Clemenpons', 'Clemenules', 'Navelina' and 'Nova' cultivars). Each treatment consisted of 4 replications with 10 fruit each. Degreened and non-degreened inoculated fruit were incubated at 20ºC and 90% RH for 7 d. Fruit were examined after 3 and 7 d to determine the incidence (% of infected wounds) and severity (diameter of lesion in mm measured only in infected fruit) of the molds.

2.5. Effect on disease development

To evaluate the effect of degreening on fungal development, fruit were inoculated with the pathogens as previously described 2 h before ethylene degreening. For each cultivar, these experiments were performed with fruit harvested at all different CI previously mentioned. After commercial degreening, degreened and control fruit were stored under two different conditions: (i) incubation at 20ºC and 90% RH for 7 d, and (ii) cold storage at 5ºC and 90% RH for 2 weeks. Stored fruit were periodically examined to determine disease incidence and severity. For each pathogen, treatment and storage condition, 4 replications of 10 fruit each were used.

2.6. Effect on citrus fruit quality

Non-inoculated oranges and mandarins were used for quality assessment. Fruit external and internal quality were determined just after harvest (initial quality) and 2-3 d after degreening on degreened and non-degreened (control) fruit (final quality).

2.6.1. External quality

Rind color was measured using Hunter parameters (L, a, b) with a colorimeter (Model Minolta CR-300, Konica Minolta Business Technologies, Inc., Tokio, Japan). A color index (CI) was calculated:

CI = 1000a/Lb (Jiménez-Cuesta et al., 1981). For each treatment, three measurements on the equatorial area of 25 fruit were performed.

Firmness of 20 fruit per treatment was determined using an Instron Universal Testing Machine (Model 4301, Instron Corp., Norwood, MA, USA). Each fruit was compressed between two flat surfaces closing together at the rate of 5 mm min^{-1}. The machine gave the deformation (mm) after application of a load of 1 kg to the equatorial region of the fruit. Results were expressed as percentage of deformation related to initial diameter. Rind break resistance was measured on 30 fruit per treatment using the same machine. Each fruit was compressed with a 5 mm diameter steel rod until the fruit rind was broken, and the necessary pressure (kg) was measured.

Rind oil release pressure (kg) of 20 fruit per treatment was determined using a fruit pressure tester with a 8 mm diameter tip (Model FT327, Facchini, Alfonsine, Italy). Each fruit was wrapped with filter paper and then compressed with the tester until essential oil stains appeared. The necessary pressure (kg) was annotated.

2.6.2. Internal quality

The fruit internal quality was only determined in clementine mandarins (cvs. 'Clemenpons' and 'Clemenules'). The juice from 3 previously weighed samples of 8 fruit each was extracted with a rotatory citrus squeezer and filtered through a 0.8 mm diameter sieve. The following fruit internal quality parameters were determined: SSC was measured with a digital refractometer (Model DR-101, Optic Ivymen System, Barcelona, Spain) and expressed as percentage. TA was determined from a 5 mL aliquot by titration with 0.1 N NaOH with phenolphthalein indicator and results were given as g of citric acid per 100 mL (%). MI was calculated as the SSC/TA ratio. In all cases, two replicated measures were performed with each juice sample. Juice yield was expressed as percentage of juice (mL) per fruit weight (g).

2.7. Statistical analysis

Data from disease assessment and fruit quality parameters were analyzed by analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus, version 5.1). Data on disease incidence were transformed to the arcsine of the square root of the proportion of infected fruit to assure the homogeneity of variances. Statistical significance was judged at the level $P \leq 0.05$. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was applied to separate means. Shown values are non-transformed data.

3. RESULTS

3.1. Effect of ethylene degreening on fruit susceptibility to disease

No significant effect of commercial degreening was observed on the incidence of both GM and BM on early season mandarins and 'Navelina' oranges, artificially inoculated 2 h after degreening with *P. digitatum* or *P. italicum* and incubated at 20ºC and 95-100% RH for 7 d (Fig. 1). Similarly, the severity of the molds was not affected by commercial degreening (Fig. 1).

3.2. Effect of ethylene degreening on disease development in fruit previously inoculated with *P. digitatum* or *P. italicum*

No significant effect of commercial degreening was observed on the incidence of both GM and BM on 'Clemenpons' (initial CI of -0.07 and 0.9; Fig. 2), 'Clemenules' (initial CI of -6.5, -3.6, and 2.2; Fig. 4) and 'Nova' (initial CI of 12.3; Fig. 8) mandarins, and 'Navelina' oranges (initial CI of -5.3, 1.1, and 1.7; Fig. 6) inoculated with *P. digitatum* or *P. italicum* about 2 h before degreening and incubated at 20ºC and 90% RH for 7 d. Likewise, no effect was found on the incidence of both molds on 'Clemenpons', 'Clemenules' and 'Nova' mandarins, and 'Navelina' oranges stored at 5ºC for 14 d, with the exception of 'Clemenules' mandarins with initial CI of -3.6, in which GM incidence on degreened and non-degreened fruit was 100 and 85%, respectively (Fig. 4); and 'Navelina' oranges with initial CI of -5.3 and 1.7, in which GM incidence on degreened and non-

degreened fruit was 90 and 63% and 98 and 70%, respectively (Fig. 6).

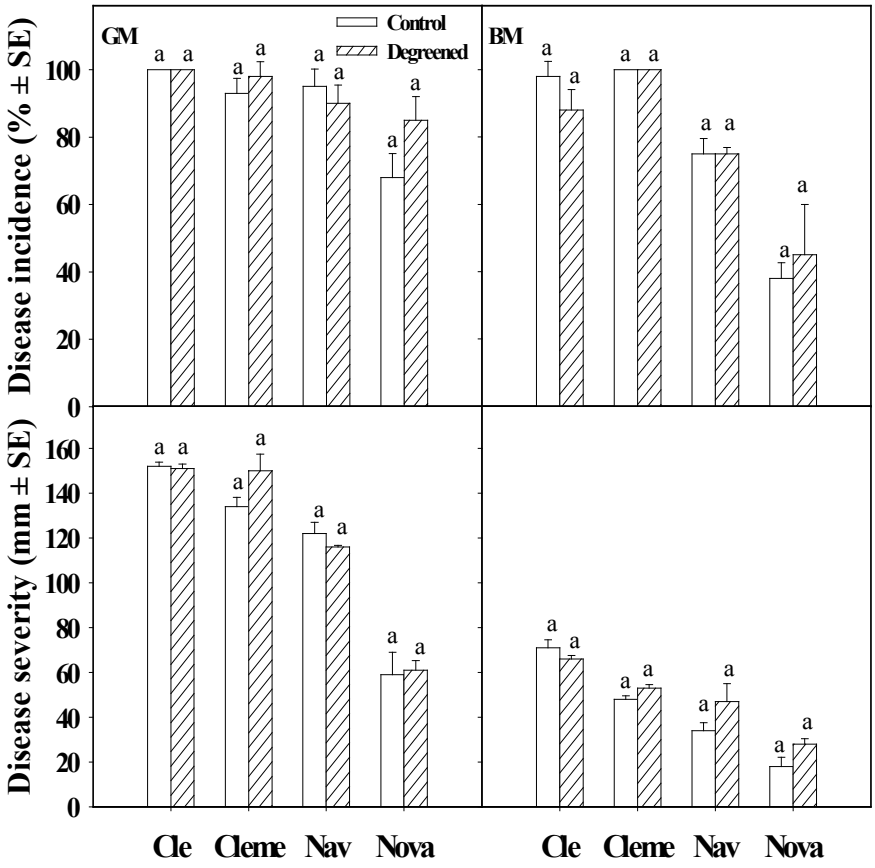

Figure 1. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 72 h) on the incidence and severity of green (GM) and blue (BM) molds on fruit of several citrus cultivars artificially inoculated 2 h after degreening, and incubated at 20ºC and 90% RH for 7 d. 'Clemenpons' (Cle), 'Clemenules' (Cleme), 'Navelina' (Nav), and 'Nova' fruit were degreened with initial color index (CI = 1000a/Lb; Hunter parameters) of -0.07, -6.5, -5.3, and 12.3, respectively. For each disease and cultivar, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05). Disease incidence data were arcsine transformed. Non-transformed means are shown.

On ‘Clemenpons’ mandarins with the initial CI of -0.07 and 0.9, commercial degreening 2 h after fungal inoculation had no significant effect on the severity of the molds on fruit incubated at 20°C for 7 d (Fig. 3). On ‘Clemenpons’ mandarins cold-stored at 5ºC for 14 d, commercial degreening did not significantly affect the severity of the molds with the exception of BM on mandarins with an initial CI of 0.9, in which lesion diameters were 33 and 27 mm on degreened and control fruit, respectively (Fig. 3).

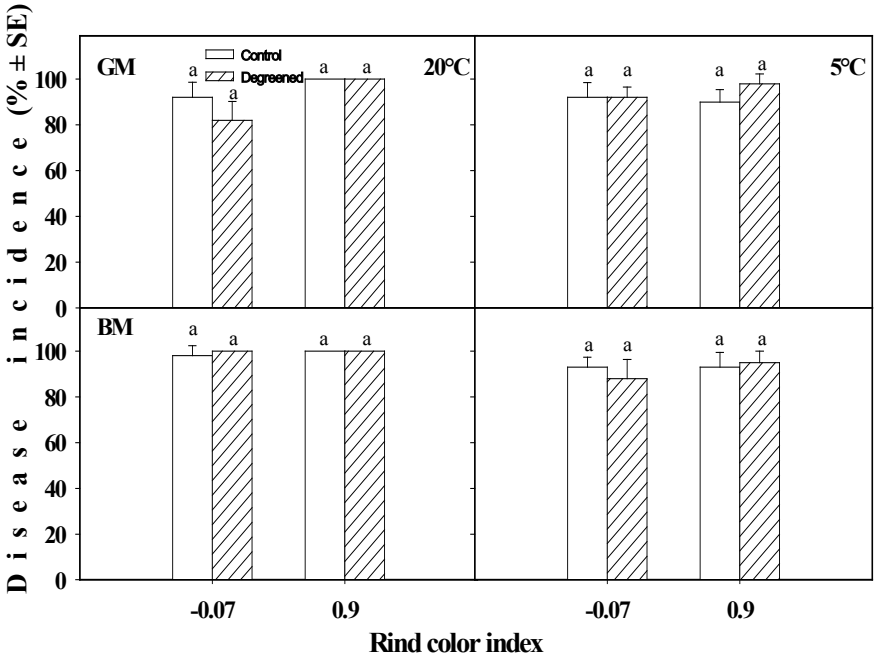

Figure 2. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 72 h) on the incidence of green (GM) and blue (BM) molds on ‘Clemenpons’ mandarins harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20ºC and 90% RH for 7 d or 5ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher’s protected LSD test (P = 0.05) applied to arcsine-transformed data. Non-transformed means are shown.

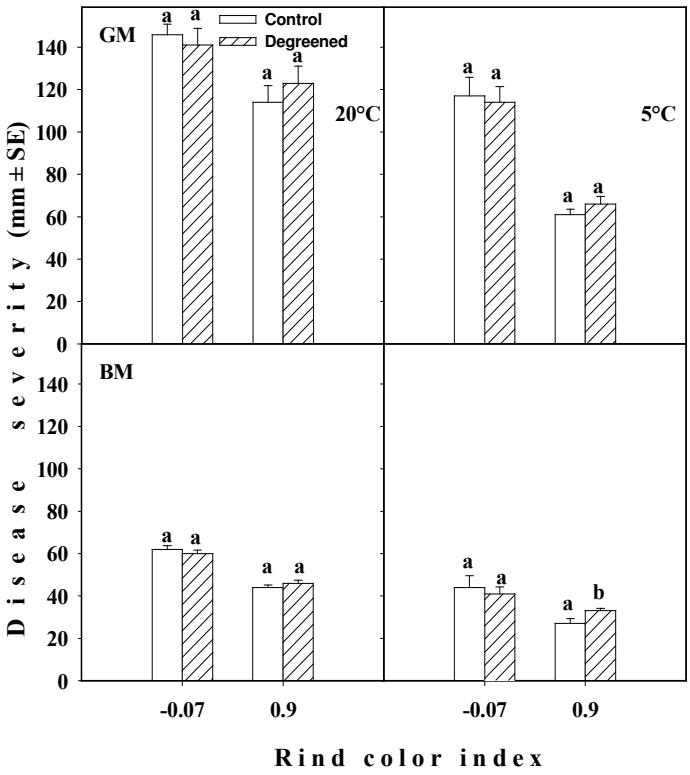

Figure 3. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 72 h) on the severity of green (GM) and blue (BM) molds on 'Clemenpons' mandarins harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20ºC and 90% RH for 7 d or 5ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05).

On 'Clemenules' mandarins, the effect of commercial ethylene degreening on disease severity was dependent on the initial rind CI. Degreening treatment significantly increased the severity of the molds on fruit incubated at 20°C for 7 d, with the exception of mandarins with an initial CI of -6.5 (Fig. 5). On mandarins cold-stored at 5ºC for 14 d, commercial degreening significantly increased the severity of

the molds on mandarins with an initial CI of -3.6, with GM and BM severity values of 49 and 34 mm and 28 and 19 mm on degreened and non-degreened fruit, respectively. No significant effect was observed on fruit with an initial CI of -6.5 (Fig. 5).

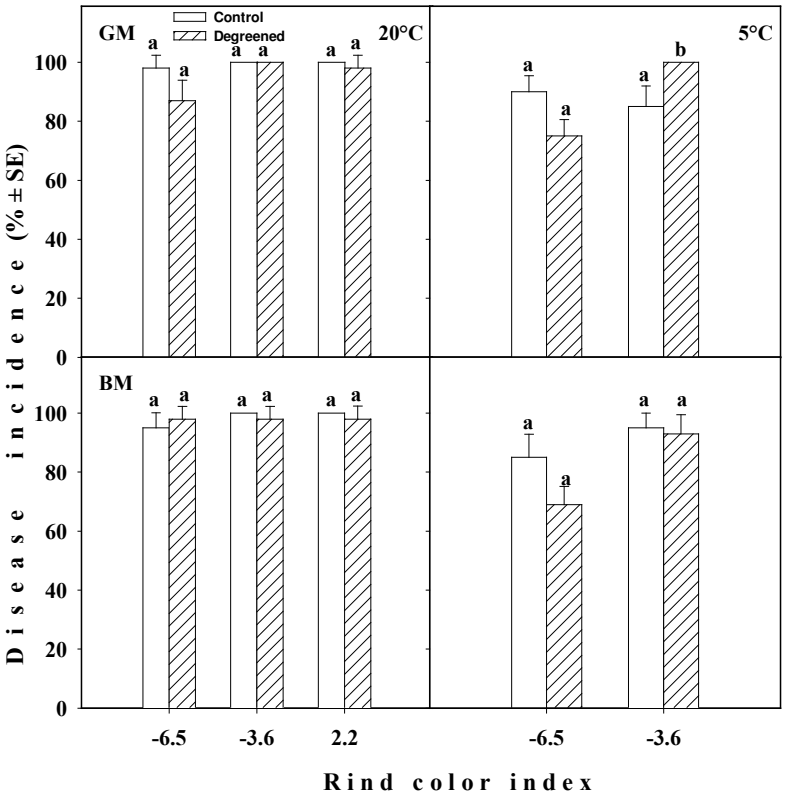

Figure 4. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 4 d) on the incidence of green (GM) and blue (BM) molds on 'Clemenules' mandarins harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20 ºC and 90% RH for 7 d or 5 ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05) applied to arcsine-transformed data. Non-transformed means are shown.

On ‘Navelina’ oranges, commercial degreening had a significant effect on the severity of the molds, and it was dependent on the initial rind CI. Degreening treatment significantly increased the severity of the molds on fruit incubated at 20 °C for 7 d, with the exception of mandarins with an initial CI of -5.3 for both molds and of 1.1 for BM (Fig. 7).

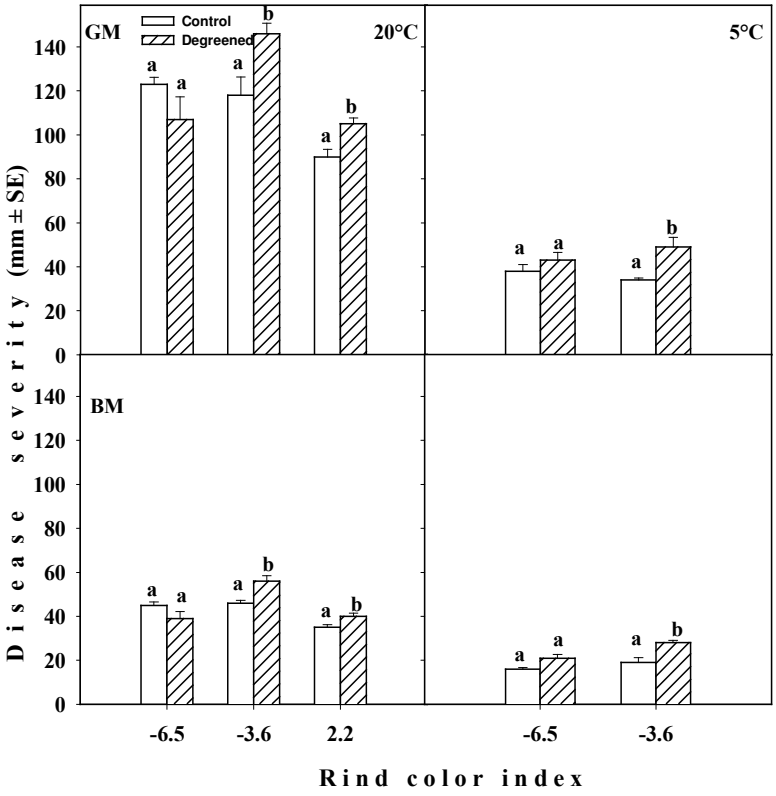

Figure 5. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 4 d) on the severity of green (GM) and blue (BM) molds on ‘Clemenules’ mandarins harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20ºC and 90% RH for 7 d or 5ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher’s protected LSD test (P = 0.05).

On mandarins cold-stored at 5ºC for 14 d, commercial degreening significantly increased the severity of the molds on mandarins with all three initial rind CI (-5.3, 1.1, 1.7), with the exception of mandarins with an initial rind CI of -5.3 for BM (Fig. 7).

On 'Nova' hybrid mandarins degreened with an initial rind CI of 12.3, ethylene degreening did not significantly affect the severity of the molds stored neither at 20ºC for 7 d nor at 5°C for 14 d, with the exception of GM severity, in which, lesion diameters were 52 and 37 mm on degreened and control fruit, respectively (Fig. 8).

3.3. Effect of ethylene degreening on citrus fruit quality

In general, the external quality attributes of citrus cultivars with different initial rind CI were not influenced by ethylene degreening. Although significant differences between degreened and control fruit were found in few cases, the practical impact of such differences was minimal (Table 1). For instance, 'Clemenules' mandarin fruit degreened with an initial rind CI of -6.5 were significantly more firm (lower deformation) than non-degreened fruit, but the deformation values were 5.48 and 6.02%, respectively. Rind break resistance was significantly higher on degreened 'Clemenules' mandarins with an initial rind color index of -6.5 (1.7 kg) than on control fruit (1.3 kg), but the force difference was only of 0.4 kg (Table 1). Rind oil release pressure was significantly lower on degreened 'Clemenpons' mandarins and 'Navelina' oranges, with the initial rind CI of 0.9 (3.99 kg) and -5.3 (5.40 kg), respectively, than on control fruit (4.93 and 5.40 kg, respectively).

No significant effect of commercial degreening was observed on internal quality attributes (TA, SSC, MI, and juice yield) of 'Clemenpons' and 'Clemenules' clementine mandarins (Table 2).

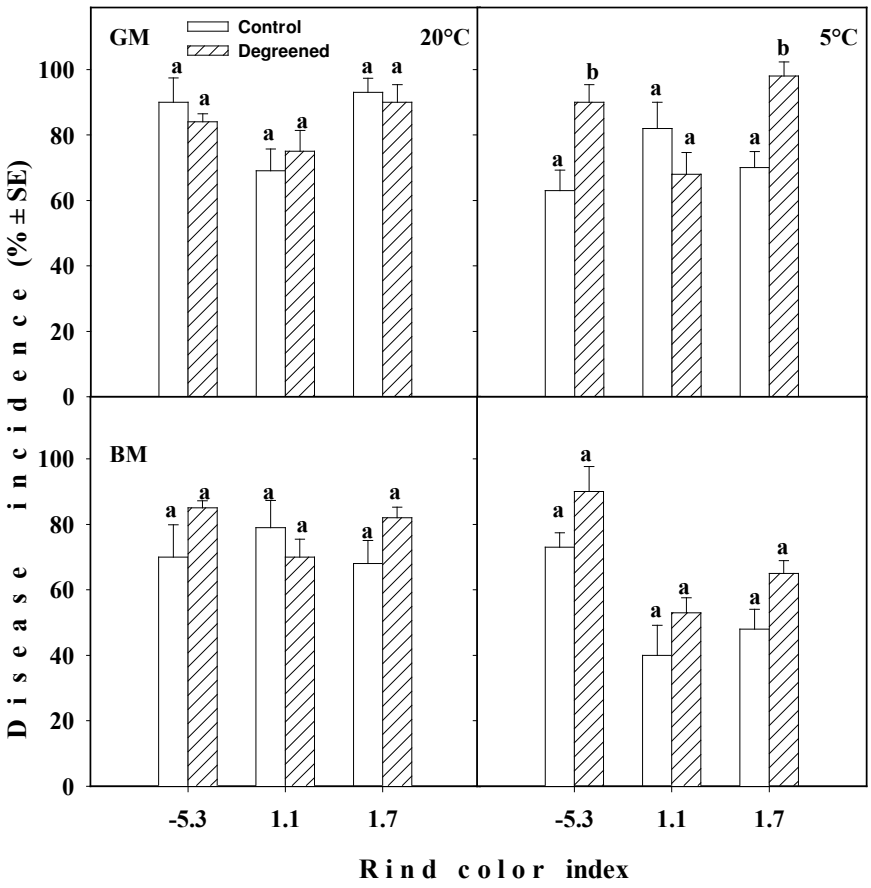

Figure 6. Effect of commercial degreening (2 μL L^{-1} ethylene at 21ºC and 95-100% RH for 3 d) on the incidence of green (GM) and blue (BM) molds on 'Navelina' oranges harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20ºC and 90% RH for 7 d or 5ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05) applied to arcsine-transformed data. Non-transformed means are shown.

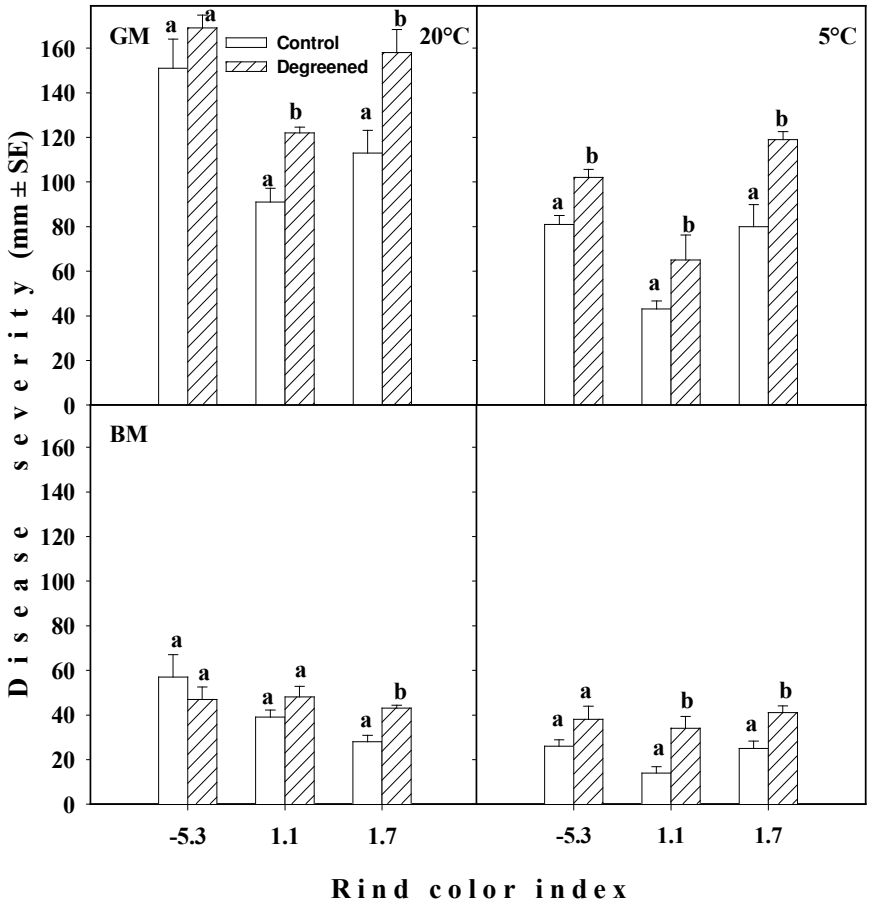

Figure 7. Effect of commercial degreening (2 μL L^{-1} ethylene at 21ºC and 95-100% RH for 3 d) on the severity of green (GM) and blue (BM) molds on 'Navelina' oranges harvested with different color index (CI = 1000a/Lb; Hunter parameters), artificially inoculated 2 h before degreening, and stored at either 20ºC and 90% RH for 7 d or 5ºC and 90% RH for 14 d. For each disease, storage temperature and CI, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05).

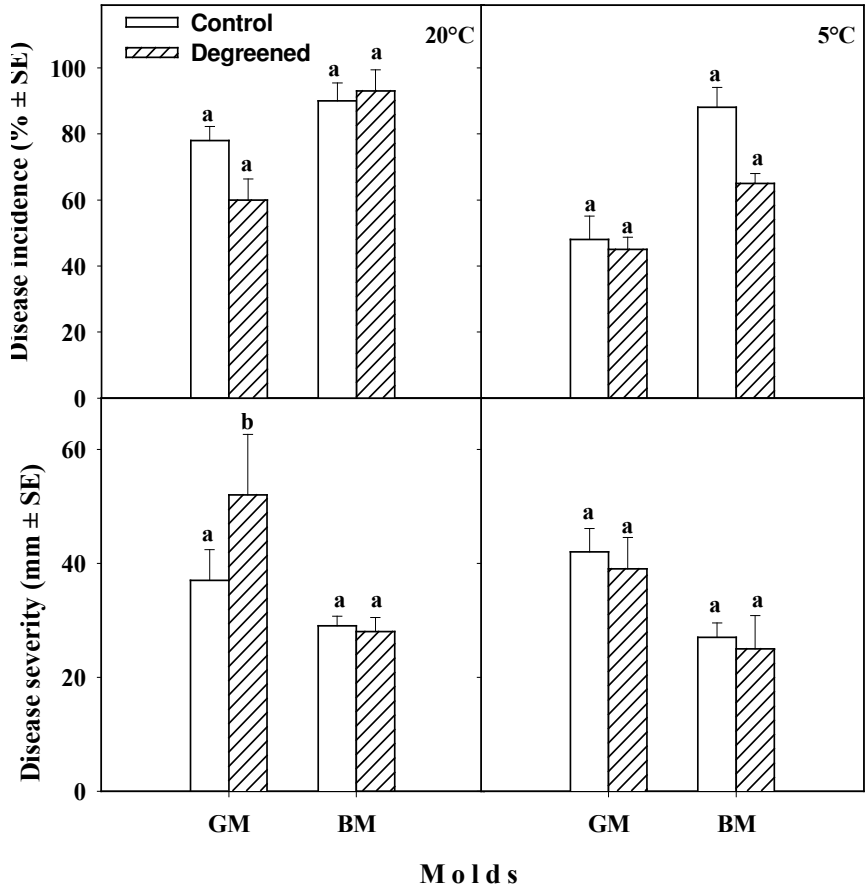

Figure 8. Effect of commercial degreening (2 µL L^{-1} ethylene at 21ºC and 95-100% RH for 3 d) on the incidence and severity of green (GM) and blue (BM) molds on 'Nova' mandarins artificially inoculated 2 h before degreening, and stored at either 20 ºC and 90% RH for 7 d or 5 °C and 90% RH for 14 d. Fruit were degreened with initial color index (CI = 1000a/Lb; Hunter parameters) of 12.3. For each disease and storage temperature, columns with the same letters are not significantly different according to Fisher's protected LSD test (P = 0.05). Disease incidence data were arcsine transformed. Non-transformed means are shown.

Table 1. Effect of commercial degreening on external quality attributes of citrus fruit.

Cultivar	Initial CI	Firmness (% deformation)			Rind break resistance (kgf)			Rind oil release pressure (kgf)		
		At harvest	Control	Degreened	At harvest	Control	Degreened	At harvest	Control	Degreened
'Clemenpons'	-0.07	6.27	8.71a	8.85a	1.23	1.13a	1.09a	-	-	-
	0.9	5.17	5.89a	5.37a	1.35	1.43a	1.27a	4.22	4.93a	3.99b
'Clemenules'	-6.5	4.26	6.02a	5.48b	1.8	1.3a	1.7b	-	-	-
	-3.6	4.42	5.91a	5.87a	1.56	1.46a	1.40a	5.15	5.30a	5.20a
	-0.6	3.44	4.76a	4.53a	1.35	1.57a	1.34a	4.60	4.60a	4.90a
	2.2	3.28	4.56a	4.10a	1.49	1.46a	1.40a	3.75	4.80a	4.46a
'Navelina'	-5.3	1.09	1.51a	1.49a	2.72	2.36a	1.93b	5.90	5.87a	5.40b
	1.1	1.63	2.63a	2.40a	2.11	2.06a	1.80a	5.18	5.76a	5.53a
	1.7	1.60	2.40a	2.03a	2.33	1.86a	1.83a	5.64	5.23a	5.86a
'Nova'										
	12.3	1.62	2.20a	2.26a	2.17	1.62a	1.70a	5.09	5.75a	5.73a

Firmness and rind oil release pressure were measured on 20 fruit per treatment; rind break resistance was measured on 30 fruit per treatment.
CI= Color index (CI=1000a/Lb; Hunter parameters).
-, not determined.
For each cultivar and initial CI, mean values within rows followed by the different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA.

Table 2. Effect of commercial degreening on internal quality attributes of clementine mandarin fruit

Treatments	TA (% citric acid)	SSC (%)	Maturity index (MI)[a]	Juice yield (%)
'Clemenpons'				
At harvest	0.84	11.03	13.21	50.65
Control	1.31a	11.02a	8.41a	52.77a
Degreened	1.36a	11.43a	8.39a	52.24a
'Clemenules'				
At harvest	0.86	10.53	12.26	45.87
Control	1.31a	10.15a	7.75a	46.80a
Degreened	1.27a	10.47a	8.26a	48.96a

[a] MI= SSC/TA (SSC= soluble solids concentration; TA= titratable acidity).
For each cultivar, columns followed with the different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA.

4. DISCUSSION

This research work has determined the effect of commercial degreening with ethylene gas at the standard Spanish conditions on fruit susceptibility and development of GM and BM in early season commercially valuable mandarins and oranges, as well as on the most important citrus fruit quality attributes. In general, commercial degreening before fungal inoculation with *P. digitatum* or *P. italicum* had no significant effect on the susceptibility to postharvest GM and BM on mandarins and oranges harvested with low CI and incubated at 20°C for 7 d (Fig. 1). The implications of these results for the Spanish citrus industry are positive because, not being more susceptible, degreened fruit will not require special or additional preventive antifungal treatments to protect the fruit from infections by *Penicillium* spp. that may take place after degreening in the packinghouse facilities or storage rooms (Fig. 1). According to previous literature, not only the cultivar, but also the degreening treatment conditions and the type of pathosystem clearly affect the influence of degreening on citrus fruit susceptibility to postharvest disease. Thus, a decrease in fruit susceptibility to GM was observed when oranges were degreened with 5 μL L^{-1} of ethylene at 30°C and 90-96% RH for 3 d (Brown, 1973). In contrast, these degreening

conditions significantly increased the fruit susceptibility of 'Robinson' tangerines to anthracnose caused by *C. gloeosporioides* (Brown, 1975). In work by El-kazzaz et al. (1983a) with oranges, the application of exogenous ethylene before fungal inoculation significantly decreased BM severity on 'Valencia' oranges. These authors suggested that longer degreening times reduced glucosamine content, and consequently more resistance to the disease, considering that glucosamine is an indicator of fungal growth. Likewise, these results showed no polyphenol oxidase (PPO) activity in both inoculated and non-inoculated fruit, but phenylalanine ammonia-lyase (PAL) activity increased significantly only in the rind of control fruit exposed to 1,000 μL L^{-1} of ethylene for 6 d.

Results from this research work showed that the influence of commercial degreening on the development of GM and BM on previously inoculated fruit was dependent not only on the cultivar but also on the initial fruit rind CI and the storage conditions in which the fruit were held after the 72-h degreening period. Furthermore, the effect on the incidence of the molds (% of infected fruit) was generally different than that on the severity of the molds (lesion diameter). In general, no significant effect on the incidence of both molds was noticed on mandarins and oranges with different initial CI artificially inoculated with *P. digitatum* or *P. italicum* before degreening and incubated at 20ºC for 7 d after degreening. These findings are similar to those obtained by Plaza et al. (2004), who observed that degreening with 5-10 μL mL^{-1} of ethylene at 20ºC and 90-95% RH had no effect on the incidence of BM or GM on 'Clemenules' mandarins inoculated before degreening. Also, degreening with ethylene gas (5 μL L^{-1} of ethylene for 2 d) did not significantly affect the incidence of GM on 'Valencia' oranges (McCornack, 1971). Since the variable disease incidence measures the amount of infections that take place from free conidia deposited in the infection courts (rind wounds) during the artificial inoculation procedure, it is clear that ethylene exposure did not affect the germination ability of these spores, which remained very high during incubation at 20ºC (Fig. 2, 4, 6). This is in agreement with previous results in which the presence or absence of ethylene had no effect on the pathogenicity or virulence of the fungus on green-mold-infected citrus fruit (Chalutz, 1979; Mullins et al., 2000). However, in some of

our trials the effect of commercial degreening was different on fruit stored at 5°C for 14 d. Particularly, the treatment favored GM incidence on 'Clemenules' mandarins with an initial CI of -3.6 and 'Navelina' oranges with initial CI of -5.3 and 1.7, while it did not affect the incidence of GM or BM in the rest of cases. Hence, it seems that in these particular cases, the interaction between previous ethylene exposure and prolonged storage at low temperature somehow affected the susceptibility of the fruit host allowing a higher amount of spores to germinate and cause viable infections. In any case, none of our trials showed a noticeable induction of fruit resistance to disease following commercial ethylene degreening of previously inoculated fruit. These results differ from those by Porat et al. (1999), who found a 10% decrease in the incidence of molds caused by *P. digitatum* and *P. italicum* in response to the application of 10 μL L^{-1} ethylene for 60 h on 'Shamouti' oranges naturally infected and stored for 4 weeks at 20ºC. Further, the incidence of GM was reduced by degreening oranges (5 μL L^{-1} of ethylene at 30°C and 90-96% RH) for 3 d (Brown, 1973). This dissimilar behavior can be logically attributed to important differences in the degreening treatment conditions, specifically to different amounts of ethylene and length and temperature of exposure. In fact, some research established an involvement of ethylene perception in promoting defense responses in citrus fruit infected by *P. digitatum* through the accumulation of defense-related mRNAs (Marcos et al., 2005) and global results of transcriptomic analysis of citrus fruit peel tissue reveal fundamental effects of phenylpropanoids and ethylene on induced resistance (Ballester et al., 2011).

Regarding the effect of commercial degreening on disease severity, it was observed in these experiments that it highly depended on the cultivar and particularly on initial rind CI, while the effect of storage conditions (incubation at 20ºC or storage at 5°C) was less relevant (Figs. 3, 5, 7). The variable disease severity quantifies the growth rate of the pathogen once the infection has been initiated. Hence, only the fruit with values of disease incidence different than zero were considered for its calculation. Therefore, the effect of ethylene degreening on this variable reflects the effect of the treatment not on free spores and their capacity to germinate and initiate infection in rind wounds, but on the ability of fungal hyphae to grow and

multiply in the infection court. The fact that this mycelial growth chiefly depends on the fruit host natural resistance to disease, defined basically by the genotype and the physical and physiological condition (Palou et al., 2007, 2008), can explain the differences in disease severity that were observed in this work. On 'Clemenpons' clementines with different initial rind CI, degreening treatment did not affect the severity of the molds, but it significantly increased that of fruit with higher initial CI of 0.9 for BM on fruit stored at 5°C (Fig. 3). On 'Clemenules' mandarins with lower initial CI of -6.5, ethylene degreening did not affect the severity of both GM and BM, while it significantly increased that of fruit with higher initial CI of -3.6 or 2.2 (Fig. 5). On 'Navelina' oranges with initial CI of -5.3, degreening exposure did not affect mold severity, but the treatment significantly increased the severity of the molds on fruit with higher initial CI of 1.1 and 1.7 (Fig. 7). It may be therefore concluded from these observations, the results obtained in the previous susceptibility trials performed with fruit with low CI (Fig. 1), and from the general lack of influence on the variable disease incidence that the effect of commercial degreening with ethylene on the development of penicillium molds relied more on the natural resistance of each cultivar and on particular fruit condition than on a primary influence of the gas on the pathogenic fungus itself. While the rind condition of the greenest mandarins and oranges (more immature, lower initial rind CI) was not affected or positively affected by commercial degreening in terms of susceptibility to decay, exposure to exogenous ethylene of less green citrus fruit (more mature, higher initial CI) significantly increased rind susceptibility to disease, presumably through an acceleration of the degradation of rind constitutive compounds associated with natural resistance (Porat, 2008). It is known that the content of major compounds with antifungal activity naturally present in the peel of citrus fruit, mostly phenolic compounds such as flavanones, flavones, etc. that act as the first line of defense against pathogens, is higher in immature fruit and decreases as the fruit ripen and age to senescence, either while remaining in the tree or after harvest (Ortuño et al., 2006; Palou et al., 2007). Some postharvest treatments of different nature have shown the ability to maintain for longer the effective levels of these constitutive antifungals, typically by an enhancement of the activity of defense-related enzymes such as PAL, β-1,3-glucanase (GLU), peroxidase (POD), or PPO (Ben-

Yehoshua et al., 1992; Lu et al., 2013). Since the application of exogenous ethylene can alter or accelerate the natural processes of fruit development, ripening and senescence (Kader, 1985), it could be possible that ethylene degreening contributed to a significant loss of bioactive compounds in the rind of more mature fruit (El-Kazzaz et al., 1983b; Sdiri et al., 2012b). The influence of the genotype, fruit condition and treatment conditions on the effect of ethylene exposure on disease severity can further be explained by contradictory results in the literature. In contrast to our findings, work reported by El-kazzaz et al. (1983b), showed that exposure to ethylene at concentrations of 1, 10, 100 or 1000 μL L^{-1} for 6 d did not affect lesion diameters on 'Navel' oranges inoculated with *P. italicum* and incubated at 20ºC for 6 d. These authors, however, did not characterize the initial maturity of the oranges used for the trials.

In this work, ethylene degreening treatment had, in general, no practical effect on external quality attributes of citrus fruit, which is in agreement with results reported in other research works (Porat et al., 1999; Carvalho et al., 2006; Sdiri et al., 2012b). One exception was the fruit firmness of 'Clemenules' mandarins with an initial rind CI of -6.5, which was slightly increased by ethylene degreening. This result coincided with the findings reported by Plaza et al. (2004) on the same cultivar. Likewise, we observed that ethylene degreening had no significant effect on internal quality attributes of 'Clemenpons' and 'Clemenules' mandarin fruit. These findings are similar to those obtained on fruits of these cultivars degreened at 2 μL L^{-1} for 5 d followed by a cold-quarantine at 1°C for 16 d (Sdiri et al., 2012b) or on 'Satsuma' mandarin degreened at 4 μL L^{-1} for 5 d (Tietel et al., 2010). In addition, a variety of results obtained with different citrus species and cultivar also support our findings (Porat et al., 1999; Abad et al., 2003; Plaza et al., 2004; Carvalho et al., 2006; Mayuoni et al., 2011a). As one exception, ethylene degreening decreased acidity levels in 'Mosambi' oranges (Ladaniya and Singh, 2001).

We concluded that the effect of commercial degreening with exogenous ethylene on the development of citrus penicillium molds relied more on fruit condition than on a direct effect of ethylene on the growth of the pathogenic fungi. While the rind condition of the greenest (more immature) mandarins was not affected by commercial

degreening, exposure to exogenous ethylene of less green (more mature) fruit significantly increased rind disease severity in some cultivars, presumably through a senescence effect on the fruit rind.

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CAPÍTULO 2

Evaluation of postharvest treatments with chemical resistance inducers to control green and blue molds on orange fruit

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

Preventive and curative activities of postharvest treatments of selected chemical resistance inducers to control postharvest green (GM) and blue (BM) molds on oranges (cvs. 'Valencia' or 'Lanelate') artificially inoculated with *Penicillium digitatum* and *P. italicum*, respectively, were evaluated. *In vivo* primary screenings to select the most effective chemicals and concentrations of benzothiadiazole (BTH), *β*-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), sodium silicate (SSi), salicylic acid (SA), acetylsalicylic acid (ASA) and harpin were tested. Chosen inducers were tested afterwards as dip treatments (immersion of 60 or 150 s at 20 ºC) with oranges artificially inoculated before or after the treatment and incubated for 7 d at 20 °C: INA at 0.03 mM, SA at 0.25 mM, BABA at 0.3 mM and BTH at 0.9 mM. Although it was an effective treatment, SSi at 1,000 mM was discarded because of potential phytotoxicity to the fruit rind. Preventive or curative postharvest dips at room temperature had no effect or only reduced the development of GM and BM very slightly. Therefore, these treatments cannot be recommended for inclusion in postharvest decay management programs for citrus packinghouses.

Key words: *Penicillium digitatum*, *P. italicum*, induced disease resistance, alternative decay control, sodium silicate, BTH, INA, BABA, SA, ASA, harpin

1. Introduction

Significant decay losses can occur after harvest, during storage and marketing of citrus fruit, primarily due to green mold (GM), caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and blue mold (BM), caused by *P. italicum* Wehmer (Eckert and Eaks, 1989). Currently, these diseases are primarily controlled by the application of conventional synthetic fungicides such as imazalil or thiabendazole (Holmes and Eckert, 1999; Palou et al., 2008). However, the need to produce residue-free fruit demanded by some markets, and resistance development to fungicides by these plant pathogens, are factors that restrict the fungicide usage worldwide (Brent and Hollomon, 2007).

One of the environmentally-friendly alternatives to control postharvest decay may be the induction of plant disease resistance, which can be defined as the ability of the plant to prevent or restrict pathogen growth and multiplication (Benhamou, 1996). Plants possess a range of defenses that can be actively expressed in response to pathogens and parasites, ranging from viruses to insect herbivores. The timing of these defense responses is critical and can be the difference between the plant being able to cope or succumbing to the challenge of a pathogen or parasite. Maintenance or induction of natural disease resistance in harvested horticultural crops using physical, biological and/or chemical elicitors has received increasing attention over recent years, and is considered a preferred strategy for disease management (Terry and Joyce, 2004). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance. In both SAR and ISR the processes that led to effective plant defense are preconditioned by prior pathogen infection or different treatments to which the plant is exposed (Vallad and Goodman, 2004). In contrast to SAR, induction of natural disease resistance in harvested fruits is considered a type of local acquired resistance (LAR), since resistance is manifested in the same plant tissues that received the induction treatments. Factors that affect the decline of natural disease resistance in fresh produce after harvest include: nutritional requirements for the pathogen; preformed antifungal compounds (phytoanticipins); the potential for inducible antifungal compounds (phytoalexins); and activation of fungal pathogenicity factors (Prusky, 1996). Various physical procedures, natural or synthetic chemicals and biological antagonists have been described as capable of controlling a large variety of plant diseases without having a direct antimicrobial activity themselves. Postharvest physical treatments such as UV-C light, blue light or heat (Palou, 2009; Alferez et al., 2012) or the application of biocontrol agents such as *Candida oleophila* (Droby et al., 2002) or *Pichia menbranefasciens* (Luo et al., 2012) have effectively reduced citrus penicillium molds by induction of host resistance. Several chemical compounds are known by their general ability to induce resistance in treated plants and they are generically called 'resistance inducers' (Sticher et al., 1997). Some of them, such as benzothiadiazole (BTH, Bion®, syn.: acibenzolar-S-methyl), *β*-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid

(INA), sodium silicate (SSi), acetylsalicylic acid (ASA), salicylic acid (SA), and harpin protein (Messenger®), have been repeatedly tested in plant vegetative parts or directly to fruits in order to induce resistance against postharvest diseases of fresh produce.

BTH was developed as a plant activator for commercial use in a wide range of crops. In recent work with this compound, incidence and lesion diameter of blue mould (caused by *Penicillium expansum*) and black rot (caused by *Alternaria alternata*) on mature pears collected from trees sprayed three times with BTH during fruit growth were significantly decreased with respect to control fruit 17 d after artificial fungal inoculation (Cao and Jiang, 2006). It was found in another study that 7 d after fungal inoculation, the lesion area and disease incidence caused by *P. expansum* on peaches treated with BTH at 200 mg L^{-1} were lower than on non-treated fruit (Liu et al., 2005). They stated that BTH treatment enhanced the activities of phenylalanine ammonialyase (PAL), polyphenoloxidase (PPO) and peroxidase (POD) in inoculated fruit, as well as the levels of total phenolic compounds and hydrogen peroxide (H_2O_2), which are considered to play important roles in plant disease resistance. On citrus, soil applications of BTH have been used for canker control in young trees (Graham and Myers, 2012).

BABA is a non-protein amino acid, which may induce resistance in plants against a broad range of disease-causing organisms, including fungi, bacteria, viruses and nematodes (Jakab et al., 2001; Cohen, 2002). In recent research work with sweet orange fruit cv. 'Darabi', the incidence and lesion diameter of citrus BM were significantly reduced by BABA applied at 40 mM (Tavallali et al., 2008). Another study by Porat et al. (2003) revealed that the application of BABA to superficial wound sites in the rind of 'Marsh Seedless' grapefruits induced resistance to citrus GM, with 20 mM being the most effective concentration. The induction of resistance to *P. digitatum* by BABA was accompanied by the activation of various pathogen defense responses in grapefruit rind tissue, including the expression of chitinase gene and protein accumulation after 48 h, as well as an increase in PAL activity after 72 h.

INA is a plant resistance inducer that acts via the SAR signal transduction pathway, but does not induce SA accumulation during the time required for the induction of SAR gene expression (Vernooij et al., 1995). In addition it is an effective inhibitor of ascorbate peroxidase (APX) synthesis (Durner and Klessing, 1995) and in field experiments, foliar INA applications were effective against several diseases on naturally infected cotton plants (Colson-Hanks et al., 2012).

Liu et al. (2010) found that the plasma membrane of Si-treated *P. digitatum* spores was damaged, suggesting that Si could play a crucial role as indicated by the effective control of GM on citrus fruit. In addition, Youssef et al. (2012) reported that pre or postharvest treatments with SSi effectively reduced decay caused by *P. digitatum* or *P. italicum* on oranges and mandarins.

SA has been shown to be a signaling molecule involved in both local defense reactions at infection sites and the induction of systemic resistance (Durner et al., 1997). Recently, Iqbal et al. (2012) revealed antifungal effects of SA treatments not only *in vitro* against *P. digitatum* and *P. italicum*, but also *in vivo* when the chemical was applied in citrus orchards. However, control of GM and BM was not achieved with SA postharvest treatments. Similar results were obtained in 'Ponkan' mandarins when SA at 400 mg L^{-1} was applied after harvest (Zheng and Zhang, 2004). On the other hand, ASA has been reported to control foliar and tuber diseases of potatoes when applied as foliar sprays or postharvest treatments (López-López et al., 1995; Bokshi et al., 2003).

Harpin is an acidic glycine-rich protein produced by the bacterium *Erwinia amylovora* that elicits a hypersensitive response in plants. Postharvest harpin treatments effectively suppressed citrus black spot caused by the quarantine pathogen *Guignardia citricarpa* on 'Valencia' oranges in Brazil (Lucon et al., 2010). Moreover, harpin significantly reduced decay caused by *P. expansum* on apple fruit cv. 'Red Delicious' when it was applied at 80 mg L^{-1} after harvest (de Capdeville et al., 2002). It was found that the level of resistance depended on the harpin, inoculum concentration, as well as duration between treatment and fungal inoculation (de Capdeville et al., 2003).

Limited information is available on the effect of these chemical resistance inducers applied on citrus fruit after harvest and particularly against citrus GM or BM. The objective of this study was to test both preventive (treatment before fungal inoculation) and curative (treatment after fungal inoculation) activities of selected chemical inducers against postharvest penicillium molds on orange fruit. Primary screenings to select the most effective chemicals and concentrations were performed *in vivo*. Most effective concentrations of the products were tested afterwards as dip treatment of oranges artificially inoculated with the pathogens.

2. Materials and methods

2.1. Fruit

The experiments were conducted with 'Valencia' and 'Lanelate' oranges (Citrus sinensis (L.) Osbeck) collected from commercial orchards in the Valencia area (Spain). The fruit were used the same day or stored up to 1 week at 5 °C and 90% relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with fresh water and allowed to air dry at room temperature.

2.2. Chemicals

Chemicals used in this research are listed in Table 1. BABA, INA, ASA and SA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MA, USA). SSi, harpin and BTH were obtained from Fisher Scientific (Loughborough, Leicestershire, UK), Ortoquel (Barcelona, Spain) and Syngenta Agro S.A. (Madrid, Spain), respectively. A sterile mother solution of each chemical was prepared. Sterile solutions at the desired concentrations were prepared by diluting with sterile water.

2.3. Fungal inoculum

P. digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co)

plates at 25 ºC. Conidia of each fungus from 7 to 14-d old cultures were taken from the plate surface with a sterile glass rod and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 spores mL^{-1} using a hemocytometer.

2.4. *In vivo* primary screenings

Seven synthetic chemicals were tested as resistance inducers at three concentrations of active ingredient. Each concentration was tenfold the previous one (Table 1). To test the preventive activity of the chemical inducers, 30 µL of the chemical solution at the desired concentration were placed, with a micropipette, in a 1 mm wide, 2 mm deep wound made with a stainless steel rod in the equatorial region of 'Valencia' or 'Lanelate' oranges (1 wound per fruit). After 24 h at room temperature, pathogen inoculation was performed by placing with a micropipette 30 µL of conidial suspension of *P. digitatum* or *P. italicum* in a new adjacent wound (about 2 mm of separation between wounds) because it was observed in preliminary trials that the original wounds were naturally healed during the time between treatment and inoculation, and the inoculated fungi were not able to infect the old wounds. To evaluate the curative activity of the chemical inducers, 30 µL of the chemical at the corresponding concentration were placed, using a micropipette, in the same inoculation rind wound about 24 h after the inoculation of the pathogen (1 wound per fruit). Control fruit were treated in both cases with 30 µL of sterile distilled water. Treated fruit were incubated at 20 ºC and 90% RH for 6 d, after which disease incidence (% of infected fruit) and severity (lesion diameter) were assessed. For each combination of chemical, concentration, pathogen and type of tested activity, 4 replicates of 5 oranges each were used. Experiments with ASA, BABA, INA, SSi and harpin were conducted with 'Valencia' oranges, whereas experiments with BTH and SA were performed with 'Lanelate' oranges.

2.5. Small-scale trials

Chemicals and concentrations selected according to the results obtained in the *in vivo* primary screenings were assayed in small-scale trials. Forty fruit per treatment (4 replicates of 10 fruit each) were arranged in plastic cavity sockets on cardboard trays in a complete randomized design. The selected chemical inducers and concentrations were: BABA at 0.3 mM (0.03 g L^{-1}), INA at 0.03 mM (0.0057 g L^{-1}), BTH at 0.9 mM (0.2 g L^{-1}) and SA at 0.25 mM (0.034 g L^{-1}).

Table 1. Characteristics of aqueous solutions of chemical resistance inducers tested against green and blue molds in *in vivo* primary screenings with 'Valencia' and 'Lanelate' oranges.

Resistance inducer	Abbreviation	Molecular formula	pH[a]	Tested concentrations (mM)
β-aminobutyric acid	BABA	$C_4H_9NO_2$	5.8	0.03, 0.3, 3
2,6-dichloroisonicotinic acid	INA	$C_6H_3Cl_2NO_2$	3.3	0.03, 0.3, 3
Sodium silicate	SSi	Na_2SiO_3	12.1	10, 100, 1000
Harpin	-	-[b]	6.8	8, 80, 800
Benzothiadiazole	BTH	$C_8H_6N_2OS_2$	6.9	0.09, 0.9, 9
Acetylsalicylic acid	ASA	$C_9H_8O_4$	3.2	0.2, 2, 20
Salicylic acid	SA	$C_7H_6O_3$	3.1	0.75, 1.5, 3

[a] pH at 20 ºC of solutions prepared at the second tested concentration of each chemical
[b] 44 kDa protein of *Erwinia amylovora*

Stainless steel buckets containing 10 L of aqueous solution of the selected chemical inducers at the desired concentrations were used in all tests. Fruit were placed into multi-perforated wall stainless steel containers, exactly fitting in the buckets mentioned above, and completely immersed in the treatment solution for 60 or 150 s at room temperature (20 °C) or heated to 50 °C. Solutions were heated by placing the buckets in a 250-L stainless steel water tank fitted with

two electrical resistances (4.5 kW each), a thermostat, and an automatic water-recirculating system. Control fruit were dipped in deionized water alone at 20 °C for 60 s. Both preventive (treatment before inoculation) and curative (treatment after inoculation) effects were determined.

To evaluate whether the selected chemical inducers showed a preventive effect against the development of GM and BM, various experiments were conducted with 'Lanelate' oranges in which a wound was made as described above. Thereafter, the treatments were applied by dipping the fruit in the corresponding chemical solution. About 2-3 h later, when the oranges had been air-dried at room temperature, fruit were inoculated with the pathogens as previously described in a new adjacent wound (about 2 mm of separation between wounds). To evaluate whether the selected chemical inducers showed a curative effect for the control of GM and BM, fruit were wound inoculated once in the rind with *P. digitatum* or *P. italicum* at 10^5 spores mL^{-1} as previously described. About 24 h later, the treatments were applied by dipping the fruit in the corresponding inducer solution. Treated fruit were incubated at 20 ºC and 90% RH for 7 d, at which time disease incidence (% of infected fruit) and severity (lesion diameter) were assessed. Also, fruit were inspected 2-3 d after treatment to detect possible phytoxicities on the fruit rind provoked by the treatments. Experiments were repeated twice.

2.6. Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus, version 5.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence were transformed to the arcsine of the square root of the proportion of infected fruit to assure the homogeneity of variances. In some cases, reductions with respect to the control treatments were calculated as percentages. Statistical significance was judged at the level $P = 0.05$. On small-scale trials, results are means from two repeated experiments. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was applied to separate means. Shown values are non-transformed means.

3. Results

3.1. *In vivo* primary screenings

3.1.1. Preventive activity

Treatments with ASA at 0.2, 2 or 20 mM had no protective action on GM and BM compared to control fruit in *in vivo* primary screenings on 'Valencia' oranges treated. Surprisingly, the incidence and development of both molds were favored by these treatments at increasing ASA concentrations (Table 2).

In a second experiment, BABA treatments did not reduce the incidence or severity of GM on 'Valencia' oranges, but significantly reduced the incidence of BM when applied at 0.3 and 3 mM (reductions by 39 and 26%, respectively, compared to water-treated control fruit, Table 2). BM severity on treated oranges was generally lower than on control fruit, but only significantly so with BABA at 0.3 mM (lesion diameters of 12 mm). In this experiment, INA treatments significantly reduced GM and BM, with the exception of 3 mM. The incidence of GM and BM was significantly reduced by 25 and 17% and 60 and 53%, respectively, by INA treatments at 0.03 and 0.3 mM. Furthermore, INA treatments at these concentrations significantly reduced the severity of GM and BM, from lesion diameters of 63 and 20 mm on control fruit to lesions of 37 and 9 mm, respectively (Table 2).

In a third experiment with 'Valencia' oranges, the incidence of GM and BM was significantly reduced by 89 and 71%, respectively, by SSi treatment at 1,000 mM. Likewise, this treatment reduced the severity of GM and BM from control lesion diameters of 56 and 18 mm to diameters of 9 and 7 mm, respectively. However, treatment with SSi at this concentration left apparent chemical residues on the rind area surrounding the inoculation site (Table 2). In this experiment, harpin treatments at all three concentrations were incapable to reduce both GM and BM on 'Valencia' oranges inoculated 24 h after treatment (Table 2).

Table 2. Preventive activity of chemical resistance inducers at three different concentrations against green and blue molds in *in vivo* primary screenings with 'Valencia' and 'Lanelate' oranges.

Experiment Orange cultivar	Chemical resistance inducers	Concentration (mM)	Green mold		Blue mold	
			Incidence (%)	Severity (mm)	Incidence (%)	Severity (mm)
Experiment 1 'Valencia'	Control	-	75 a	68 a	80 a	22 a
	ASA	0.2	80 a	65 a	65 a	16 a
		2	95 a	68 a	85 a	17 a
		20	100 a	81 a	100 b	29 b
Experiment 2 'Valencia'	Control	-	95 b	63 b	95 c	20 bc
	BABA	0.03	80 ab	49 ab	80 bc	15 ab
		0.3	80 ab	50 ab	60 ab	12 ab
		3	80 ab	51 ab	70 ab	14 ab
	INA	0.03	70 a	37 a	40 a	9 a
		0.3	79 ab	41 ab	45 a	11 a
		3	95 b	56 b	100 c	28 c
Experiment 3 'Valencia'	Control	-	85 b	56 bc	75 bc	18 bc
	SSi	10	90 b	79 c	55 bc	9 ab
		100	80 b	61 bc	50 ab	12 abc
		1000*	10 a	9 a	20 a	7 a
	Harpin	8	100 b	79 c	85 bc	22 c
		80	83 b	49 b	85 c	19 bc
		800	80 b	66 bc	61 bc	17 abc

Experiment Orange cultivar	Chemical resistance inducers	Concentration (mM)	Green mold		Blue mold	
			Incidence (%)	Severity (mm)	Incidence (%)	Severity (mm)
Experiment 4	Control	-	100 b	53 bc	90 a	22 bc
'Lanelate'	BTH	0.09	85 ab	43 ab	90 a	19 b
		0.9	80 a	32 a	90 a	17 ab
		9	95 ab	52 bc	95 a	15 ab
	SA	0.75	95 ab	54 bc	70 a	10 a
		1.5	90 ab	50 abc	80 a	21 bc
		3	95 ab	62 c	85 a	27 c

Oranges were wounded, treated with acetylsalicylic acid (ASA), *β*-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), sodium silicate (SSi), harpin, benzothiadiazole (BTH), or salicylic acid (SA), artificially inoculated 24 h later with *Penicillium digitatum* or *P. italicum*, and incubated for 6 d at 20 °C and 90% RH. For each experiment, mean values followed by different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence was arcsine transformed. Non-transformed means are shown.

[*] Presence of SSi residues around the inoculation site.

In a fourth experiment, BTH treatment significantly induced resistance against GM on 'Lanelate' oranges only when applied at 0.9 mM, with an incidence reduction of 20% with respect to the control treatment (GM incidence of 100%). Likewise, GM severity was significantly reduced by this treatment, from lesion diameters of 53 mm on the control treatment to lesions of 32 mm on treated oranges. BM was not reduced by BTH treatments (Table 2). None of the SA treatments showed significant preventive activity against neither GM nor BM on 'Lanelate' oranges after 6 d of incubation at 20 °C and 90% RH (Table 2).

3.1.2. Curative activity

In general, the chemicals ASA, BABA, INA, harpin and SA were ineffective at all tested concentrations to control both GM and BM in *in vivo* primary screenings with 'Valencia' oranges inoculated 24 h before the treatments and incubated at 20 °C and 90% RH for 6 d (Table 3). In contrast, SSi treatment at 1,000 mM significantly reduced the incidence of GM and BM by 26 and 44%, respectively, and the severity of these molds from 55 and 29 mm to 30 and 5 mm, respectively. However, apparent SSi residues were noticed around the inoculation site after this treatment (Table 3). BTH treatments did not affect the incidence of both GM and BM on 'Lanelate' oranges, but treatments at 0.09 and 9 mM significantly reduced GM severity from 79 mm on control fruit to 62 mm on treated fruit (Table 3).

Table 3. Curative activity of chemical resistance inducers at three different concentrations against green and blue molds in *in vivo* primary screenings with 'Valencia' and 'Lanelate' oranges.

Experiment Orange cultivar	Chemical resistance inducers	Concentration (mM)	Green mold		Blue mold	
			Incidence (%)	Severity (mm)	Incidence (%)	Severity (mm)
Experiment 1 'Valencia'	Control	-	75 a	55 bc	90 a	26 a
	ASA	0.2	100 b	43 b	90 a	20 a
		2	95 b	60 c	90 a	25 a
		20	60 a	22 a	80 a	18 a
Experiment 2 'Valencia'	Control	-	100 a	65 ab	100 a	34 ab
	BABA	0.03	90 a	61 a	95 a	36 b
		0.3	100 a	79 b	95 a	32 ab
		3	95 a	63 a	100 a	33 ab
	INA	0.03	100 a	76 ab	95 a	32 ab
		0.3	95 a	77 ab	95 a	28 a
		3	100 a	79 b	100 a	35 b
Experiment 3 'Valencia'	Control	-	85 ab	55 b	85 b	29 bc
	SSi	10	95 b	60 b	90 b	23 b
		100	85 ab	55 b	85 b	24 b
		1000*	65 a	30 a	45 a	5 a
	Harpin	8	90 b	54 b	90 b	24 bc
		80	95 b	54 b	100 b	28 bc
		800	100 b	60 b	90 b	31 c

Experiment Orange cultivar	Chemical resistance inducers	Concentration (mM)	Green mold		Blue mold	
			Incidence (%)	Severity (mm)	Incidence (%)	Severity (mm)
Experiment 4 'Lanelate'	Control	-	100 b	79 c	100 a	31 a
	BTH	0.09	90 b	62 ab	95 a	32 a
		0.9	100 b	68 abc	95 a	28 a
		9	100 b	62 ab	100 a	27 a
	SA	0.75	95 ab	56 a	100 a	32 a
		1.5	100 b	76 c	100 a	31 a
		3	100 b	73 bc	95 a	28 a

Oranges were artificially wound inoculated with *Penicillium digitatum* or *P. italicum*, treated with acetylsalicylic acid (ASA), *β*-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), sodium silicate (SSi), harpin, benzothiadiazole (BTH), or salicylic acid (SA) 24 h after inoculation, and incubated for 6 d at 20 °C and 90% RH. For each experiment, mean values followed by different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence was arcsine transformed. Non-transformed means are shown.

* Presence of SSi residues around the inoculation site.

3.2. Small-scale dip trials with selected inducers

According to the results obtained in the *in vivo* primary screenings, INA at 0.03 mM, SA at 0.25 mM, BABA at 0.3 mM, and BTH at 0.9 mM were chosen as the best treatments to be assayed in preventive and curative postharvest dips in small-scale trials.

3.2.1. Preventive activity

In general, dips with INA, SA and BABA at 0.03, 0.25 and 0.3 mM, respectively, applied at 20 °C for 60 or 150 s did not significantly induce resistance against GM and BM on 'Lanelate' oranges treated, inoculated 2-3 h later, and incubated for 7 d at 20 °C and 90% RH. Only the severity of GM was significantly reduced when 0.03 mM INA was applied for 150 s (Fig. 1). Similarly, BTH at 0.9 mM applied at 20 or 50 °C for 60 or 150 s did not show preventive activity against GM and BM on 'Lanelate' oranges treated, inoculated 2-3 h later, and incubated for 7 d at 20 °C (Fig. 2).

3.2.2. Curative activity

Dips at 20 °C for 60 s in aqueous solutions of INA at 0.03 mM and BABA at 0.3 mM were more effective than dips in SA at 0.25 mM to reduce the development of GM or BM on 'Lanelate' oranges treated 24 h after the inoculation of the pathogens and incubated for 7 d at 20 °C and 90% HR. Nevertheless, these treatments only reduced the severity of GM and BM by 2 and 20%, and 12 and 14%, respectively (Fig. 3).

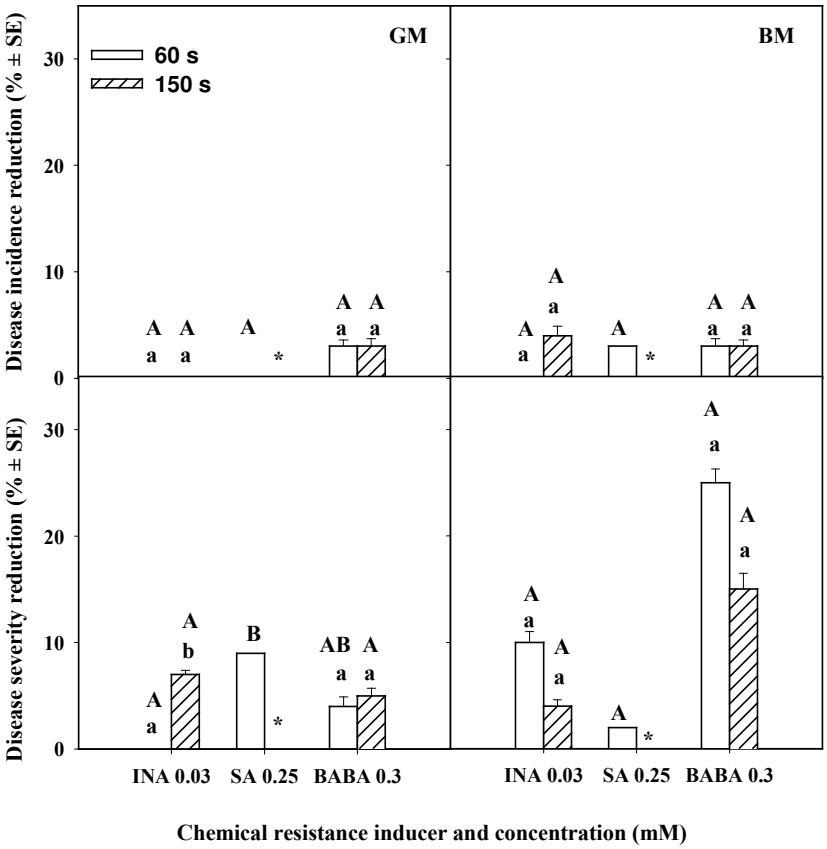

Figure 1. Preventive activity of chemical inducers against green (GM) and blue (BM) molds on wounded 'Lanelate' oranges dipped for 60 or 150 s at 20 °C, artificially inoculated 2-3 h later with *Penicillium digitatum* or *P. italicum*, and incubated for 7 d at 20 °C and 90% RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water for 60 s (incidence of 98-100% and 94-95% for GM and BM, respectively, and severity of 105-146 mm and 46 mm for GM and BM, respectively). For each mold, columns with different lowercase and capital letters indicate significantly different dip time and chemical treatment, respectively, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence data were arcsine transformed. Non-transformed means from two repeated experiments are shown. Asterisks indicate non-registered data.

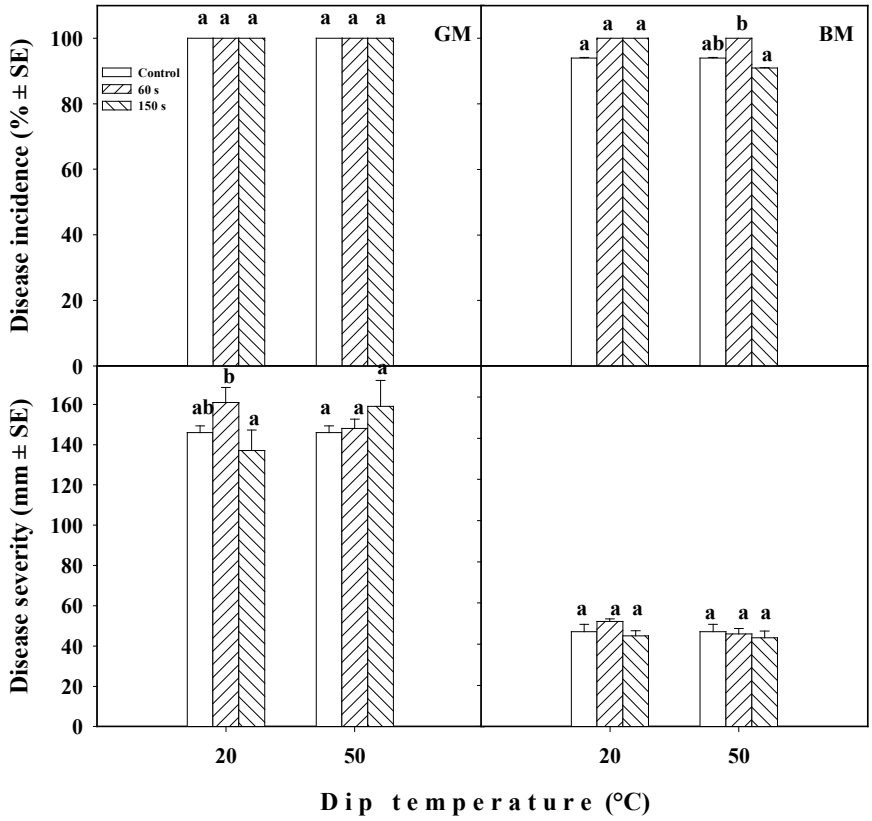

Figure 2. Preventive activity of BTH at 0.9 mM against green (GM) and blue (BM) molds on wounded 'Lanelate' oranges dipped at 20 or 50 °C for 60 or 150 s, artificially inoculated 2-3 h later with *Penicillium digitatum* or *P. italicum*, and incubated for 7 d at 20 °C and 90% RH. Control fruit were treated with water for 60 s. For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence data were arcsine transformed. Non-transformed means from two repeated experiments are shown.

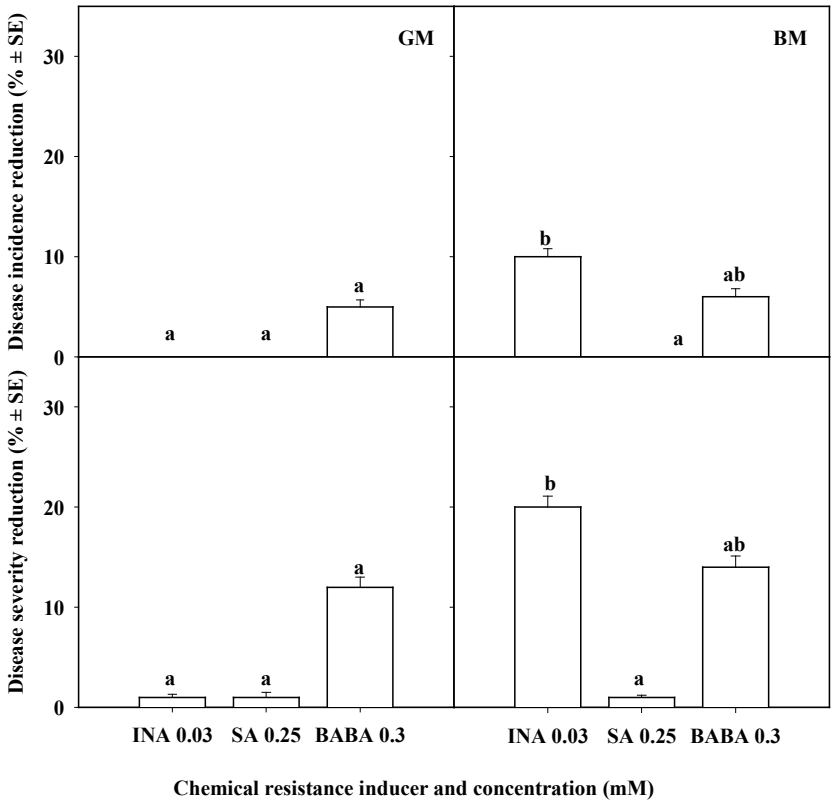

Figure 3. Curative activity of chemical inducers against green (GM) and blue (BM) molds on 'Lanelate' oranges artificially wound inoculated with *Penicillium digitatum* or *P. italicum*, dipped for 60 s at 20 °C about 24 h after inoculation, and incubated for 7 d at 20 °C and 90% RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water (incidence of 95-100 and 88-94% for GM and BM, respectively, and severity of 87-129 mm and 39-46 mm for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence data were arcsine transformed. Non-transformed means from two repeated experiments are shown.

Dips with BTH at 0.9 mM at 20 °C for 60 or 150 s had no significant effect on the development of GM and BM on 'Lanelate' oranges treated 24 h after fungal inoculation. In contrast, 150 s dips at 50 °C significantly reduced the incidence of GM and BM by 10 and 49%, respectively, and they were superior to 60 s dips at this temperature (Fig. 4).

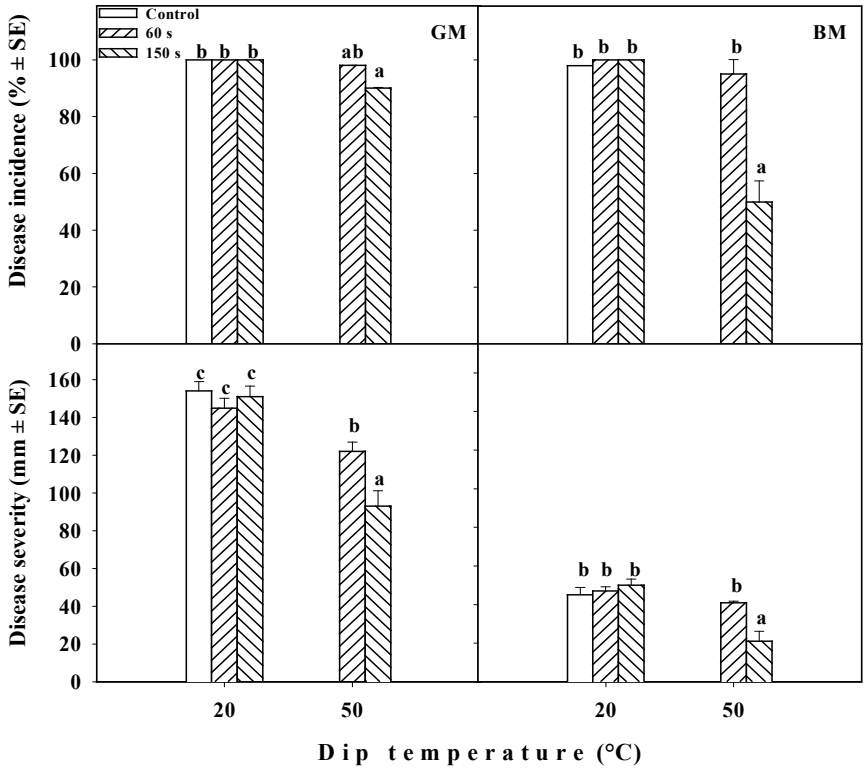

Figure 4. Curative activity of BTH at 0.9 mM against green (GM) and blue (BM) molds on 'Lanelate' oranges artificially wound inoculated with *Penicillium digitatum* or *P. italicum*, dipped at 20 or 50 °C for 60 or 150 s about 24 h after inoculation, and incubated for 7 d at 20 °C and 90% RH. Control fruit were treated with water for 60 s at 20 °C. For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence data were arcsine transformed. Non-transformed means from two repeated experiments are shown.

4. Discussion

This study reports that four (SSi, INA, BABA and BTH) of seven chemicals in a primary screened *in vivo* with 'Valencia' or 'Lanelate' oranges, applied before fungal inoculation (preventive activity), significantly induced resistance against GM and BM at least at one of the assayed concentrations. Reductions of incidence compared with the control treatments ranging from 15 to 89% and 26 to 71%, respectively. The results showed that the chemicals induced resistance in a concentration-dependent manner, and they were more effective at a certain concentration. Therefore, the inducers tested here acted probably more as plant growth regulators, which are typically more effective when applied at an optimal concentration, than as conventional fungicides, whose efficacy gradually increases as the application dose increases.

It was found in this study that the effectiveness of SSi at 1,000 mM to prevent GM and BM caused by *P. digitatum* and *P. italicum*, respectively, on 'Valencia' orange fruit (incidence reduction of 89 and 71%, respectively) was superior to that reported after treatment of citrus fruit (Liu et al., 2010; Youssef et al., 2012). It has been proposed that the mode of action of Si treatments is through a direct effect on the pathogen. Liu et al. (2010) found that the plasma membrane of Si-treated *P. digitatum* spores was damaged, leading to higher leakage of protein and sugar. However, other research works contradicted this hypothesis and attributed the antifungal action of Si to indirect effects to the fruit host. Hereto, Si can play an important role in the formation of physical and mechanical barriers to the penetration of pathogens at the cell wall level (Datnoff et al., 2001; Buonaurio et al., 2009). Si treatments may also act by eliciting biochemical defense reactions, including the accumulation of lignin, phenolic compounds and pathogenesis-related (PR) proteins in the infected plants (Epstein, 1999). In any case, the treatment with SSi at 1,000 mM was not selected for further research due to the presence of chemical residues on the fruit rind and potential phytotoxicity.

The antimicrobial activity of INA in field conditions has been demonstrated in cotton against diseases such as alternaria leaf spot (Alternaria macrospora), bacterial blight (Xanthomonas campestris pv.

malvacearum) or verticillium wilt (Verticillium dahliae) (Colson-Hanks et al., 2012). The inhibition of APX activity with consequent enhanced levels of H_2O_2 and activation of defense-related genes was proposed as a major mode of action for INA (Durner and Klessig, 1995). Previous studies have addressed the induction by INA of pathogen resistance in vegetative parts of the plant, whereas in the present study we found that INA may also increase disease resistance in reproductive organs, such as the mature rind of citrus fruit. INA at concentrations of 0.03 and 0.3 mM effectively induced resistance against GM or BM, with mold incidence reductions ranging from 17 to 60%.

In this work, BM incidence reduction (39%) by BABA on 'Valencia' orange fruit was inferior to that observed after BABA treatments on grapefruits (Porat et al., 2003) and sweet oranges (Tavallali, 2008). However, the concentration used here (0.3 mM) was much lower than that used in other studies. Postharvest BABA treatments at 0.3, 3 or 30 mM had no effect on the incidence and severity of GM on 'Valencia' oranges. However, Porat et al. (2003) found that BABA applied at 20 mM reduced GM development by 65% on treated grapefruits. These researchers and others (Jakab et al., 2001) attributed the induction of resistance by BABA treatments to different mechanisms that occur in grapefruit peel tissue, including the activation of chitinase gene expression, the biosynthesis of pathogen-related proteins or an increase in PAL activity. On the other hand, according to Ton and Mauch-Mani (2004), BABA-induced resistance against necrotrophic pathogens is based on primed callose accumulation as a response to fungal infection.

With the exception of SSi at 1,000 mM (incidence reductions of GM and BM of 26 and 44%, respectively, on fruit treated 24 h after fungal inoculation), the chemical inducers had no curative effect in *in vivo* primary screenings against GM and BM on `Valencia' and 'Lanelate' oranges, which indicated the lack of a direct antimicrobial activity against the fungi. The results on the curative activity of SSi are in agreement with findings reported by Liu et al. (2010) working with *P. digitatum*-infected citrus fruit, and Tian et al. (2005) working with jujube fruit infected with *A. alternata* or *P. expansum*.

Postharvest dips at room temperature for 60 s with chemical inducers selected in *in vivo* primary screenings (INA at 0.03 mM, SA at 0.25 mM and BABA at 0.3 mM) had no effect or only reduced very slightly the incidence and severity of GM and BM, irrespective of whether the dips were conducted before (preventive activity) or after (curative activity) the inoculation with the pathogens. In general, the effectiveness of treatments with chemical inducers applied as preventive aqueous dips was considerably lower than that obtained in *in vivo*. The main reason that may account for these differences is the longer contact time between the chemical inducer and the wounded rind tissues in the case of the primary screenings (air-dried drop) in comparison with the dips (immersion for 60 or 150 s).

Given prior literature references, one of the most unexpected results from our trials was the lack of activity of dip treatments with SA to control either GM or BM. Findings reported by Iqbal et al. (2012) showed that SA at 6 mM significantly inhibited in *in vitro* conditions the radial growth, sporulation and spore germination of both *P. digitatum* and *P. italicum*. Furthermore, these workers showed that SA applications at 8 and 10 mM reduced the severity and incidence of GM on artificially inoculated 'Lanelate' oranges compared to control fruit after 7 d of incubation at 25 °C. Similar good performance of SA treatments on the reduction of citrus penicillium decay has been additionally reported by Zheng and Zhang (2004) with 'Ponkan' mandarins or, more recently, by Aminifard et al. (2013) with 'Moro' blood oranges. The mechanism by which SA induce SAR to treated plants has not been completely elucidated yet, but one of the possible modes of action is the inhibition of the H_2O_2-degrading activity of catalases, thereby leading to an increase in the endogenous levels of H_2O_2, which is generated by photorespiration, photosynthesis, oxidative phosphorylation and the hypersensitive response-associated oxidative burst. H_2O_2, and other reactive oxygen species derived from it, are believed to serve as activators of the expression of plant defense-related genes (Durner et al., 1997; Vlot et al., 2009).

In our experimental conditions, dips with BTH at 0.9 mM did not show preventive activity against the molds on 'Lanelate' oranges and showed limited curative activity only when applied at 50 °C. This

poor performance of postharvest BTH dips is in agreement with research results obtained by Quaglia et al. (2011) with apples, which revealed that the accumulation of PR proteins activated following BTH treatments were ineffective against postharvest blue mold caused by *P. expansum*. Moreover, the lack of protection against fungal infection despite PR protein accumulation induced by treatments with BTH or other chemical inducers has previously been reported for other plant-pathogen interactions (Van Loon, 1997). Nevertheless, other researchers reported significant disease reductions after postharvest BTH treatments. Liu et al. (2005) found a reduction of up to 50% of incidence of disease caused by *P. expansum* on peaches treated with BTH at 200 mg L^{-1}. In this case, however, BTH dips were applied for 5 min and performed 60 h before artificial fungal inoculation. The authors discussed that BTH might increase the disease resistance of peach fruit by enhancing their antioxidant systems and their free radical-scavenging capabilities. Similar observations were recently made by Cao et al. (2011) after treating harvested strawberries with BTH.

In spite of some promising results obtained in *in vivo* primary screenings in the present study, in our experimental conditions postharvest dip treatments with chemical resistance inducers did not satisfactorily reduce decay caused by *P. digitatum* and *P. italicum* on citrus fruit after harvest. Therefore, such treatments are not cost-effective and they cannot be recommended for inclusion in commercial decay management programs for citrus packinghouses. However, before the current needs for non-contaminant postharvest decay control, a new research direction worthy to explore is the application of chemical inducers in citrus groves during fruit developing stages to test if resistance to postharvest diseases are effectively induced in the fruit tissues. Extensive research with a variety of horticultural commodities indicates that significant benefits in terms of disease reduction may be obtained from field treatments with chemical elicitors (Terry and Joyce, 2004; Cao and Jiang, 2006; Buonaurio et al., 2009; Iqbal et al., 2012; Youssef et al., 2012; Feliziani et al., 2013). In general, resistance to plant pathogens are more easily induced in vegetative parts of the plant than in reproductive portions, such as the rind tissues of citrus fruit (Porat et al., 2003). On the other hand, the metabolic activity of the entire plant

might result in the production of ISR or SAR, and in addition the higher metabolic activity of the fruit developing on the tree in comparison to harvested fruit might significantly improve the production of LAR.

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CAPÍTULO 3

Preventive and curative activity of postharvest potassium silicate treatments to control green and blue molds on orange fruit

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

Preventive and curative antifungal activity of postharvest treatments with potassium silicate (PSi) against green (GM) and blue (BM) molds were evaluated on oranges (cvs. 'Valencia' or 'Lanelate') artificially inoculated in rind wounds with *Penicillium digitatum* and *P. italicum*, respectively. The most effective PSi concentration, the effect of fungal inoculum concentration and the influence of temporal and spatial factors on antifungal activity were assessed in *in vivo* primary screenings. After 6 days of incubation at 20°C, significant preventive (treatment before fungal inoculation) and curative (treatment after inoculation) activity was observed with PSi at 90 mM (GM and BM incidence reductions of 23 and 52%, and 23 and 40%, respectively). In preventive tests, the effectiveness of PSi was influenced by inoculum concentration (10^3, 10^4, 10^5, or 10^6 spores mL^{-1}), but not by the distance between treatment and inoculation sites (10, 20 or 30 mm). PSi applied about 2 h before inoculation showed higher preventive activity than applied before 24, 48 or 96 h. In order to determine the best dip treatment conditions, PSi at 90 mM was tested at 20 or 50°C for 60 or 150 s in small-scale trials with 'Lanelate' oranges artificially inoculated before or after the treatment and incubated for 7 days at 20°C. Dips at 20°C for 60 s were selected and subsequently applied on inoculated 'Valencia' oranges stored at 5°C and 90% RH for up to 6 weeks. Curative postharvest dips effectively reduced the incidence and severity of both GM and BM during cold storage, while preventive dips significantly reduced the severity but not the incidence. Overall, postharvest PSi treatments showed potential as a new tool to be part of non-polluting strategies to control penicillium decay of citrus fruit.

Key words *Penicillium digitatum*, *Penicillium italicum*, Citrus sinensis, silicon, alternative postharvest disease control

Introduction

Green (GM) and blue (BM) molds caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *P. italicum* Wehmer, respectively, are major diseases responsible for postharvest losses of citrus fruit

worldwide (Palou et al. 2008). In spite of the application of fungicides and the increasing implementation of some alternative control strategies, the high incidence of GM and BM still accounts for important economical problems on stored citrus commodities. Therefore, it is necessary to further develop alternative approaches and novel technologies, including physical, chemical and biological control methods, for a cost-effective integrated control of these molds (Droby et al. 2009; Palou et al. 2008).

Silicon (Si) is considered a functional plant nutrient that plays an important role as a component of cell walls (Laing et al. 2006). In agriculture, the interest of Si is multiple because has shown value as a fertilizer and for pest and disease control. In addition, some positive effects on the reduction of physiological disorders have also been described. In the particular case of citrus fruit, it was found that postharvest Si treatments could alleviate chilling injury, especially on long-term cold-stored lemons (Mathaba et al. 2009). The earliest scientific works on the role of Si in plant disease control were reported in the 1920s and 1930s. Wagner (1940) was the first to report an interaction between Si fertilization and the incidence of cucumber powdery mildew. In Europe, more recent research demonstrated that the addition of potassium silicate (PSi; K_2SiO_3) to nutrient solutions reduced the incidence of powdery mildew and stem lesions caused by the pathogen *Botrytis cinerea* on cucumber plants (O'Neill 1991). While the role of silicified cell walls in protecting plants against pathogens may not be completely discarded, other results suggest that Si acts in the host tissue by affecting the signals between host and pathogen, resulting in a more rapid and extensive activation of plant defense mechanisms (Chérif et al. 1992a,b, 1994; Samuels et al. 1991). The specific mechanisms responsible for the protection of plants from fungal diseases by Si are not well understood. Si may act by eliciting biochemical defense reactions, including the accumulation of lignin, phenolic compounds, and pathogenesis-related proteins in infected plants (Chérif et al. 1992a; Epstein 1999). Results by Shen et al. (2010) suggested that reductions in fungal disease after treatment of field plants with low concentrations of PSi were probably not due to fungistatic effects of Si, but rather to other mechanisms such as Si acting as a physical barrier against pathogen penetration or Si-induced defense response in plants.

In contrast to PSi, whose antifungal activity has been practically assessed only as field treatments for herbaceous crops, another Si salt, sodium silicate (SSi), has been tested as postharvest treatments for some fruit or vegetable commodities. Bi et al. (2006) found that SSi at 100 mM was more effective than SSi at 25 or 50 mM to control diseases caused by *Alternaria alternata*, *Fusarium semitectum*, and *Trichothecium roseum* on artificially inoculated Hami melon fruit; SSi at 200 mM was phytotoxic. Furthermore, SSi treatments applied at 100 mM before inoculation with *T. roseum* induced lower decay incidence and severity on melons than treatments applied after fungal inoculation. According to these workers, the protection provided by SSi treatments was correlated with the activation of two families of defense-related enzymes, peroxidase and quitinase. In further research with melons, Guo et al. (2007) reported that SSi and silicon oxide, both applied as postharvest dips, reduced the severity of pink rot, caused by *T. roseum*, on Chinese cantaloupes, with lesion diameters reduced by up to fivefold when compared with the controls. In the same work, the effectiveness of SSi increased at higher concentrations and the growth of the fungus was completely inhibited at 100 mM. Few research results are available on the activity of SSi against citrus postharvest pathogens. Liu et al. (2010) found that the plasma membrane of Si-treated spores of *P. digitatum* was damaged, which suggested that Si played a crucial role as antifungal agent. This author showed that treatments with SSi significantly controlled GM caused by *P. digitatum* on citrus fruit. In addition, Youssef et al. (2012) recently reported that both preharvest and postharvest treatments with SSi were effective against *P. digitatum* or *P. italicum* on oranges and mandarins. Furthermore, we observed very recently that postharvest treatments with SSi significantly reduced penicillium molds on oranges artificially inoculated with *P. digitatum* or *P. italicum* about 24 h after treatment. However, these treatments left visible salt residues and were potentially phytotoxic to the fruit rind (Moscoso-Ramírez and Palou 2013).

Before the promising activity of Si, applied as SSi, against some important postharvest diseases and the lack of information on the performance of PSi, this research focused on the evaluation of the antifungal properties of postharvest treatments with PSi against GM and BM. Particularly, the objectives of this study were to: i) determine

in *in vivo* primary screenings the range of effective PSi concentrations for optimization of both preventive and curative activities of PSi against GM and BM on orange fruit, ii) determine the influence of temporal and spatial factors on the preventive activity of PSi against *P. digitatum*, iii) optimize the conditions for postharvest dip treatments with PSi, and iv) evaluate the effectiveness of postharvest PSi treatments during prolonged cold storage of oranges.

Material and methods

Fruit

The experiments were carried out with 'Valencia' and 'Lanelate' oranges (Citrus sinensis (L.) Osbeck) collected from commercial orchards in the Valencia area (Spain). The fruit were used the same day or stored up to 1 week at 5°C and 90% relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with fresh water and allowed to air dry at room temperature.

Fungal inoculum

P. digitatum and *P. italicum* , isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25ºC. Conidia of each fungus from 7- to14-days-old were taken from the agar surface with a sterile glass rod and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 spores ml^{-1} using a haemocytometer.

Determination of potassium silicate concentration

In vivo primary screenings were used to establish the most appropriate concentration for both preventive and curative activity of PSi against GM and BM. For preventive activity, 30 µl of solution of PSi (K_2SiO_3; syns.: potassium salt of silicic acid, potassium metasilicate; Sil-MATRIX®, 29.1% a.i. PSi, PQ Corporation, Valley Forge, Pennsylvania, USA) at 0.9, 9 or 90 mM a.i. were placed, using a micropipette, in a 1 mm wide, 2 mm long, 1 mm diameter wound made with a stainless steel rod on the equatorial region of 'Valencia' oranges. About 24 h later, 30 µl of conidial suspension of *P. digitatum* or *P. italicum* were placed, using a micropipette, in a new identical wound adjacent to the first one (about 1-2 mm of separation between wounds). No good decay rate had been obtained in control fruit in previous experiments when the fungal inoculation had been performed in the same wound inflicted 24 h before, probably due to wound lignification during this period of time. To evaluate the curative activity, 30 µl of PSi solution at 0.9, 9, 30, 90 or 150 mM were placed, using a micropipette, in the same inoculation rind wound about 24 h after the inoculation of the pathogen. Control fruit were treated with 30 µl of sterile distilled water. In all cases, 4 replicates of 5 oranges each were used for each treatment. Treated fruit were stored at 20ºC and 90% RH for 6 days, at which time disease incidence (% of infected fruit) and severity (lesion diameter) were evaluated.

Influence of inoculum concentration

To evaluate the influence of inoculum concentration of *P. digitatum* on the preventive or curative activity of PSi, concentrations of 10^3, 10^4, 10^5 and 10^6 spores ml^{-1} of *P. digitatum* were prepared following the procedure described above. Thirty µl of PSi solution at 90 mM (13.9 g l^{-1}) were placed, using a micropipette, in a 1 mm wide, 2 mm long, 1 mm diameter wound made with a stainless steel rod on the equatorial region of 'Lanelate' oranges. For preventive and curative activity, 30 µl of conidial suspension of *P. digitatum* at the desired inoculum concentration were placed, using a micropipette, in the same rind wound about 2 h after and before, respectively. Control fruit were treated with 30 µl of sterile distilled water. Each treatment was applied to 4 replicates of 5 oranges each. Treated fruit were stored

at 20ºC and 90% RH for 6 days, at which time disease incidence and severity were recorded. The experiment was repeated once.

Assessment of temporal and spatial characteristics of preventive activity

An experiment with 'Valencia' oranges was conducted in order to temporally characterize the preventive activity of PSi. Thirty µl of PSi aqueous solution at 90 mM were placed, using a micropipette, in a 1 mm wide, 2 mm long, 1 mm diameter wound made with a stainless steel rod in the equator of the fruit. Subsequently, 30 µl of a 10^5 spores ml^{-1} *P. digitatum* conidial suspension were pipetted about 2, 24, 48 or 96 h after treatment in a new adjacent wound (about 2 mm of separation between wounds). Each treatment was applied to 4 replicates of 5 oranges each. Treated fruit were stored at 20ºC and 90% RH for 6 days, at which time disease incidence and severity were evaluated. The experiment was repeated once.

Another experiment was designed to evaluate the spatial influence on the preventive activity of PSi. Fifty µl of PSi aqueous solution at 90 mM were pipetted in a wound made with a stainless steel rod in the fruit equatorial region as described above. About 2 h after treatment, 30 µl of a 10^4 spores ml^{-1} *P. digitatum* conidial suspension were placed in a new rind wound inflicted at a distance of 10, 20 or 30 mm from the initial treatment wound. Control fruit were treated with sterile distilled water and then inoculated in the same rind wound. Four replicates of 5 oranges each were used per treatment. Treated fruit were stored at 20ºC and 90% RH for 6 days, at which time disease incidence and severity were recorded. The experiment was repeated once.

Determination of dip treatment conditions

Small-scale trials were conducted using 'Lanelate' oranges to establish the best dip treatment conditions to resemble potential commercial applications in citrus packinghouses. Fungal inoculation in one rind wound per fruit at a concentration of 10^5 spores ml^{-1} of *P. digitatum* or *P. italicum* was carried out following the procedure previously described. Dips were performed in stainless steel buckets

containing 10 L of 90 mM aqueous solution of PSi. This concentration of PSi was selected according to previous results obtained in the *in vivo* primary screenings. When needed, PSi solutions were heated by placing the buckets in a 250-L stainless steel water tank fitted with two electrical resistances (4.5 kW each), a thermostat, and an automatic water-recirculating system. Fruit were placed into 18 L multi-perforated wall stainless steel containers, exactly fitting in the above-mentioned buckets, and completely immersed in the treatment solution for 60 or 150 s at 20 or 50°C. To assess preventive activity, dips were performed about 2 h before fungal inoculation and control fruit were dipped for 60 s in water alone at both 20 and 50°C. To assess curative activity, dips were performed about 24 h after fungal inoculation and control fruit were dipped in water alone at 20°C for 60 s. After treatment, fruit were not rinsed with tap water. Forty fruit per treatment (4 replicates of 10 fruit each) were arranged in plastic cavity sockets on cardboard trays. Treated fruit were incubated at 20°C and 90% RH. Disease incidence and severity were assessed after 7 days of incubation. Potential fruit phytotoxicity caused by PSi or heat was visually assessed after 3 days at 20°C. For this purpose, fruit were classified into one of four categories, depending on rind appearance: 0 = no rind damage; 1 = slight brownish blemishes present (<10% fruit surface); 2 = moderate brownish blemishes present (10% <fruit surface<25%) and 3 = severe rind injury (>25% fruit surface). A ponderate rind pitting index (0–3 scale) was calculated for each treatment. These trials were performed twice.

Effectiveness on long-term cold-stored fruit

Both preventive and curative activities of postharvest PSi dips against GM and BM were evaluated on 'Valencia' oranges subjected to long-term cold storage. Stainless steel buckets containing 10 L of 90 mM PSi aqueous solution were used to dip the fruit at 20ºC for 60 s. Fruit were inoculated and treated or vice versa as described before and stored up to 6 weeks at 5ºC and 90% RH in a cold room in the IVIA CTP facilities. Control fruit were dipped in water alone at 20°C for 60 s. Forty fruit per treatment (4 replicates of 10 fruit each) were used. Disease incidence and severity were assessed after 2, 4, and 6 weeks at 5ºC.

Statistical analysis

Data were analyzed by analyses of variance (ANOVA) with Statgraphics software (Statgraphics Plus, v. 5.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence were transformed to the arcsine of the square root of the proportion of infected fruit to assure the homogeneity of variances. In some cases, reductions with respect to the control treatments were calculated as percentages. Statistical significance was judged at the level $P = 0.05$. Unless otherwise stated, results are means from two repeated experiments. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was applied to separate means. Shown values are non-transformed means.

Results

Potassium silicate concentration

Among the concentrations of PSi evaluated in this set of *in vivo* experiments to prevent the molds, the concentration of 90 mM significantly inhibited the development of GM and BM on 'Valencia' oranges and was clearly superior to the concentrations of 0.9 and 9 mM (Fig. 1). The incidence of GM and BM was reduced by 23 and 52%, respectively, by PSi at 90 mM after 6 days of incubation at 20°C. Furthermore, PSi at 90 mM effectively reduced the severity of GM and BM by 74 and 67%, respectively (Fig. 1).

In the curative tests, the most effective treatment was also PSi at a concentration of 90 mM, which significantly reduced the incidence of GM and BM on 'Valencia' oranges by 23 and 40%, respectively, after 6 days of incubation at 20°C (Fig. 1). Likewise, the severity of GM and BM was effectively reduced by 57 and 66%, respectively, by treatment at 90 mM. Conversely, PSi treatments at 0.9, 9, 30 and 150 mM did not significantly reduce or reduced very slightly the incidence and severity of both molds (Fig. 1).

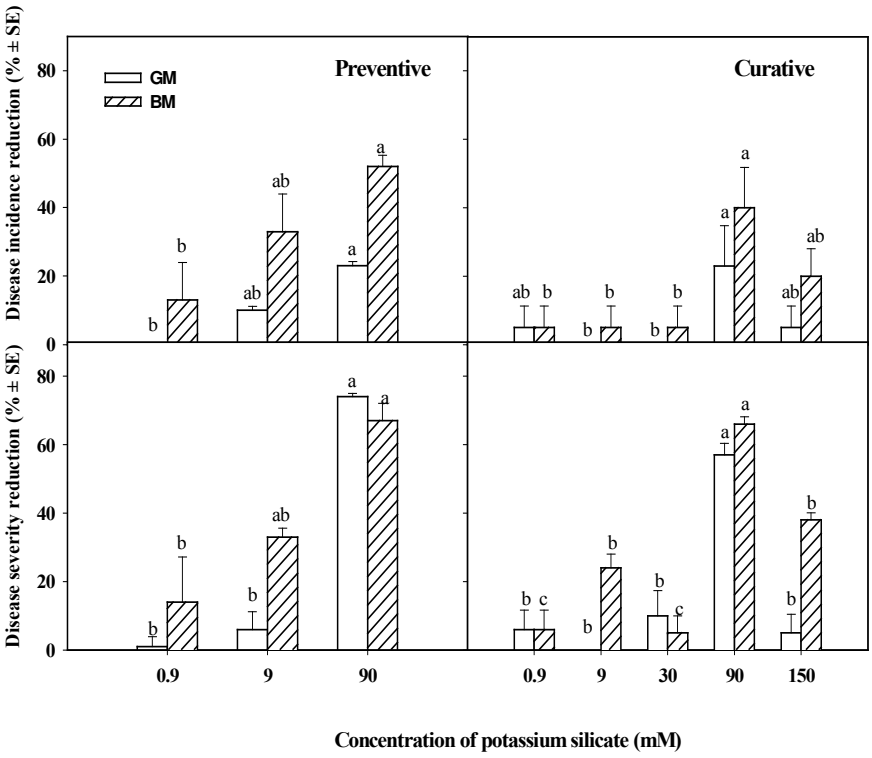

Figure 1. Preventive and curative activity of potassium silicate (PSi) at different concentrations against green (GM) and blue (BM) molds in *in vivo* primary screenings with 'Valencia' oranges. In preventive tests, oranges were wounded, treated with 30 µl of PSi solution at different concentrations and artificially inoculated 24 h later with *Penicillium digitatum* or *P. italicum*. In curative tests, fungal inoculation was performed 24 h before the application of PSi. Treated fruit were incubated for 6 days at 20°C and 90% RH. Disease incidence and severity reductions were determined with respect to control fruit treated with sterile water (incidence of 75-100% and 80-100% for GM and BM, respectively, and severity of 55-93 mm and 22-35 mm for GM and BM, respectively). For each mold, columns followed by different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence was arcsine transformed. Non-transformed means are shown.

Influence of inoculum concentration

Regardless the inoculum concentration of *P. digitatum*, the preventive activity of PSi at 90 mM on 'Lanelate' orange fruit was clearly significant. On PSi-treated fruit GM incidence was reduced by

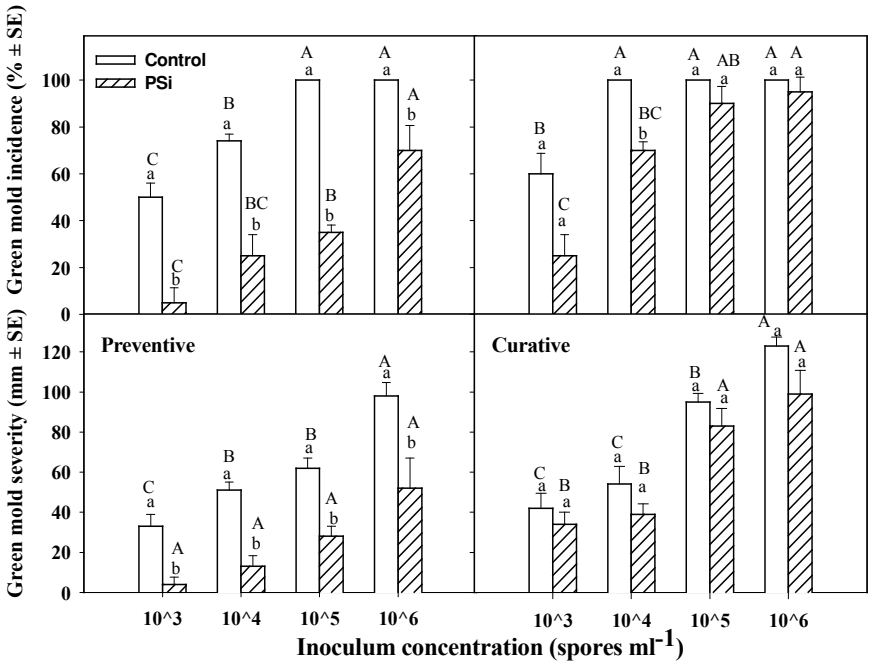

Figure 2. Influence of inoculum concentration on the preventive and curative activity of postharvest treatments with 90 mM potassium silicate (PSi) against green mold on 'Lanelate' oranges. In preventive tests, oranges were wounded, treated with 30 µl of PSi solution at 90 mM and artificially inoculated about 2 h later with *Penicillium digitatum*. In curative tests, fungal inoculation was performed about 2 h before the application of PSi. Control fruit were treated with sterile distilled water. Treated fruit were incubated for 6 days at 20°C and 90% RH. For each inoculum concentration and treatment, columns with different lowercase and capital letters, respectively, are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Results are means from two experiments. Disease incidence was arcsine transformed. Non-transformed means are shown.

90, 66, 65, and 30% at concentrations of *P. digitatum* of 10^3, 10^4, 10^5, and 10^6 spores ml^{-1}, respectively, while it was 50, 74, 100, and 100% on control fruit (Fig. 2). Similarly, GM lesion diameters were significantly reduced from 33, 51, 62 and 98 mm on control fruit to lesions of 4, 13, 28 and 52 mm on PSi-treated fruit when the oranges had been inoculated with concentrations of 10^3, 10^4, 10^5 and 10^6 spores ml^{-1} of *P. digitatum*, respectively (Fig. 2).

In the curative tests, the effectiveness of PSi treatments at 90 mM to reduce GM incidence was lower and there were only significant differences with the control treatment when an inoculum concentration of *P. digitatum* of 10^4 spores ml^{-1} was used (GM incidence reduction of about 30%; Fig. 2). Likewise, GM severity was not significantly reduced by PSi treatment regardless of the inoculum concentration (Fig. 2).

In general, both GM incidence and severity significantly and expectedly increased on both control and PSi-treated fruit as the concentration of *P. digitatum* used for inoculation increased. The main exception was GM incidence on control fruit used on curative trials, which already reached 100% with an inoculum concentration of 10^4 spores ml^{-1} (Fig. 2).

Influence of temporal and spatial characteristics on preventive activity

The preventive activity of PSi at 90 mM against GM on 'Valencia' oranges incubated at 20°C for 6 days was significantly different only when the fruit were inoculated with *P. digitatum* about 2 h after the treatment. GM incidence and severity were 35, 95, 95 and 100% and 11, 69, 68 and 93 mm, respectively, when the time interval between treatment and inoculation were of 2, 24, 48 and 96 h (Table 1).

On the other hand, the performance of PSi at 90 mM against GM on 'Valencia' oranges was not affected by the distance between the treatment and inoculation sites (10, 20 or 30 mm). None of the PSi preventive treatments at these distances significantly reduced green mold in comparison with water-treated control fruit (Table 1).

Table 1. Influence of temporal and spatial factors on the preventive activity of potassium silicate (PSi) against citrus green mold on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* after treatment application

Treatments	Geen mold	
	Incidence (%)	Severity (mm)
Time between PSi treatment and fungal inoculation (h)		
2	35 b	11 b
24	95 a	69 a
48	95 a	68 a
96	100 a	93 a
ıPSi treatment application site and fungal inoculation site (mm)		
Control (water)	85 a	79 a
10	95 a	80 a
20	90 a	79 a
30	90 a	77 a

Fruit were treated in a rind wound with 30 µl of PSi at 90 mM, inoculated with *P. digitatum* and incubated at 20°C and 90% RH for 6 days. Means followed by different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Results are means from two experiments. Green mold incidence was arcsine transformed. Non-transformed means are shown.

Dip treatment conditions

A concentration of aqueous solution of 90 mM PSi was selected as the most effective in the previous *in vivo* primary screenings. Thus, this concentration was used in this subsequent set of trials. Dips of PSi at 90 mM at 20 or 50°C for 60 or 150 s significantly prevented the incidence of GM and BM on 'Lanelate' oranges inoculated about 2 h after treatment and incubated for 7 days at 20°C. On fruit dipped for 60 or 150 s at 20 or 50°C, PSi at 90 mM significantly reduced GM incidence by 37 and 27% and 50 and 55%, respectively, with respect to water-treated control treatments (GM incidence of 100%; Fig. 3). On fruit dipped for 60 or 150 s at 20 or 50°C, PSi treatment significantly reduced BM incidence by 18 and 37% and 40 and 28%,

respectively, with respect to the controls (Fig. 3). Likewise, the severity of both molds was similarly reduced by PSi at 90 mM on these preventive tests. GM and BM lesion diameters were reduced from 105 and 46 mm on control fruit to 50-70 and 15-20mm, respectively, on PSi-treated oranges (Fig. 3). Thus, mold development on PSi-treated oranges was not significantly affected by immersion time.

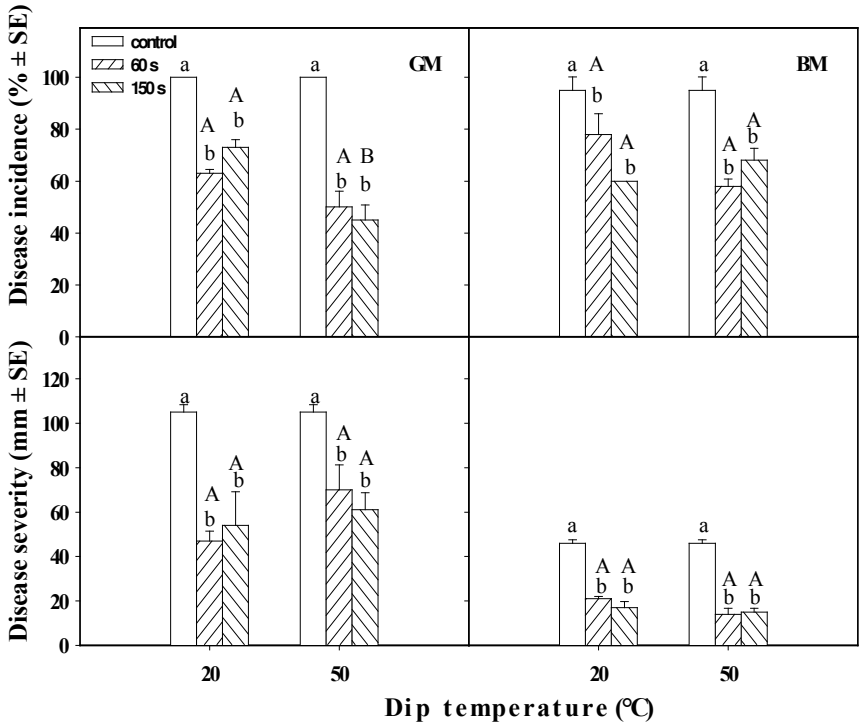

Figure 3. Preventive activity of dips with 90 mM potassium silicate (PSi) against green (GM) and blue (BM) molds on artificially wounded 'Lanelate' oranges treated for 60 or 150 s at 20 or 50°C, inoculated about 2 h later with *Penicillium digitatum* or *P. italicum*, and incubated for 7 days at 20°C and 90% RH. Control fruit were dipped in water at 20 or 50°C for 60 s. For each mold and dip temperature and for each mold and PSi dip time, columns with different lowercase and capital letters, respectively, are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Results are means from two experiments. Disease incidence was arcsine transformed. Non-transformed means are shown.

In tests to assess the curative activity, dips with PSi at 90 mM applied at 20 or 50°C for 60 or 150 s significantly reduced the incidence of GM and BM on 'Lanelate' oranges inoculated 24 h before treatment and incubated for 7 days at 20°C. On fruit dipped for 60 or 150 s at 20 or 50°C, PSi at 90 mM reduced the incidence of GM by 35 and 38% and 40 and 62%, respectively. There was, therefore, a significant effect of immersion time, but only when the dips were performed at 50ºC (Fig. 4).

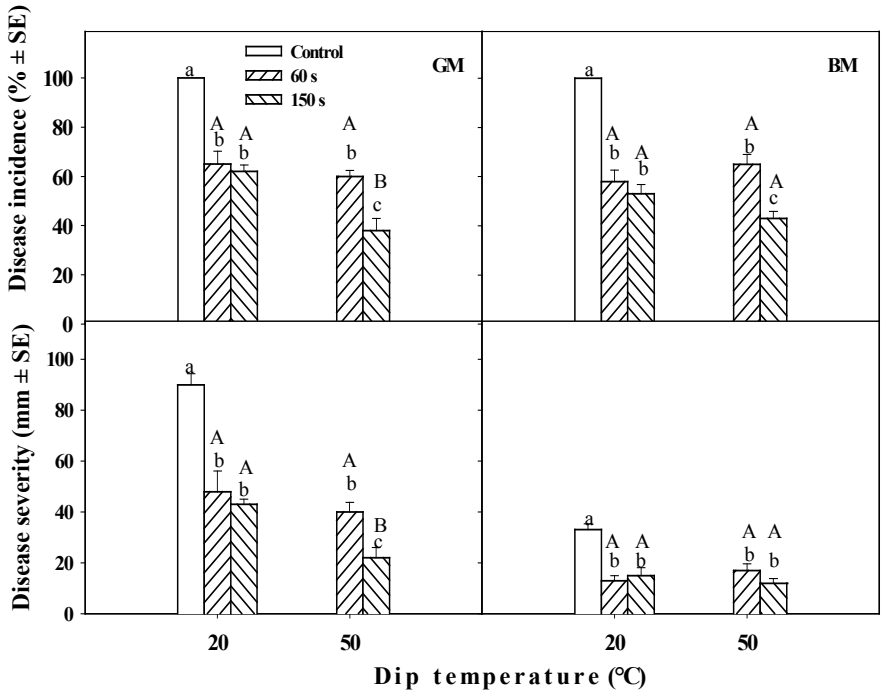

Figure 4. Curative activity of dips with 90 mM potassium silicate (PSi) against green (GM) and blue (BM) molds on 'Lanelate' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, dipped for 60 or 150 s at 20 or 50°C, about 24 h later and incubated for 7 days at 20°C and 90% RH. Control fruit were treated with water at 20ºC for 60 s. For each mold and for each mold and PSi dip time, columns with different lowercase and capital letters, respectively, are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Results are means from two experiments. Disease incidence was arcsine transformed. Non-transformed means are shown.

This pattern was also observed for the incidence of BM, with reductions of 42 and 47%, and 35 and 57%, respectively, on fruit dipped for 60 or 150 s at 20 or 50°C (Fig.4). Likewise, the severity of both molds also followed very similar patterns, and significant differences between immersion times were only observed for GM on oranges dipped at 50ºC (Fig. 4).

As a conclusion of both preventive and curative tests, PSi dips at 20°C for 60 s were selected for use in subsequent experiments.

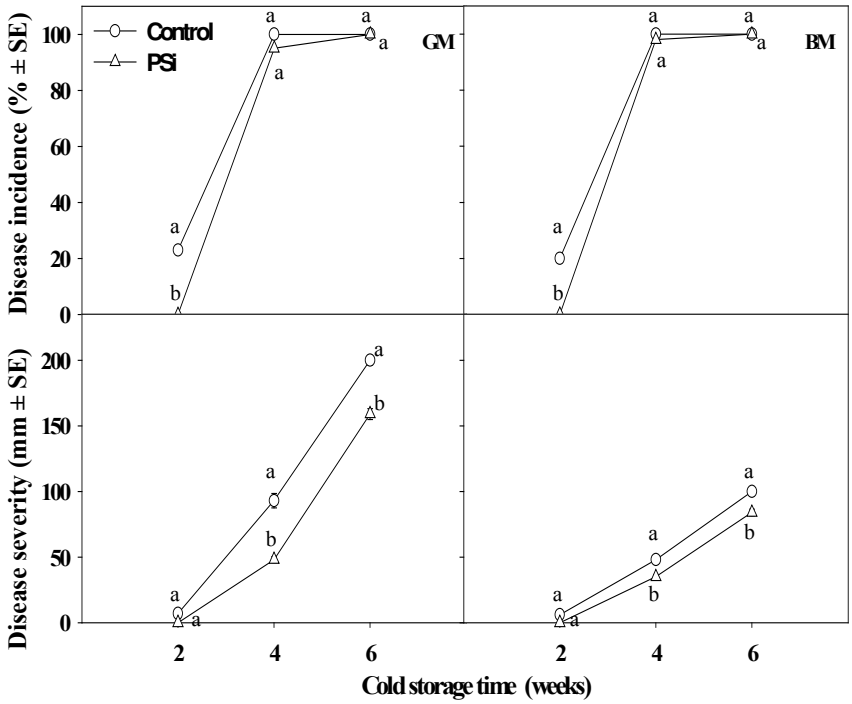

Figure 5. Preventive activity of potassium silicate (PSi) dips at 90 mM against green (GM) and blue (BM) molds on wounded 'Valencia' oranges dipped for 60 s at 20°C, artificially inoculated with *Penicillium digitatum* or *P. italicum* about 2 h later, and cold-stored at 5°C and 90% RH for 6 weeks. For each mold and evaluation date, means with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence was arcsine transformed. Non-transformed means are shown.

Effectiveness on long-term cold-stored fruit

After 2 weeks of storage at 5°C, the incidence of both GM and BM on 'Valencia' oranges artificially inoculated about 2 h after treatment (preventive activity) was totally prevented by the application of PSi at 90 mM as 60 s dips at 20°C. At this time, however, the incidence of GM and BM on control fruit was only of 23 and 20%, respectively. After 4 and 6 weeks of cold storage, PSi at 90 mM showed no protective effect, since the incidence of GM and BM was not significantly different from that on control fruit (Fig. 5). In general, PSi treatments significantly reduced the severity of GM and BM throughout the entire storage period of 6 weeks at 5°C. GM lesion diameters after 4 and 6 weeks on control and PSi-treated oranges were 93 and 48 mm, and 200 and 159 mm, respectively. In the case of BM, these diameters were 48 and 35 mm, and 100 and 84 mm, respectively (Fig. 5).

In the curative tests, the incidence of both GM and BM on 'Valencia' oranges artificially inoculated 24 h before treatment, and stored up to 6 weeks at 5°C and 90% RH were significantly reduced by the application of PSi at 90 mM as 60 s dips at 20°C. At the end of the cold storage period, the incidence of both GM and BM was significantly reduced by up to 45% with respect to the control fruit (GM and BM incidence of 100%) (Fig. 6). The treatment with PSi at 90 mM also effectively reduced the severity of GM and BM during cold storage at 5°C. GM and BM lesion diameters at the end of the 6-week cold storage period on control and PSi-treated oranges were 200 and 98 mm, and 100 and 48 mm, respectively (Fig. 6).

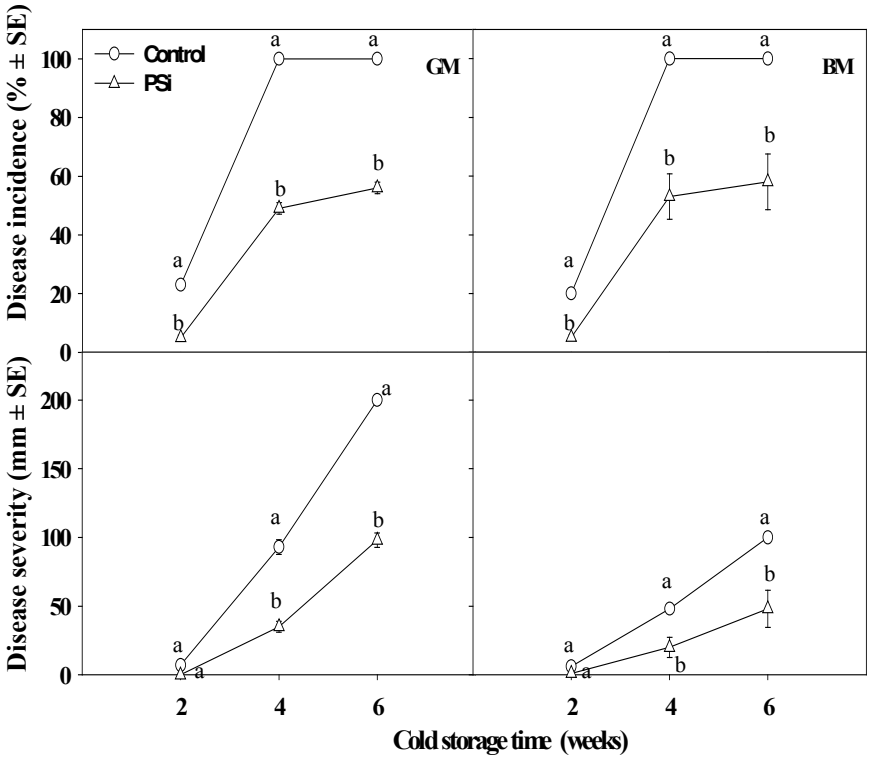

Figure 6. Curative activity of potassium silicate (PSi) dips at 90 mM against green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum* in rind wounds, dipped 24 h later for 60 s at 20°C, and cold stored at 5°C and 90% RH for 6 weeks. For each mold and evaluation date, means with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence was arcsine transformed. Non-transformed means are shown.

Discussion

This study reports that postharvest treatments with Si in the form of PSi aqueous solutions applied to 'Valencia' or 'Lanelate' oranges both before (preventive activity) and after (curative activity) artificial fungal inoculation, showed significant antifungal activity against citrus GM and BM. Results from *in vivo* primary screenings showed that PSi treatments inhibited mold development in a concentration-dependent manner, and they were more effective at a concentration of 90 mM. Therefore, PSi acted here probably more as a plant growth regulator, which are typically more effective when applied at an optimal concentration, than as a conventional fungicide, whose efficacy gradually increases as the application dose increases. This hypothesis was supported by the fact that in the screenings for curative action, disease reduction was lower after PSi application at 150 than at 90 mM (Fig. 1). Results from these tests showed that the effectiveness of PSi at 90 mM to prevent GM or BM on 'Valencia' orange fruit (incidence reduction of 23 and 52%, respectively) was slightly inferior or similar to that reported after sodium silicate treatments on citrus (Liu et al. 2010), and it was also similar to that observed on jujube fruit (Tian et al. 2005). Further, it was found in previous work in our laboratory that preventive treatments with SSi at 1000 mM reduced GM and BM on artificially inoculated oranges by 70-90%, but this treatment caused injuries in the fruit rind (Moscoso-Ramírez and Palou 2013). It can be therefore concluded that, from a practical point of view, postharvest PSi treatments are superior to SSi treatments for the purpose of postharvest disease reduction. In addition, PSi treatments not exceeding 1% (wt/vol) were exempted in the USA from the requirement of a tolerance for residues in or on all food commodities when applied or used as a fungicide, insecticide or miticide (US EPA 2006). Moreover, aqueous PSi was also included in the list of synthetic substances allowed for use in organic crop production. The only requirement is that the silica used for the manufacture of PSi must be sourced from naturally occurring sand (USDA AMS NOP 2010).

It has been proposed that the mode of action of Si treatments is through a direct effect on the pathogen. For instance, Liu et al. (2010) reported damage on the plasma membrane of Si-treated *P. digitatum*

spores, leading to higher leakage of proteins and sugars. However, other research works contradicted this hypothesis and attributed the antifungal action of Si to indirect effects to the fruit host. Hereto, Si can play an important role on the formation of physical and mechanical barriers to the penetration of pathogens at the cell wall level (Buonaurio et al. 2009; Datnoff et al. 2001). Si treatments may also act by eliciting biochemical defense reactions, including the accumulation of lignin, phenolic compounds and pathogenesis-related (PR) proteins in infected plants (Epstein 1999). According to our results, we can hypothesize that in the case of citrus penicillium molds, the mode of action of postharvest PSi treatments for disease control might be a combination of both direct effects on the pathogen *P. digitatum* or *P. italicum* and indirect effects on the fruit host. Curative tests were performed with oranges artificially inoculated in rind wounds with a conidial suspension about 24 h before the treatment. This is the usual procedure to simulate the most common natural infections on laboratory assays to test the efficacy of postharvest fungicides against citrus GM and BM (Eckert and Brown 1986). Since the spore suspensions were freshly prepared the same inoculation day and the inoculated fruit were kept at a constant temperature of 20ºC during the 24-h period between inoculation and treatment, the effect of the treatment was mostly on recently germinated conidia (on germ tubes or young hyphae), although a variable proportion could still be ungerminated conidia. On the other hand, preventive tests were carried out with oranges wounded, treated and inoculated about 2 or 24 h after treatment, depending on the experiment. It seems that PSi treatment effectively conferred by some mechanism some degree of resistance to the fruit rind, since significant disease reductions were observed on both primary screenings and dip trials with a PSi concentration of 90 mM.

In any case, both preventive and curative effects were, as expected, dependent on the concentration of pathogenic inoculum. Early research showed that this factor clearly influenced the rate of successful infections when citrus rind wounds are inoculated with *P. digitatum* or *P. italicum* (Eckert and Eaks 1989). This was confirmed in this work, as disease incidence and severity on control fruit consistently increased as inoculum density increased (Fig. 2). Conversely, the protective action of PSi consistently decreased as the

inoculum load increased (GM incidence reductions after 6 days at 20ºC of 90, 70 and 30% on oranges inoculated with 10^3, 10^5 and 10^6 spores ml^{-1} of *P. digitatum*). This effect on the incidence of GM was not as pronounced in the tests to assess the influence on the curative activity of PSi, probably because control fruit was already 100% decayed with a concentration of 10^4 spores ml^{-1} of *P. digitatum*. In contrast, the effect of inoculum density in these tests was clear for GM severity, which steadily increased on both control and PSi-treated oranges as the inoculum load increased.

The period of time between treatment and inoculation (temporal factor) affected the preventive performance of PSi only when it was of about 2 h. In this case, GM incidence and severity were significantly lower than with the rest of time intervals (Table 1). We assume that after 2 h, a large amount of active PSi residues might be present into the treated rind wound and they might adversely affect the viability of ungerminated spores that had just been inoculated into the adjacent wound. In contrast, time intervals of 24, 48 and 96 h between treatment and inoculation were too long to maintain equivalent proportions of active residues in the rind tissue. It appears, in addition, that during these time periods the treatment did not induce any defense or protection mechanisms against *P. digitatum* important enough to significantly reduce disease on inoculated fruit. The importance of the timing of defense responses in phenomena related to acquired or induced plant resistance to pathogens is critical, as it has been suggested by some researchers (Vallad and Goodman 2004). Our data also showed that PSi locally applied to a rind wound after harvest had no systemic activity and GM incidence and severity were not reduced on inoculated wounds located at 10, 20, or 30 mm from the treatment site. Therefore, the preventive action of postharvest PSi treatments would be a type of local acquired resistance (LAR), since resistance is only manifested in the same plant tissues that receive the resistance induction treatments. In contrast, most of preharvest or field applications with chemical resistance inducers typically induce systemic acquired resistance (SAR) or induced systemic resistance (ISR), in which pathogen resistance is produced in plant organs other than those that had been directly treated (Edreva 2004; Vallad and Goodman 2004). The production of SAR or ISR is favored by the high metabolic activity of the plant growing in the field. In general,

resistance to plant pathogens are more easily induced in vegetative parts of the plant than in reproductive portions, such as the rind tissues of citrus fruit (Porat et al. 2003).

In this study, variable results were obtained when postharvest PSi treatments at 90 mM were applied with micropipette into wounds in *in vivo* primary screenings or as aqueous dips in laboratory trials. With the exception of BM incidence in preventive tests, disease incidence was generally higher on dip-treated oranges than on fruit treated with a solution drop. This could be explained by different penetration capability of the product into rind wounds and different length of contact between the product and the treated fruit.

In general, dip times and temperatures did not consistently influence the effectiveness of PSi dips. Therefore, dips at room temperature (20°C) for 60 s were selected and applied on subsequent trials with long-term cold stored oranges. This is a result that might facilitate the commercial adoption of postharvest PSi treatments in citrus packinghouses, since implementation and application costs of non-heated solutions would be considerably lower than that of solutions heated to temperatures of 40-50ºC. In our work, the curative effectiveness of these PSi dips to reduce the incidence of GM and BM was similar to that obtained by Bi et al. (2006) using SSi treatments to control melon pink rot caused by *T. roseum*. GM and BM severity on oranges was reduced by up to two-fold by PSi dips at room temperature for 60 s in either preventive or curative tests. Although these reductions were significant, they were lower than those obtained by Guo et al. (2007) after dipping Chinese cantaloupes in SSi solutions to control pink rot (severity reduction of about five-fold).
In preventive tests with long-term cold-stored fruit at 5°C, we found that PSi at 90 mM applied as 60 s dips at 20°C did not significantly reduce the incidence of GM and BM after 4 or 6 weeks of storage, but it was effective to reduce the severity of both molds. In contrast, in curative tests, this same treatment effectively reduced both incidence and severity of GM and BM with respect to control fruit (Figs. 5, 6). Therefore, before the overall performance of cold-stored oranges treated with PSi, this treatment could be recommended for long-term storage of citrus fruit at commercial refrigeration temperatures.

The primary findings of this research work were that postharvest PSi treatments showed significant preventive and curative antifungal activity against citrus penicillium molds. Considering that Si is the second most abundant atom in the earth's crust and it is readily available, the cost of Si treatments are relatively inexpensive and, in any case, will be lower than that of other new alternative strategies for citrus postharvest disease control. Although large-scale semicommercial trials are needed for efficacy assessment before commercial implementation, Si treatments show great potential as part of non-polluting integrated disease management programs. According to Laing et al. (2006), the application of Si in crops provides a viable component of integrated management of insect pests and diseases because it leaves no insecticide residues in food or the environment, and it can be easily integrated with other pest management practices, including biological control.

Acknowledgments

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CAPÍTULO 4

Characterization of postharvest treatments with sodium methylparaben to control citrus green and blue molds

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] CLARA MONTESINOS-HERRERO[1] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

The curative antifungal activity of postharvest sodium methylparaben (SMP) treatments against citrus green (GM) and blue (BM) molds was characterized on different citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *Penicillium italicum* and incubated at 20°C and 90% RH for 7 d or stored at 5°C and 90% RH for 8 weeks plus 7 d of shelf-life at 20°C. Effective concentrations were selected in *in vivo* primary screenings with 'Valencia' oranges. SMP at 200 mM was tested at 20, 50 or 62°C for 30, 60 or 150 s in small-scale trials to determine the best dip treatment conditions. Dips of 200 mM SMP at 20°C for 60 s were selected and applied alone or in combination with 25 μL L^{-1} of the conventional fungicide imazalil (SMP + IMZ 25). Imazalil at the very low concentrations of 25 (IMZ 25) or 50 μL L^{-1} (IMZ 50) were also tested. Effectiveness of SMP alone at 20°C for 60 s was significantly higher on oranges (cvs. 'Valencia' and 'Lanelate') than on mandarins (cvs. 'Clemenules', 'Nadorcott' and 'Ortanique'), with GM and BM incidence reductions of up to 88% after 7 d at 20°C. SMP was compatible with IMZ 25 and consistently improved its performance, irrespective of citrus cultivars and storage conditions. All treatments were less effective on 'Clemenules' mandarins. On 'Valencia' oranges stored for 8 weeks at 5°C and 7 d at 20°C, the combined treatment was significantly more effective than the single treatments (reductions of GM and BM incidence of about 50-60% and 90-95%, respectively). In additional tests, 200 mM SMP dips at 20°C for 60 s did not prevent GM on 'Valencia' oranges wounded, treated, inoculated with *P. digitatum* 24 h later, and incubated at 20°C for 7 d. In contrast, the treatments IMZ 25 and SMP + IMZ 25 showed significant preventive activity. It can be concluded from these results that SMP aqueous solutions, especially applied at room temperature, might be an interesting nonpolluting control alternative to be included in citrus postharvest disease control programs in the future.

Key words: *Penicillium digitatum*, *Penicillium italicum*, oranges, mandarins, food additives, alternative disease control, imazalil

1. INTRODUCTION

Postharvest green mold (GM), caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., and postharvest blue mold (BM), caused by *Penicillium italicum* Wehmer, are the most economically important postharvest diseases of citrus in Spain, California, and all citrus production areas characterized by low summer rainfall (Ecker and Eaks, 1989; Palou et al., 2007). Blue mold is especially important on citrus fruit kept under cold storage (Brown and Ecker, 2000; Palou et al., 2002b). Currently, these diseases are primarily controlled by application of synthetic fungicides such as imazalil or thiabendazole (Holmes and Eckert, 1999; Palou et al., 2002a). However, resistance development to fungicides by plant pathogens is a factor limiting fruit production worldwide duo to the decrease in efficacy of fungicides (Brent and Hollomon, 2007). Furthermore, in the development and use of chemical fungicides for postharvest disease control, considerable attention must be given to the preservation of the global environment.

Alternative methods that have been pursued for the control of postharvest diseases include biological control, physical methods such as heat or radiations, and the use of safe low-toxicity chemicals such as food additives (Palou et al., 2002b, 2008; Smilanick et al., 2008; Montesinos-Herrero et al., 2009; Valencia-Chamorro et al., 2009).

Food additives are widely utilised for controlling food pH, taste and texture. Some of them such as parabens also have a broad-spectrum antimicrobial activity and are commonly used as food preservatives (Arslan et al., 2009). Parabens and some of their salts are classified as “generally regarded as safe” (GRAS) compounds and approved for use in foods by the US Food and Drug Administration (FDA) and European Union (EU) regulations (Milss et al., 2004). The main advantages of using salt compounds as fungicides include their relatively low mammalian toxicity, a broad spectrum of modes of action, and relatively low cost (Milss et al., 2004). The antimicrobial activity of parabens increases as the chain length of the alkyl group increases, but since their aqueous solubility decreases, the sodium salts of parabens are also frequently used in formulations (Giordano et al., 1999).

One of the paraben sodium salts, sodium methylparaben (SMP), has demonstrated a potential to control postharvest pathogens on some fruit species like strawberry or citrus (Yildirm and Yapici, 2007; Valencia-Chamorro et al., 2009). However, in these works SMP has been investigated in *in vitro* conditions or in applications incorporated to edible coatings. Thus, very little information is available about the use of aqueous solutions of SMP applied as potential postharvest antifungal treatments, particularly against *Penicillium* molds of citrus fruit. The present research had the following aims: (1) to evaluate the curative activity of SMP at different concentrations against GM and BM, (2) to optimize the SMP dip treatment conditions, (3) to determine the SMP compatibility with low doses of imazalil under selected dip conditions, (4) to determine the effectiveness of SMP treatments on economically important citrus species and cultivars, (5) to evaluate the curative activity of SMP treatments on long-term cold-stored citrus fruit, and (6) to evaluate the preventive activity of SMP treatments against citrus postharvest GM and BM.

2. MATERIALS AND METHODS

2.1. Fruit

The experiments were conducted with 'Valencia' and 'Lanelate' oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' (synonyms: 'Nules', 'Clementina de Nules') clementine mandarins (*Citrus reticulata* Blanco), and 'Nadorcott' (*C. reticulata* x *C. sinensis*; synonyms: 'Afourer', 'W. Murcott') and 'Ortanique' [*C. reticulata* x (*Citrus sinensis* x *C. reticulata*); synonym: 'Topaz'] hybrid mandarins. Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored up to 1 week at 5°C and 90% relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with tap water and allowed to air dry at room temperature.

2.2. Fungal inoculation

P. digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were

cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25ºC. Conidia of each fungus from 7 to 14-days-old cultures were taken from the agar surface with a sterile glass rod and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 or 10^6 spores mL^{-1} using a haemocytometer. Unless otherwise stated, the tip of a stainless steel rod, 1 mm wide and 2 mm in length, was immersed in the conidial suspension and inserted in the fruit rind afterwards. Fruit were inoculated at two opposite points in the equatorial zone, one with *P. digitatum* and the opposite with *P. italicum.* Inoculated fruit were kept in a temperature-controlled room at 20ºC for 24 h, until treatment.

2.3. Curative activity

2.3.1. *In vivo* primary screenings

SMP (Methyl 4-hydroxybenzoate sodium salt; Merck KgaA, Darmstadt, Germany; Table 1; Fig. 1) was tested at nine concentrations to control citrus postharvest GM and BM on fruit previously inoculated with the pathogens. A sterile mother solution of SMP was prepared at a concentration of 250 mM. Sterile solutions at concentrations of 0.1, 1, 10, 40, 70, 100, 150, 200 and 250 mM SMP were prepared by diluting with sterile water. Inoculum preparation was carried out following the procedure described above. Thirty µL of conidial suspension of *P. digitatum* or *P. italicum* at a concentration of 10^6 spores mL^{-1} were placed in rind wounds using a micropipette. About 24 h after the inoculation of the pathogen, 30 µL of SMP solution at the corresponding concentration were placed, using a micropipette, in the same inoculation rind wound. Control fruit were treated with 30 µL of sterile distilled water. For each combination of concentration of SMP and pathogen, 4 replicates of 5 'Valencia' oranges each were used. Treated fruit were incubated at 20ºC and 90% RH for 3 and 6 d, at which time disease incidence (% of infected fruit) and severity (lesion diameter) were determined. Severity was assessed over the entire fruit sample, not only over infected and symptomatic fruit.

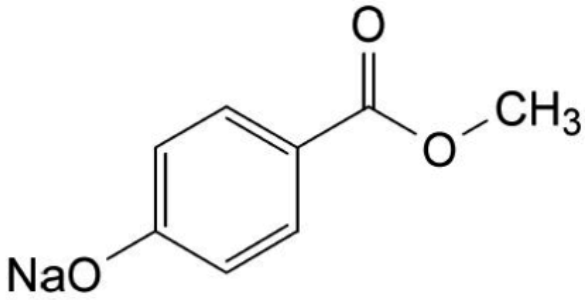

Figure 1. Chemical structure of sodium methylparaben.

Table 1. Physicochemical properties of sodium methylparaben

Property	Description
Formula	$C_8H_7NaO_3$ (Fig. 1)
Synonyms	Methyl 4-hydroxybenzoate, sodium salt Methyl *p*-hydroxybenzoate, sodium salt 4-Hydroxybenzoic acid methyl ester, sodium salt Sodium 4-carbomethoxyphenolate Methyl-4-oxide-benzoate, sodium salt
Physical state	White crystalline powder
Molecular weight	174.13
Melting point (°C)	131
Boiling point (°C)	270-280
Solubility in water	Soluble
pH	9.5-10.5 (10% Aqueous solution)
Refractive index	1.525
Stability	Stable under normal conditions. Hygroscopic

Source: Merck KgaA, Darmstadt, Germany

2.3.2. Dip treatment conditions

Small-scale trials were conducted using 'Valencia' oranges to establish the best dip treatment conditions. Fungal inoculation at a concentration of 10^6 spores mL^{-1} of *P. digitatum* or *P. italicum* was carried out following the procedure mentioned in the 'fungal inoculation' section. Stainless steel buckets containing 10 L of aqueous solution of 200 mM (30.43 g L^{-1}; 3% w v^{-1}) SMP were used. This concentration of SMP was selected according to previous results obtained in the *in vivo* primary screenings and preliminary dip treatment tests. When needed, solutions were heated by placing the buckets in a 250-L stainless steel water tank fitted with two electrical resistances (4.5 kW each), a thermostat, and an automatic water-recirculating system. Fruit were placed into 18 L multi-perforated wall stainless steel containers, exactly fitting in the above-mentioned buckets, and completely immersed in the treatment solution for 30, 60 or 150 s at 20, 50 or 62°C. After treatment, fruit were rinsed for 5 s with tap water at low pressure in order to eliminate paraben salt residues. Control fruit were dipped in water alone at 20°C. Sixty fruit per treatment (3 replicates of 20 fruit each) were arranged in plastic cavity sockets on cardboard trays. Treated fruit were incubated at 20°C and 90% RH for 7 d. Disease incidence was assessed after 7 d of incubation. Potential fruit phytotoxicity caused by SMP or heat was visually assessed after 3 d at 20°C. For this purpose, fruit were classified into one of four categories, depending on rind appearance: 0 = no rind damage; 1 = slight brownish blemishes present (<10% fruit surface); 2 = moderate brownish blemishes present (10% <fruit surface<25%) and 3 = severe rind injury (>25% fruit surface). A ponderate rind pitting index (0–3 scale) was calculated for each treatment.

2.3.3. Combination with low doses of imazalil

To determine the effect of the combination of the paraben salt with low doses of the chemical fungicide imazalil (IMZ; (±)-1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl)-1H--imidazole; Fecundal-S 7.5% EC; Fomesa Fruitech S.L., Valencia, Spain) to control green and blue molds, the following treatments were applied to 'Valencia'

oranges inoculated at a fungal concentration of 10^6 spores mL^{-1}: (1) water (control), (2) 200 mM SMP (SMP), (3) 25 µL L^{-1} IMZ (IMZ 25), (4) 50 µL L^{-1} IMZ (IMZ 50), and (5) combination of 200 mM SMP with 25 µL L^{-1} IMZ (SMP + IMZ 25). Aqueous solutions of both chemicals were mixed into 10 L buckets and manually stirred with a clean plastic rod. IMZ was used at two doses considerably lower than those recommended for commercial applications (500-1,000 µL L^{-1}). Fungal inoculation and dip treatments were performed following the procedure mentioned above. Dip conditions were temperature of 20ºC and immersion time of 60 s. After treatment, only fruit treated with SMP were rinsed with tap water for 5 s. Each treatment was applied to 3 replicates of 20 fruit each. Disease incidence and pathogen sporulation (% of lesions showing spores) were determined after 7 d of incubation at 20ºC and 90% RH. Fruit phytotoxicities were assessed after 3 d at 20ºC. The experiment was repeated twice.

2.3.4. Effectiveness on major citrus species and cultivars

To assess whether the effectiveness of curative treatments was dependent on the host fruit, 'Valencia' and 'Lanelate' oranges and 'Nadorcott', 'Ortanique' and 'Clemenules' mandarins were subjected to the following treatments: (1) water (control), (2) 200 mM SMP, (3) 25 µL L^{-1} IMZ (IMZ 25), or (4) 200 mM SMP + 25 µL L^{-1} IMZ (SMP + IMZ 25). All treatments were applied as dips at 20 ºC for 60 s. The experimental design, fungal inoculation (10^6 spores mL^{-1}) and dip treatments followed the same procedures previously described. Treated fruit were not rinsed with tap water, with the exception of fruit treated with SMP alone. Treated fruit were incubated at 20 ºC and 90% RH for 7 d, at which time disease incidence and pathogen sporulation were assessed.

2.3.5. Effectiveness on long-term cold-stored fruit

To evaluate the curative capability of SMP against GM and BM on 'Valencia' oranges subjected to long-term cold storage, an experiment was conducted using inoculated fruit (10^6 spores mL^{-1}) treated with: (1) water (control), (2) 200 mM SMP, (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 200 mM SMP + 25 µL L^{-1} IMZ (SMP + IMZ 25). All treatments were applied as dips at 20ºC for 60 s. Each treatment was

applied to 3 replicates of 20 fruit each. Treated fruit were not rinsed with tap water, with exception of fruit treated only with SMP. Treated fruit were stored up to 8 weeks at 5ºC and 90% RH. Following the refrigeration period, the fruit were subjected to 7 d of shelf-life at 20ºC and 70-80% RH. Disease incidence and severity and pathogen sporulation were assessed after 2, 4, 6, and 8 weeks at 5ºC plus 7 d at 20ºC.

2.4. Preventive activity

To evaluate whether SMP treatments or combinations showed a preventive effect on the control of GM and BM, an experiment was conducted with 'Valencia' oranges in which a 1 mm wide, 2 mm deep wound was made with a stainless steel rod on the equatorial region of each fruit to simulate natural wounds. Then, the following treatments were applied by dipping the fruit in 200 mM aqueous solution at 20ºC for 60 s: (1) water (control), (2) 200 mM SMP (SMP), (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 200 mM SMP + 25 µL L^{-1} IMZ (SMP + IMZ 25). About 24 h later, fruit were inoculated with the tip of a stainless steel rod, 1 mm wide and 2 mm in length that had been immersed in a 10^5 spores mL^{-1} conidial suspension of *P. digitatum* and inserted in a new adjacent wound (about 2 mm of separation between wounds). Each treatment consisted of 3 replicates of 20 fruit each. After inoculation, treated fruit were incubated at 20ºC and 90% RH for 7 d, at which time disease incidence and severity and pathogen sporulation were assessed. The experiment was repeated twice.

2.5. Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus version 4.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence and pathogen sporulation were transformed to the arcsine of the square root of the proportion of infected or sporulated fruit to assure the homogeneity of variances. In some cases, reductions with respect to the controls were calculated as percentages. Statistical significance was judged at the level $P = 0.05$. On repeated experiments, means from both trials are presented since the factor 'trial' was not significant. When appropriated, the Fisher's Protected Least Significant Difference

(LSD) test was used to separate means. Shown values are non-transformed means.

3. RESULTS

3.1. Curative activity

3.1.1. *In vivo* primary screenings

Among the concentrations of SMP evaluated in this set of experiments, the following concentrations completely inhibited the development of GM and BM on 'Valencia' oranges: 150, 200 and 250 mM (Fig. 2). Moreover, the concentrations of 40, 70 and 100 mM SMP significantly reduced the incidence of GM and BM by 90, 59 and 58%, and 84, 84 and 58%, respectively, after 6 d of incubation at 20°C. In contrast, the concentrations of 0.1, 1 and 10 mM SMP were not effective to reduce the incidence of the molds on 'Valencia' oranges (Fig. 2). Likewise, concentrations of 40 and 70 mM SMP effectively reduced the severity of GM and BM by 82 and 60%, and 66 and 47%, respectively, and concentrations of 0.1, 1, 10 and 100 mM SMP did not significantly reduce the severity of both molds (Fig. 2).

3.1.2. Dip treatment conditions

Concentrations of 150 and 200 mM SMP were selected as the best in the previous *in vivo* primary screenings. Preliminary dip treatments (data not shown) showed that 200 mM were superior to 150 mM and, thus, this concentration was selected for use in subsequent trials. SMP at 200 mM applied at 20 or 50°C for 150 s significantly reduced the incidence of GM in comparison with fruit dipped for 30 or 60 s at the same temperatures (Fig. 3). On fruit dipped for 150 or 60 s at 62°C, SMP was significantly more effective than on fruit dipped for 30 s at the same temperature, with GM reductions after 7 d at 20°C of 97, 88 and 67%, respectively. On the other hand, on fruit dipped for 30, 60 or 150 s at 20°C, SMP reduced the incidence of BM by 77, 87 and 88%, respectively, and not being these values significantly different one from each other. A similar trend was observed for BM on fruit dipped

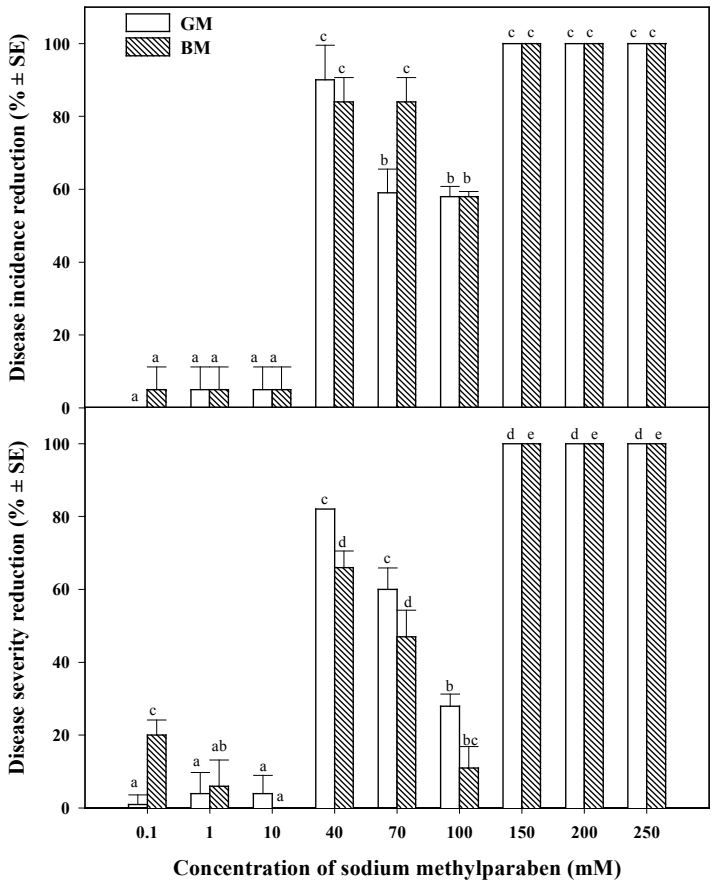

Figure 2. Curative activity of sodium methylparaben (SMP) at different concentrations against green (GM) and blue (BM) molds in *in vivo* primary screenings with 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later, and incubated for 6 d at 20°C and 90% RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water (incidence of 90-100% for both molds and severity of 89-125 mm and 36-45 mm for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Incidence values were arcsine-transformed. Non-transformed means are shown.

for 30 or 150 s at 50°C (Fig. 3). Irrespective of treatment time, SMP dips were more effective at 62°C, but oranges dipped at this temperature showed moderate phytotoxic injuries (rind pitting scale = 2, 10-25% of fruit surface). In general dips at 50°C did not improve the performance of those at 20°C (Fig. 3). Therefore, SMP dips at 20°C for 60 s were selected for use in further experiments.

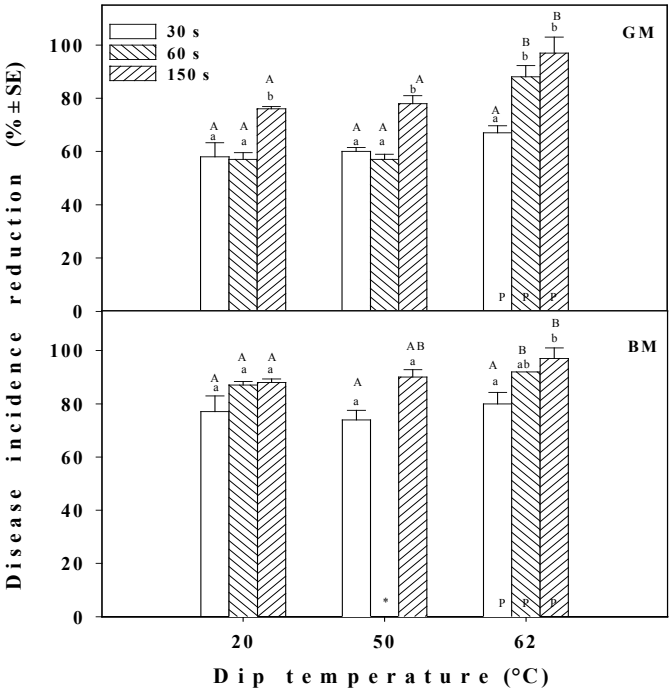

Figure 3. Effect of dip temperature and time on the effectiveness of 200 mM sodium methylparaben (SMP) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later, and incubated for 7 d at 20°C and 90% RH. Inoculated fruit were rinsed with tap water for 5 s after treatment. Reductions of disease incidence were determined with respect to control fruit treated with water (incidence of 100% for both molds for all temperatures and times). For each mold, columns with different lowercase and capital letters indicate significantly different dip time and dip temperature, respectively, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown. 'P' indicates appearance of phytotoxicities on the fruit rind. Asterisk indicates non-registered data.

3.1.3. Combination with low doses of imazalil

Dips of 'Valencia' oranges at 20°C for 60 s with the combination SMP + IMZ 25 significantly enhanced the control of GM if compared to the other treatments, with a GM incidence reduction after 7 d at 20°C of 97%. SMP, IMZ 25 and IMZ 50 reduced GM incidence by 63, 50 and 68%, respectively, and did not significantly differ from each other (Fig. 4). In the case of BM, the treatments SMP, IMZ 50 and SMP + IMZ 25 reduced the disease incidence by 80-95%, with no significant differences between them. IMZ 25 was the least effective treatment against BM and reduced the incidence by 51%. On the other hand, all treatments exerted a high anti-sporulant activity, especially against *P. italicum*, with sporulation reduction with respect to the control treatment ranging from 70 to 100% (Fig. 4).

3.1.4. Effectiveness on major citrus species and cultivars

Overall, the effectiveness of 200 mM SMP aqueous treatment applied alone at 20°C for 60 s to control GM and BM was significantly higher on oranges than on mandarins, with the exception of GM on 'Nadorcott' mandarins. After incubation at 20°C for 7 d, SMP applied alone significantly reduced the incidence of GM and BM by 63 and 38% and 88 and 57% on 'Valencia' and 'Lanelate' oranges, respectively (Fig. 5). Conversely, SMP reduced the incidence of GM and BM by less than 20% on 'Ortanique' and 'Clemenules' mandarins. SMP applied alone reduced the incidence of GM and BM by 51 and 8%, respectively, on 'Nadorcott' mandarins. Similarly, IMZ 25 and the combined treatments were generally more effective to reduce the incidence of GM on oranges than on mandarins, the main exception being the combined treatment on 'Ortanique' mandarins (90% incidence reduction). These treatments reduced the incidence of BM especially on 'Valencia' oranges and were much less effective on 'Clemenules' mandarins (Fig. 5). On the other hand, all three treatments presented an important anti-sporulant activity on most of the cultivars after 7 d at 20°C. In general, irrespective of the dip treatment, sporulation of both *P. digitatum* and *P. italicum* was higher on 'Clemenules' mandarins than on the rest of citrus cultivars (Fig. 5).

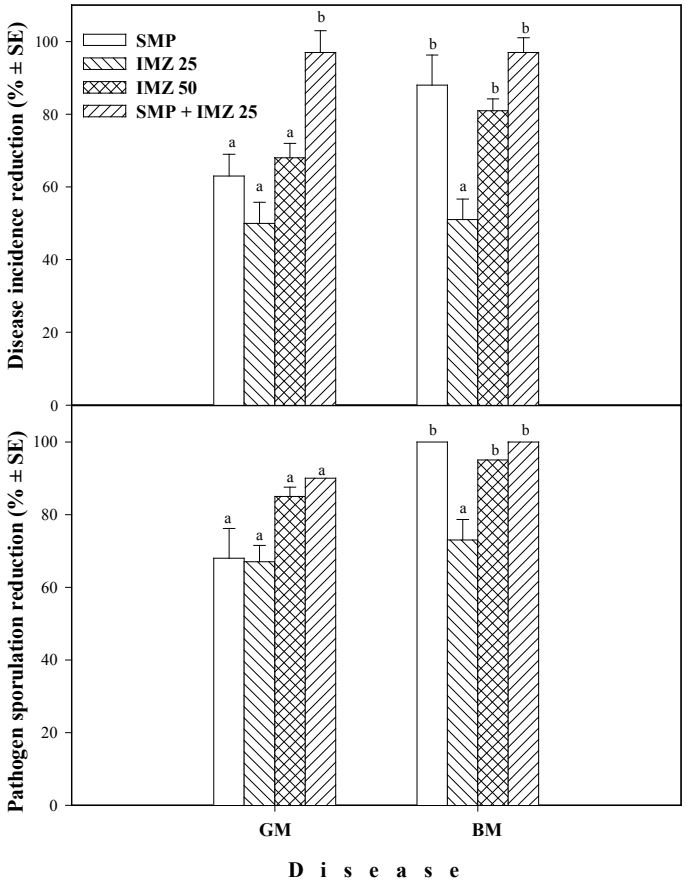

Figure 4. Effectiveness of 200 mM sodium methylparaben alone (SMP), 25 µL L^{-1} fungicide imazalil (IMZ 25), 50 µL L^{-1} imazalil (IMZ 50) and combination of 200 mM SMP and 25 µL L^{-1} IMZ (SMP + IMZ 25) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 100 and 98% for GM and BM, respectively, and pathogen sporulation of 100 and 91-98% for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means from two experiments are shown.

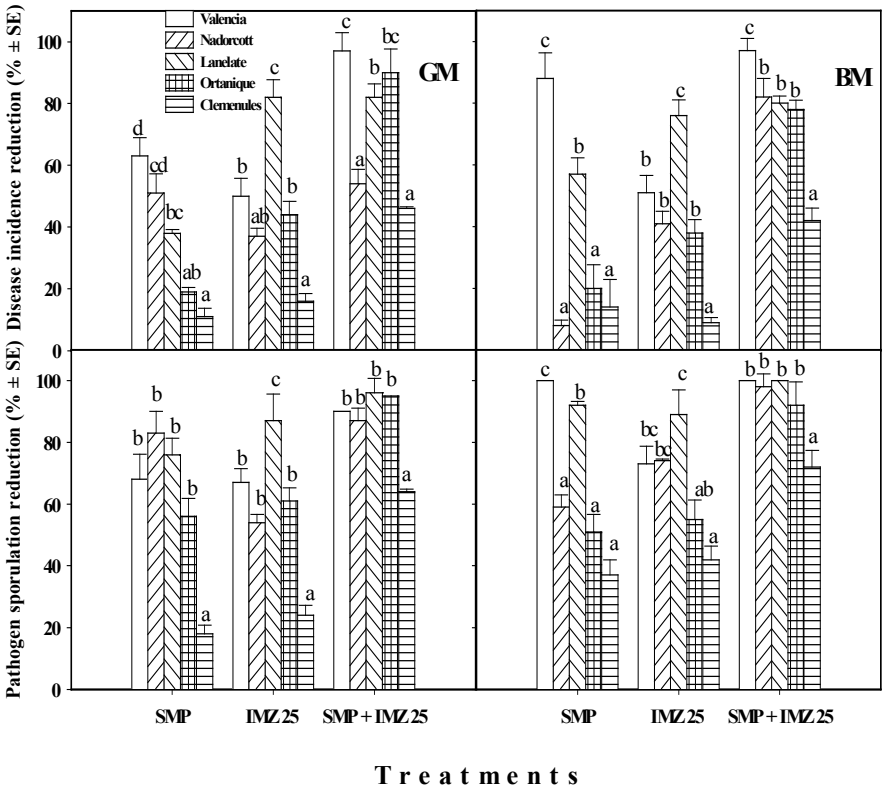

Figure 5. Incidence and sporulation of green (GM) and blue (BM) molds on citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, dipped 24 h later in water (control), 200 mM sodium methylparaben alone (SMP), 25 µL L^{-1} fungicide imazalil (IMZ 25), or 200 mM SMP combined with 25 µL L^{-1} imazalil (SMP + IMZ 25) for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 93-100 and 95-100% for GM and BM, respectively, and pathogen sporulation of 80-100 and 65-100% for GM and BM, respectively for all cultivars). For each mold and dip treatment, columns with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown.

3.1.5. Effectiveness on long-term cold-stored fruit

The incidence of both GM and BM on 'Valencia' oranges stored up to 8 weeks at 5°C and 90% RH followed by 7 d of shelf-life at 20°C were effectively reduced by the application of SMP, IMZ 25 and SMP + IMZ 25 as 60 s dips at 20°C (Fig. 6). The combined treatment was significantly more effective than the single treatments (reductions of GM and BM incidence after 8 weeks at 5°C of about 50-60% and 90-95%, respectively). After 8 weeks at 5°C plus 7 d of shelf-life at 20°C, the incidence of GM and BM on fruit treated with SMP + IMZ 25, SMP, and IMZ 25 was 16, 47 and 44%, and 19, 59 and 53%, respectively, while decay was 100% on control fruit (Fig. 6).

In general, all three treatments reduced the severity of GM and BM during the entire cold storage period at 5°C and the combination SMP + IMZ 25 was superior to the single treatments. GM lesion diameters after 4 weeks at 5°C on control, SMP-, IMZ 25-, and SMP + IMZ 25-treated oranges were about 70, 12, 26, and 0 mm, respectively. In the case of BM, these diameters were 33, 8, 15, and 3 mm, respectively. Severity on control fruit was annotated until maximum values of 130 and 115 mm for GM and BM, respectively (Fig. 6). Similarly, all three treatments significantly prevented pathogen sporulation on decayed 'Valencia' oranges stored at 5°C, being the combined treatment more effective than SMP or IMZ 25 alone (Fig. 6).

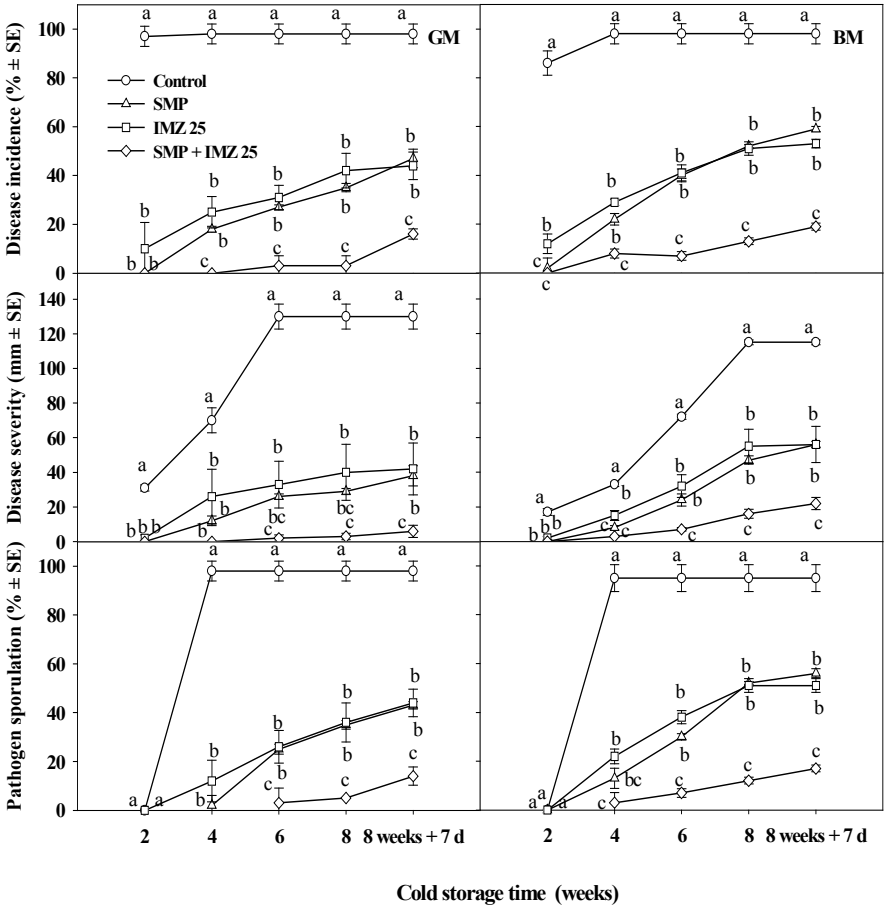

Figure 6. Incidence, severity and sporulation of green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, dipped 24 h later in water (control), 200 mM sodium methylparaben alone (SMP), 25 µL L^{-1} fungicide imazalil (IMZ 25), or 200 mM SMP combined with 25 µL L^{-1} IMZ (SMP + IMZ 25) for 60 s at 20°C, and cold stored at 5°C and 90% RH for 8 weeks followed by 7 d of shelf-life at 20°C. For each mold and evaluation date, means with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means are shown.

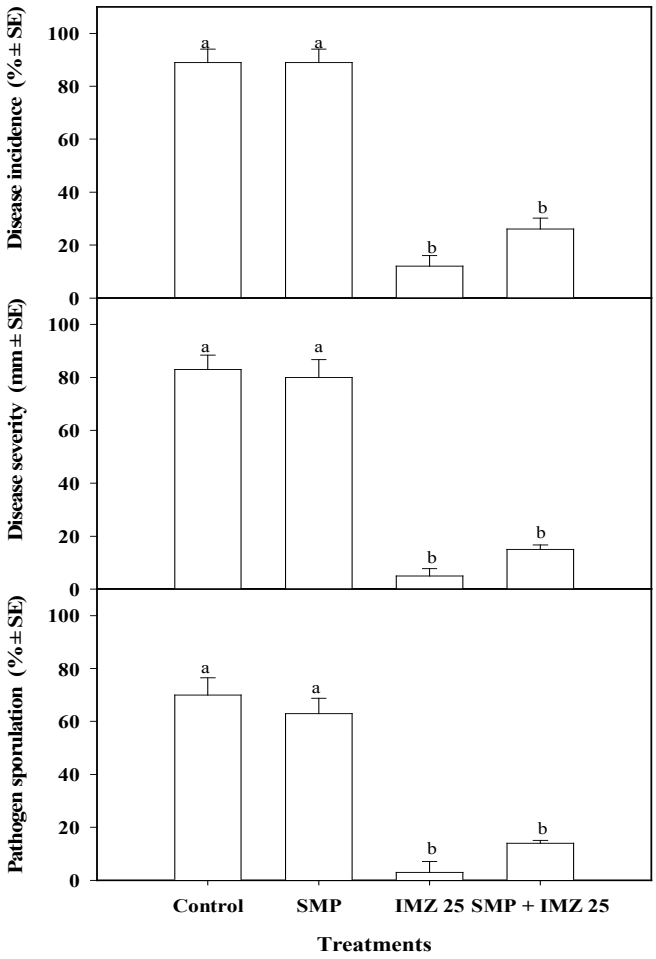

Figure 7. Preventive activity of sodium methylparaben (SMP) at 200 mM, 25 µL L^{-1} fungicide imazalil (IMZ 25), or 200 mM SMP combined with 25 µL L^{-1} IMZ (SMP + IMZ 25) against green mold on 'Valencia' oranges treated, artificially inoculated 24 h later with *Penicillium digitatum*, and incubated for 7 d at 20°C and 90% RH. Control fruit were treated with water and inoculated. Columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means from two experiments are shown.

3.2. Preventive activity

The treatments IMZ 25 and SMP + IMZ 25 effectively prevented GM on wounded and treated 'Valencia' oranges inoculated in new adjacent wounds and incubated at 20°C for 7 d. SMP treatment, however, showed no preventive activity under these experimental conditions and did not reduce the incidence of GM with respect to the control fruit (89%; Fig. 7). On the other hand, similar results were obtained for disease severity and pathogen sporulation (Fig. 7).

4. DISCUSSION

It has been characterized in this work the antifungal activity of postharvest SMP treatments against citrus GM and BM. Firstly, we have conducted an *in vivo* preliminary study in order to select the most effective concentration of SMP to inhibit the development of GM and BM on previously inoculated oranges (curative activity). A concentration of 200 mM SMP was chosen, among the wide range of concentrations tested, to be used in further small-scale trials. Secondly, a set of experiments was designed to determine the treatment conditions for dips in SMP aqueous solutions, and dips at 20°C for 60 s were selected. It seems from this result that the synergy between heat (water heated at non-phytotoxic temperatures) and SMP for disease control is lower than that between heat and other food additives also tested as alternative control means (Palou et al., 2001; Montesinos-Herrero et al., 2009). According to our results, this is due to the elevated control level obtained with SMP dips at room temperature (20°C; Fig. 3) and this is a factor that may facilitate the commercial adoption of postharvest SMP treatments in citrus packinghouses, since implementation and application costs of non-heated solutions would be considerably lower. Furthermore, as shown in the tests at 62ºC, there is a risk of rind phytotoxicity when heated SMP solutions are used. In this work, the curative activity of SMP applied alone at 200 mM (3% w v^{-1}) with the same dip and incubation conditions to 'Valencia' oranges or 'Clemenules' mandarins was equiparable or even higher than that of heated solutions of sodium carbonate, potassium benzoate, and sodium bicarbonate at similar concentrations (2-3%) (Palou et al., 2001; 2002a,b; 2007). However, it

was slightly inferior to that of heated solutions of potassium sorbate or sodium benzoate at these concentrations (Palou et al., 2002b; Montesinos-Herrero et al., 2009). Although depending on amounts and sources, the price of SMP (industrial product) may be slightly higher than that of other GRAS compounds, it could be used as an alternative treatment in citrus packinghouses, especially when there are technical or economical problems to effectively heat large volumes of antifungal treatment solutions.

Potential antifungal activity of SMP against postharvest pathogens causing fruit decay has been demonstrated by scarce *in vitro* previous studies: SMP showed a strong inhibitory effect on mycelial growth and conidia germination of *Botrytis cinerea* at the high dose of 1,000 μg mL^{-1} (Yildirm and Yapici, 2007), and films containing SMP inhibited the growth of *P. digitatum* and *P. italicum* in dichloran rose-bengal chloramphenicol agar (DRBC) (Valencia-Chamorro et al., 2008). Furthermore, it has also been supported by an *in vivo* research work in which edible fruit coatings containing SMP as an ingredient significantly reduced the incidence of both GM and BM on 'Clemenules' mandarins artificially inoculated with the pathogens 24 h before treatment, and incubated at 20°C for 7 d (Valencia-Chamorro et al., 2009).

The effectiveness of 200 mM SMP applied in aqueous dips was considerably lower than that obtained in *in vivo* primary screenings (GM and BM incidence reduction of 100%; Fig. 2), especially in the case of GM. This was possibly due to the increased contact time of the SMP drop with the rind wound inoculated with *P. digitatum* or *P. italicum* with respect to the dip contact time. In fact, at 20 and 50°C, GM was more satisfactorily controlled by longer immersion times (Fig. 3). On the other hand, rinsing the treated fruit with fresh water could also slightly reduce the treatment efficacy, as it has been shown, for instance, in work with clementines and other antifungal salts like sodium carbonates (Larrigaudière et al., 2002; Palou et al., 2007). However, in other cases, rinsing the fruit at low pressure did not significantly reduce the efficacy of GRAS compounds (Smilanick et al., 1999) and it was an effective method to avoid potential phytotoxicities and negative effects on fruit quality (loss of weight and

rind firmness), especially on fruit that will be cold-stored for long periods (Larrigaudière et al., 2002).

Overall, we demonstrated in this work that SMP and the fungicide IMZ are compatible treatments that might be used in combination to control the development of GM and BM on citrus fruit. SMP, therefore, may be useful to reduce fungicide residues on fruit surface and also to establish a management program to diminish the risks of proliferation of *Penicillium* resistant strains in citrus packinghouses. A synergistic activity was observed when SMP was combined with the fungicide IMZ at low dosages and in general the control of both GM and BM was improved in comparison with that provided by SMP and IMZ applied alone. The effectiveness of the combination (SMP + IMZ 25) was comparable to that of potassium sorbate combined with 25 μL L^{-1} IMZ applied to oranges or mandarins in previous research (Smilanick et al., 2008; Montesinos-Herrero et al., 2009).

Regarding the effectiveness of SMP to control GM and BM on major citrus species and cultivars, we have generally observed that the treatments were more effective on oranges than on mandarins. These differences on treatment effectiveness clearly show the strong influence that intrinsic fruit characteristics may have on either fruit susceptibility to infection by *P. digitatum* and *P. italicum* or on fruit response to SMP application. In fact, the influence of the type of fruit on the performance of other food additives or GRAS substances was also reported in previous studies. Palou et al. (2001, 2002a) found that treatments with aqueous solutions of sodium bicarbonate or sodium carbonate for 150 s were significantly less effective against green and blue molds on mandarins than on oranges. Similar observations were reported by Montesinos-Herrero et al. (2009) regarding potassium sorbate treatments. In general, the inhibitory ability of low toxicity antifungal compounds such as SMP or other food additives depends on the presence of residues of the compound within the wound infection courts occupied by the fungus and on interactions between this residue and constituents of the rind (Smilanick et al., 1999; Palou et al., 2001, 2002b). Apparently, the nature of such interactions would be different according to the citrus species and cultivar as a consequence of different flavedo and albedo characteristics or presence of different constituents (preformed antifungal compounds)

in the rind. Additionally, such constituents and their concentration in the rind would be determined by not only the genotype but also the fruit physical and physiological condition. On the one hand, these factors determine the natural fruit susceptibility to decay; mature citrus fruits are typically more susceptible to decay than immature ones because, among other possible causes, their level of preformed antifungal compounds is lower. On the other hand, the biosynthesis and/or accumulation of antifungal compounds as a response to different postharvest treatments is also lower in mature fruit (del Río and Ortuño, 2004; Ben-Yehoshua and Porat, 2005). It is known that an indirect mechanism of action of certain postharvest treatments such as heat (Ben-Yehoshua and Porat, 2005) or solutions of some GRAS compounds (Venditti et al., 2005) is the induction in the treated fruit tissues of disease resistance. According to these considerations, the relatively poor performance of SMP and the other tested treatments on 'Clemenules' and in some cases on 'Nadorcott' mandarins could be explained by the weak physical condition of their rind and perhaps a reduced ability to synthesize antifungal compounds. At commercial maturity, the rind of these mandarin cultivars is typically soft and thin and they can ripen to an overmature stage during the postharvest phase easier and faster than other citrus species and cultivars such as 'Valencia' or 'Lanelate' oranges.

It is generally believed that SMP has an inhibitory effect on membrane transport and mitochondrial function processes, and its antimicrobial activity is higher against fungi than bacteria (Soni et al., 2005). SMP might interfere on both the germinative and vegetative phases of microbial development, but spore germination is much more susceptible to SMP than vegetative growth in fungi (Watanabe and Takesue, 1976). Paraben salts like SMP are more soluble in water than their correspondent parabens. In aqueous solutions, they act as phenolic weak acids. When a weak acid is dissolved in water, equilibrium is established between undissociated acid molecules and charged ions, the proportion of undissociated acid increasing with lower pH. Currently, the most accepted theory for preservative action suggests inhibition via depression of internal pH. Undissociated acid molecules are lipophilic and pass readily through the plasma membrane by diffusion. In the cytoplasm (approximately pH 7.0), acid molecules dissociate into charged anions and protons. These cannot

pass across the lipidic bilayer and accumulate in the cytoplasm, thus lowering pH and inhibiting metabolism (Krebs et al., 1983). Since the carboxylic acid ester is neutral and not acidic, parabens do not have the same pH dependence than benzoic acid or other organic acids. In general, SMP is effective in acid, neutral and slightly alkaline solutions (pH 4.5-7.5) (Rosen and Berke, 1973; Soni et al., 2005). The toxicity of SMP aqueous solutions at its natural pH of 10.2 is low with respect at a pH of 5 (77% of undissociated acid proportion). However, when applied to citrus fruit they become more active within the wounds in the albedo tissue because of the relatively low pH in these wounds. Rind pH of citrus fruit ranges from 4 to 6 depending on the species and cultivar (Smilanick et al., 1999, 2005; Prusky et al., 2004), and according to this, SMP would be more effective against *P. digitatum* or *P. italicum* on fruit with lower rind pH. However, such rind pH differences cannot explain alone the different effectiveness of the treatments among different citrus species and cultivars.

Overall, we found that SMP applied alone, IMZ 25, and SMP + IMZ 25 all were very effective to reduce the incidence of GM and BM on inoculated and long-time cold-stored 'Valencia' oranges (Fig. 6). Among these treatments, the combination was significantly superior (GM and BM incidence reductions higher than 80%) to the single treatments (GM and BM incidence reductions of 60 and 20-50%, respectively) after 8 weeks at 5°C plus 7 d at 20°C. The effect of these treatments on disease severity and pathogen sporulation showed a very similar trend. In general, disease control was slightly lower for BM than GM, which might be explained because *P. italicum* is better adapted to grow at temperatures below 10 °C than *P. digitatum* (Brown and Eckert, 2000). In any case, citrus fruit treated with SMP or combinations could be used for long-term storage at commercial refrigeration temperatures.

It is clear in this study that SMP applied alone to oranges as dips at 20ºC for 60 s showed no preventive or protective effect to control GM in new rind wounds inflicted 2 mm away from the original wounds. Both GM incidence and severity and sporulation of *P. digitatum* were not reduced by application of SMP 24 h prior to fungal inoculation. New adjacent wounds were done because it was observed in preliminary trials that original wounds in control fruit were naturally

healed during the time between treatment and inoculation, and the inoculated fungus had not the ability to infect the old wounds. In contrast, preventive treatments with IMZ 25 (alone or combined with SMP) greatly reduced GM after 7 d of incubation at 20°C (Fig. 7). This result indicates that SMP, unlike IMZ, completely lacks systemic activity and it is not able to spread itself through flavedo and albedo tissues even a distance as short as 2 mm. Therefore, the postharvest treatment will not provide protective effect against infections that may take place in new wounds inflicted during fruit handling in citrus packinglines or storage rooms. Similar results have been reported for other food additives or GRAS substances tested as postharvest antifungal treatments such as sodium carbonate, sodium bicarbonate or potassium sorbate (Usall et al., 2008; Montesinos-Herrero et al., 2009). This is a clear disadvantage of these alternative control means with respect to conventional fungicides that can only be overcome by the integration of treatments with different modes of action.

Information gathered from this study provides an important basis for further research work into the uses of SMP for control of citrus postharvest *Penicillium* molds. Due to the high effectiveness of aqueous solutions, especially at room temperature, and the compatibility with the fungicide IMZ applied at low doses, SMP treatments may be new tools to include in integrated disease management programs, especially suitable for citrus packinghouses either suffering high levels of fungicide resistant strains of *Penicillium* spp. or working with certain citrus cultivars destined to international markets with low or zero pesticide residue tolerances.

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CAPÍTULO 5

Control of citrus postharvest penicillium molds with sodium ethylparaben

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] CLARA MONTESINOS-HERRERO[1] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

The curative antifungal activity of postharvest sodium ethylparaben (SEP) treatments against citrus green (GM) and blue (BM) molds was determined on different citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *Penicillium italicum* and incubated at 20°C and 90% RH for 7 d or stored at 5°C and 90% RH for 8 weeks plus 7 d of shelf-life at 20°C. The best concentration was selected in *in vivo* primary screenings with 'Valencia' oranges. SEP at 80 mM was tested at 20, 50 or 62°C for 30, 60 or 150 s in small-scale trials to determine the best dip treatment conditions. Dips of 80 mM SEP at 20°C for 60 s were selected and applied alone or in combination with 25 μL L^{-1} of the conventional fungicide imazalil (SEP + IMZ 25). Imazalil at the very low concentrations of 25 (IMZ 25) or 50 μL L^{-1} (IMZ 50) was also tested. Effectiveness of SEP alone at 20°C for 60 s was significantly higher on oranges (cvs. 'Valencia' and 'Lanelate') than on mandarins (cvs. 'Clemenules', 'Nadorcott' and 'Ortanique'), with GM and BM incidence reductions of up to 57-73% after 7 d at 20°C. SEP was compatible with IMZ 25 and consistently improved its performance, irrespective of citrus cultivars and storage conditions. All treatments were less effective on 'Clemenules' mandarins. On 'Valencia' oranges stored for 8 weeks at 5°C and 7 d at 20°C, the combined treatment was significantly more effective than the single treatments (reductions of GM and BM incidence of about 96-93% and 55-39%, respectively). In additional tests, SEP, IMZ 25 and the combination applied at 20°C for 60 s prevented GM on 'Valencia' oranges treated, inoculated with *P. digitatum* 24 h later and incubated at 20°C for 7 d. It can be concluded from these results that SEP might be an integrating nonpollutant control alternative to be included in citrus postharvest disease control programs in the future.

Key words: *Penicillium digitatum*, *Penicillium italicum*, oranges, disease incidence, food additives, nonpollutant disease control.

1. INTRODUCTION

Postharvest green mold (GM), caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., and postharvest blue mold (BM), caused by *Penicillium italicum* Wehmer, are the most economically important postharvest diseases of citrus in Spain, California, and all citrus production areas characterized by low summer rainfall (Ecker and Eaks, 1989; Palou et al., 2007). BM is especially important on citrus fruit kept under cold storage, but GM may cause 60-80% of decay under ambient conditions (Brown and Ecker, 2000; Palou et al., 2002b). However, the severity of losses due to these fungi varies depending upon production area, cultivar, climatic conditions and postharvest handling practices (Iqbal et al., 2012). Currently, these diseases are primarily controlled by application of synthetic fungicides such as imazalil (IMZ) or thiabendazole (Holmes and Ecker, 1999; Palou et al., 2002a), but the efficacy of fungicides is decreasing due to development of resistance by pathogens, which is limiting fruit production worldwide (Brent and Hollomon, 2007). Furthermore, in the development and use of chemical fungicides for postharvest disease control, considerable attention must be given to the preservation of the global environment.

Parabens are widely used as preservatives in food, cosmetic and pharmaceutical products. Acute, subchronic, and chronic studies in rodents indicate that parabens can be considered as low-toxicity compounds. Parabens are rapidly absorbed, metabolized, and excreted (Mills et al., 2004; Soni et al., 2005), and they are classified as "generally regarded as safe" (GRAS) compounds, approved for use in foods by the US Food and Drug Administration (FDA) and European Union (EU) regulations (Mills et al., 2004). The antimicrobial activity of the parabens increases as the chain length of the alkyl group increases; their aqueous solubility, however, decreases, so the sodium salts of parabens are also frequently used in formulations (Giordano et al., 1999). It is generally believed that their inhibitory effects on membrane transport and mitochondrial function processes are key on their mode of action (Soni et al., 2005). Therefore, the use of parabens might be considered as one of the nonpolluting alternative methods to conventional fungicides for the control of postharvest diseases of

horticultural products (Palou et al., 2008; Montesinos-Herrero et al., 2009; Valencia-Chamorro et al., 2009).

One of the paraben sodium salts, sodium ethylparaben (SEP), has demonstrated to be useful to control postharvest pathogens on citrus fruit (Valencia-Chamorro et al., 2008, 2009). However, in these studies, SEP was tested in *in vitro* conditions or in applications incorporated to edible coatings. Thus, little information is available about the use of aqueous solutions of SEP applied as potential postharvest antifungal treatments, particularly against penicillium molds of citrus fruit. The present research had the following aims: (1) to preliminarily evaluate the curative activity of SEP at different concentrations against GM and BM, (2) to optimize the SEP dip treatment conditions, (3) to determine the SEP compatibility with low doses of imazalil under selected dip conditions, (4) to determine the effectiveness of SEP treatments on economically important citrus species and cultivars, (5) to evaluate the curative activity of SEP treatments on long-term cold-stored citrus fruit and (6) to evaluate the preventive activity of SEP treatments against citrus postharvest GM.

2. MATERIALS AND METHODS

2.1. Fruit

The experiments were conducted with 'Valencia' and 'Lanelate' oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' (synonyms: 'Nules', 'Clementina de Nules') clementine mandarins (*Citrus reticulata* Blanco), and 'Nadorcott' (*C. reticulata* x *C. sinensis*; synonyms: 'Afourer', 'W. Murcott') and 'Ortanique' [*C. reticulata* x (*Citrus sinensis* x *C. reticulata*); synonym: 'Topaz'] hybrid mandarins. Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored up to 1 week at 5°C and 90% relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with tap water and allowed to air dry at room temperature.

2.2. Fungal inoculation

P. digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25ºC. Conidia of each fungus from 7 to 14-day-old cultures were taken from the agar surface with a sterile rod and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 or 10^6 spores mL^{-1} using a haemocytometer. With the exceptions of *in vivo* primary screenings and preventive activity tests, the fruit inoculation procedure was as follows: the tip of a stainless steel rod, 1 mm wide and 2 mm in length, was immersed in the conidial suspension and inserted in the fruit rind afterwards. Fruit were inoculated at two opposite points in the fruit equatorial zone, one with *P. digitatum* and the opposite with *P. italicum*. Inoculated fruit were kept in a temperature-controlled room at 20ºC for 24 h, until treatment.

2.3. Curative activity

2.3.1. *In vivo* primary screenings

SEP (Ethyl 4-hydroxybenzoate sodium salt; Merck KgaA, Darmstadt, Germany; Table 1, Fig. 1) was tested at eight concentrations to control citrus postharvest GM and BM on fruit previously inoculated with the pathogens. A sterile mother solution of SEP was prepared at a concentration of 100 Mm. Sterile solutions at concentrations of 0.1, 1, 10, 20, 30, 40, 70 and 100 mM SEP were prepared by diluting with sterile water. Inoculum preparation was carried out following the procedure described above. In this case, 30 µL of conidial suspension of *P. digitatum* or *P. italicum* were placed, using a micropipette, on rind wounds made with the stainless steel rod described above. Different sets of fruit were used for each pathogen. About 24 h after the inoculation of the pathogen, 30 µL of SEP solution at the above mentioned concentrations were placed, using a micropipette, in the same inoculation rind wound. Control fruit were treated with 30 µL of sterile distilled water. For each combination of

concentration of SEP and pathogen, 4 replicates of 5 'Valencia' oranges each were used. Treated fruit were incubated at 20ºC and 90% RH for 6 d, at which time disease incidence (% of infected fruit) and severity (lesion diameter) were determined. Severity was assessed over the entire fruit sample, not only over infected and symptomatic fruit.

Table 1. Physicochemical properties of sodium ethylparaben

Property	Description
Formula	$C_9H_9NaO_3$ (Fig. 1)
Molecular weight (g/mol)	188.1631
Synonyms	*p*-hydroxybenzoic acid ethyl ester sodium salt Sodium ethyl-*p*-hydroxybenzoate Sodium 4-ethoxycarbonylphenoxide Ethyl-4-hydroxy benzoic acid sodium salt Sodium 4-(ethoxycarbonyl) phenolate benzoic acid 4-hydroxy-, ethyl ester, sodium salt
E number	E-215
Physical state	White hygroscopic crystalline powder
Melting point (°C)	115-118
Boiling point (°C)	297-298
Solubility in water	Soluble
PH	9.5-10.5 (10% w/v aqueous solution)

Source: Merck KgaA, Darmstadt, Germany

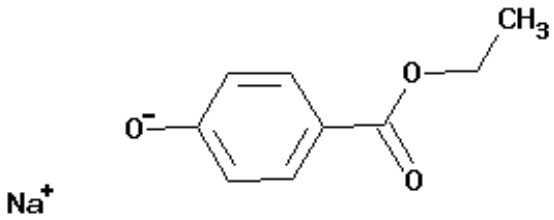

Figure 1. Chemical structure of sodium ethylparaben

2.3.2. Dip treatment conditions

Small-scale trials were conducted using 'Valencia' oranges to establish the best dip treatment conditions. Fungal inoculation with a concentration of 10^6 spores mL^{-1} was carried out following the procedure mentioned above.

Stainless steel buckets containing 10 L of aqueous solution of 80 mM (13.29 g/L; 1.3% w/v) SEP were used. This concentration of SEP was selected according to previous results obtained in the *in vivo* primary screening tests. When needed, solutions were heated by placing the buckets in a 250-L stainless steel water tank fitted with two electrical resistances (4.5 kW each), a thermostat, and an automatic water-recirculating system. Fruit were placed into 18 L multi-perforated wall stainless steel containers, exactly fitting in the above mentioned buckets, and completely immersed in the treatment solution for 30, 60 or 150 s at 20, 50 or 62°C. After treatment, the fruit were rinsed for 5 s with tap water at low pressure in order to eliminate paraben salt residues. Control fruit were dipped in water alone at 20°C. Sixty fruit per treatment (3 replicates of 20 fruit each) were arranged in plastic cavity sockets on cardboard trays. Treated fruit were incubated at 20°C and 90% RH for 7 d. Disease incidence was assessed after 7 d of incubation. Potential fruit phytotoxicity caused by SEP or heat was visually assessed after 3 d at 20°C. For this purpose, fruit were classified into one of four categories, depending on rind appearance: 0 = no rind damage; 1 = slight brownish blemishes present (<10% fruit surface); 2 = moderate brownish blemishes present (>10% and <25% fruit surface) and 3 = severe rind injury (>25% fruit surface). A ponderate rind pitting index (0–3 scale) was calculated for each treatment.

2.3.3. Combination with low doses of imazalil

In order to determine the effect of the combination of the paraben salt with low doses of the chemical fungicide imazalil (IMZ; (±)-1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl)-1H-imidazole;
Fecundal 7.5% EC; Fomesa Fruitech S.L., Valencia, Spain) to control

green and blue molds, the following treatments were considered: (1) water (control), (2) 80 mM SEP (SEP), (3) 25 μL L^{-1} IMZ (IMZ 25), (4) 50 μL L^{-1} IMZ (IMZ 50) and (5) combination of 80 mM SEP with 25 μL L^{-1} IMZ (SEP + IMZ 25). Aqueous solutions of both chemicals were mixed into 10 L buckets and manually stirred with a clean plastic rod. IMZ was used at two doses considerably lower than those recommended for commercial applications. This experiment was conducted using 'Valencia' oranges. Fungal inoculation and dip treatments were performed following the procedure mentioned above. Dip conditions were temperature of 20ºC and immersion time of 60 s. After treatment, only fruit treated with SEP were rinsed with tap water for 5 s. Each treatment was applied to 3 replicates of 20 fruit each. Disease incidence and pathogen sporulation (% of lesions showing spores) were determined after 7 d of incubation at 20ºC and 90% RH. Pathogen sporulation was assessed over the entire fruit sample, not only over infected and symptomatic fruit. Fruit phytotoxicities were assessed after 3 d at 20ºC. The experiment was repeated twice.

2.3.4. Effectiveness on major citrus species and cultivars

To assess whether the effectiveness of curative treatments was dependent on the host fruit, different citrus species and cultivars were subjected to the following treatments: (1) water (control), (2) 80 mM SEP (SEP), (3) 25 μL L^{-1} IMZ (IMZ 25), or (4) 80 mM SEP + 25 μL L^{-1} IMZ (SEP + IMZ 25). All treatments were applied as dips at 20ºC for 60 s. The trials were conducted with commercially important orange ('Valencia' and 'Lanelate') and mandarin cultivars ('Nadorcott', 'Ortanique' and 'Clemenules'). The experimental design, fungal inoculation and dip treatments followed the same procedures previously described. Treated fruit were not rinsed with tap water, with the exception of fruit treated with SEP alone. Treated fruit were incubated at 20ºC and 90% RH for 7 d, at which time disease incidence and pathogen sporulation were assessed.

2.3.5. Effectiveness on long-term cold-stored fruit

The curative capability of SEP against GM and BM on 'Valencia' oranges subjected to long-term cold storage was evaluated in an

experiment using inoculated fruit treated with: (1) water (control), (2) 80 mM SEP (SEP), (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 80 mM SEP + 25 µL L^{-1} IMZ (SEP + IMZ 25). All treatments were applied as dips at 20ºC for 60 s. Treated fruit were not rinsed with tap water, with exception of fruit treated only with SEP. Treated fruit were stored up to 8 weeks at 5ºC and 90% RH. Following the refrigeration period, the fruit were held for 7 d of shelf-life at 20ºC and 70-80% RH. Disease incidence and severity and pathogen sporulation were assessed after 2, 4, 6, and 8 weeks at 5ºC and 8 weeks at 5ºC plus 7 d at 20ºC.

2.4. Preventive activity

To evaluate whether SEP treatments or combinations showed a preventive effect on the control of GM and BM, an experiment was conducted with 'Valencia' oranges in which a 1 mm wide, 2 mm deep wound was made with a stainless steel rod on the equatorial region of each fruit to simulate natural wounds. Then, the following treatments were applied by dipping the fruit in 80 mM aqueous solution at 20ºC for 60 s: (1) water (control), (2) 80 mM SEP (SEP), (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 80 mM SEP + 25 µL L^{-1} IMZ (SEP + IMZ 25). About 24 h later, fruit were inoculated with the tip of a stainless steel rod, 1 mm wide and 2 mm in length which had been immersed in a 10^5 spores mL^{-1} conidial suspension of *P. digitatum* and inserted in a new adjacent wound (about 2 mm of separation between wounds). Each treatment consisted of 3 replicates of 20 fruit each. After inoculation, treated fruit were incubated at 20ºC and 90% RH for 7 d at which time disease incidence and severity and pathogen sporulation were assessed. The experiment was repeated twice.

2.5. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus 4.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence and pathogen sporulation were transformed to the arcsine of the square root of the proportion of infected or sporulated fruit to assure the homogeneity of variances. In some cases, reductions with respect to the controls were calculated as percentages. Statistical significance was judged at the

level $P = 0.05$. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was used to separate means. Shown values are non-transformed means.

3. RESULTS

3.1. Curative activity

3.1.1. *In vivo* primary screenings

Among the concentrations of SEP evaluated in this set of experiments, treatment with 70 mM SEP was the most effective since it completely inhibited the development of GM and BM on 'Valencia' oranges after 6 d of incubation (Fig. 2). Concentrations of 20 and 40 mM SEP totally inhibited the development of GM, but not that of BM. Moreover, treatments with 20, 30, 40 and 100 mM SEP significantly reduced the incidence of BM by 87, 62, 93 and 80%, respectively, but those with 0.1 and 1 mM SEP were not effective to reduce the incidence of both molds on 'Valencia' oranges (Fig. 2). Similarly, treatments with 10, 20, 30, 40, 70 and 100 mM SEP significantly reduced the severity of both molds. The most effective concentrations against GM and BM were 20, 40 and 70 mM and 30 and 70 mM, respectively (Fig. 2).

3.1.2. Dip treatment conditions

In the previous *in vivo* primary screenings, the concentration of 70 mM SEP was the most effective among those tested. However, the efficacy of an additional preliminary dip treatment with 80 mM SEP (data not shown) showed to be slightly superior to that of 70 mM and, therefore, this concentration was selected for its use in subsequent trials. In general, dip treatments with 80 mM SEP applied at 20 or 50°C for 30, 60, or 150 s similarly reduced the incidence of GM on treated fruit (Fig. 3). On fruit dipped for 60 or 150 s at 62°C, SEP was significantly more effective than on fruit dipped for 30 s at the same temperature, with GM reductions of 93, 97 and 78%, respectively, after 7 d at 20°C. On the other hand, on fruit dipped for 150 s at 20 or 62°C, SEP significantly reduced the incidence of BM in comparison

with fruit dipped for 30 s, with reductions of 85 and 95%, and 57 and 83%, respectively. A different trend was observed for BM on fruit dipped for 30, 60 or 150 s at 50°C, since these values were not significantly different one from each other (Fig. 3).

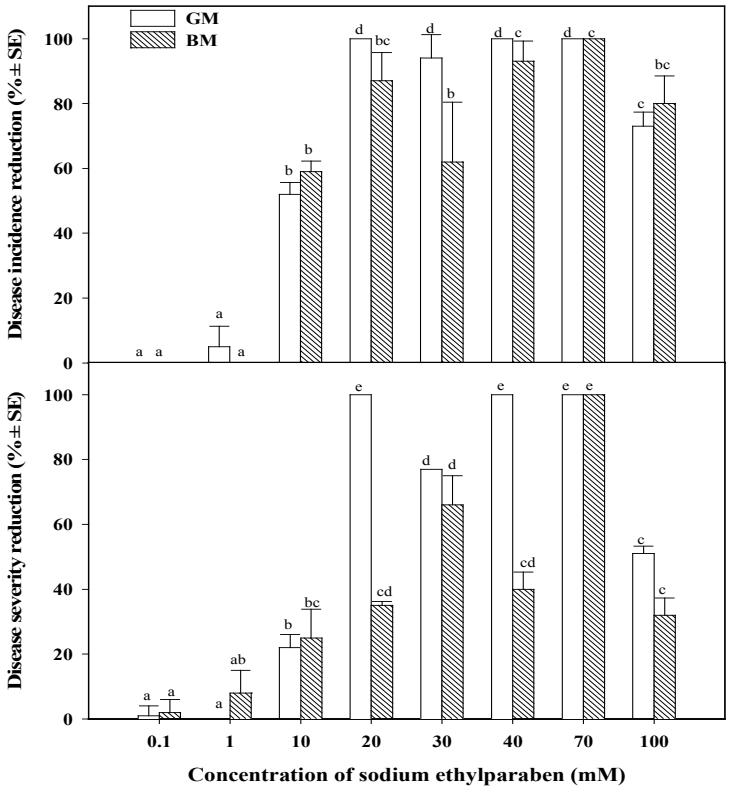

Figure 2. Curative activity of sodium ethylparaben (SEP) at different concentrations against green (GM) and blue (BM) molds in *in vivo* primary screenings with 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later, and incubated for 6 d at 20°C and 90% RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water (incidence of 80-100% and 50-90%, and severity of 94-126 mm and 39-62 mm for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Incidence values were arcsine-transformed. Non-transformed means are shown.

In most cases, SEP dips were more effective at 62°C, but oranges dipped at this temperature showed slight phytotoxic injuries on up to 37% of treated fruit (data not shown). Generally, dips at 50°C did not improve the performance of those at 20°C. Also, dips at 20°C for 150 s were not significantly better than those applied for 60 s at the same temperature (Fig. 3). Therefore, SEP dips at 20°C for 60 s were selected for use in further experiments.

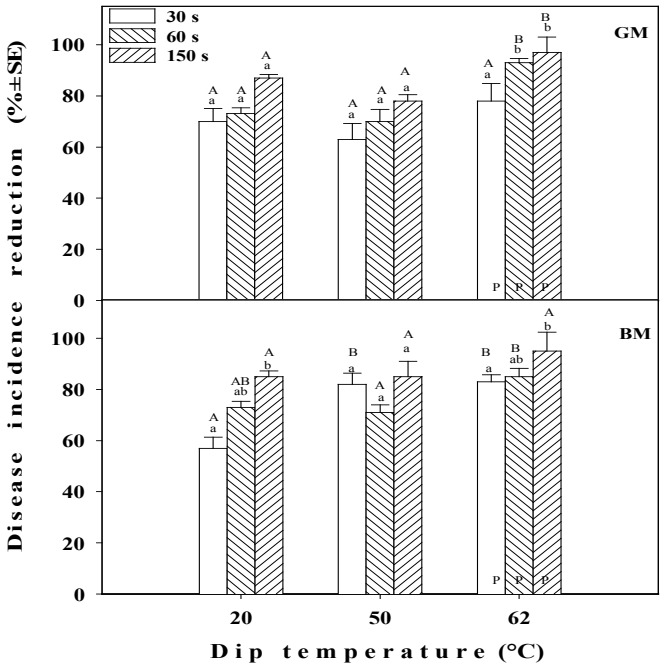

Figure 3. Effect of dip temperature and time on the effectiveness of 80 mM sodium ethylparaben (SEP) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later, and incubated for 7 d at 20°C and 90% RH. Inoculated fruit were rinsed with tap water for 5 s after treatment. Reductions of disease incidence were determined with respect to control fruit treated with water (incidence of 100% for both molds for all temperatures and times). For each mold, columns with different lowercase and capital letters indicate significantly different dip time and dip temperature, respectively, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown. 'P' indicates appearance of slight phytoxicities on the fruit rind.

3.1.3. Effect of combination with low doses of imazalil

Dips of 'Valencia' oranges at 20°C for 60 s with the combination SEP + IMZ 25 significantly enhanced the control of GM in comparison with the rest of treatments, with GM incidence reductions after 7 d at 20°C of 93%. SEP, IMZ 25 and IMZ 50 reduced GM incidence by 73, 50 and 68%, respectively, and did not significantly differ from each other (Fig. 4). Likewise, the SEP + IMZ 25 treatment significantly improved the control of BM, when compared with those of SEP and IMZ 25 treatments. IMZ 50 was equiparable to the combined treatment against BM, and reduced the incidence by 81%. On the other hand, all treatments exerted a high antisporulant activity, especially against *P. italicum*, with sporulation reductions with respect to the control treatment ranging from 68 to 97% (Fig. 4).

3.1.4. Effectiveness on major citrus species and cultivars

Overall, the effectiveness of 80 mM SEP aqueous treatment applied alone at 20°C for 60 s to control GM and BM was significantly higher on oranges than on mandarins. After incubation at 20°C for 7 d, SEP applied alone significantly reduced the incidence of GM and BM by 73 and 57% and 72 and 60% on 'Valencia' and 'Lanelate' oranges, respectively (Fig. 5). Conversely, SEP reduced the incidence of GM and BM by less than 20% on 'Nadorcott', 'Ortanique' and 'Clemenules' mandarins. Similarly, IMZ 25 and the combined treatment were more effective to reduce the incidence of GM and BM on oranges than on mandarins. The combined treatment was superior to the single treatments for the control of both GM and BM, especially on mandarin cultivars (Fig. 5). On the other hand, all three treatments presented an important antisporulant activity on most of the cultivars after 7 d at 20°C. In general, irrespective of the dip treatment, the reduction of sporulation of both *P. digitatum* and *P. italicum* was higher on oranges than on mandarins. The combination SEP + IMZ 25 generally reduced pathogen sporulation more effectively than the individual treatments (Fig. 5).

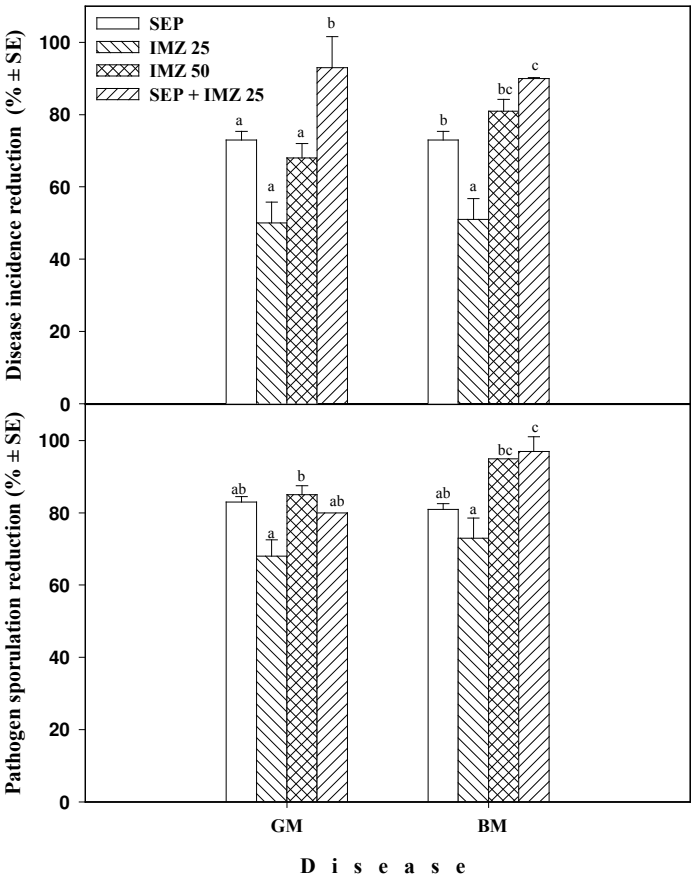

Figure 4. Effectiveness of 80 mM sodium ethylparaben alone (SEP), 25 µL L^{-1} imazalil (IMZ 25), 50 µL L^{-1} imazalil (IMZ 50) and combination of 80 mM SEP and 25 µL L^{-1} IMZ (SEP + IMZ 25) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, treated 24 h later for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 100 and 98-100%, and pathogen sporulation of 100 and 98% for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown.

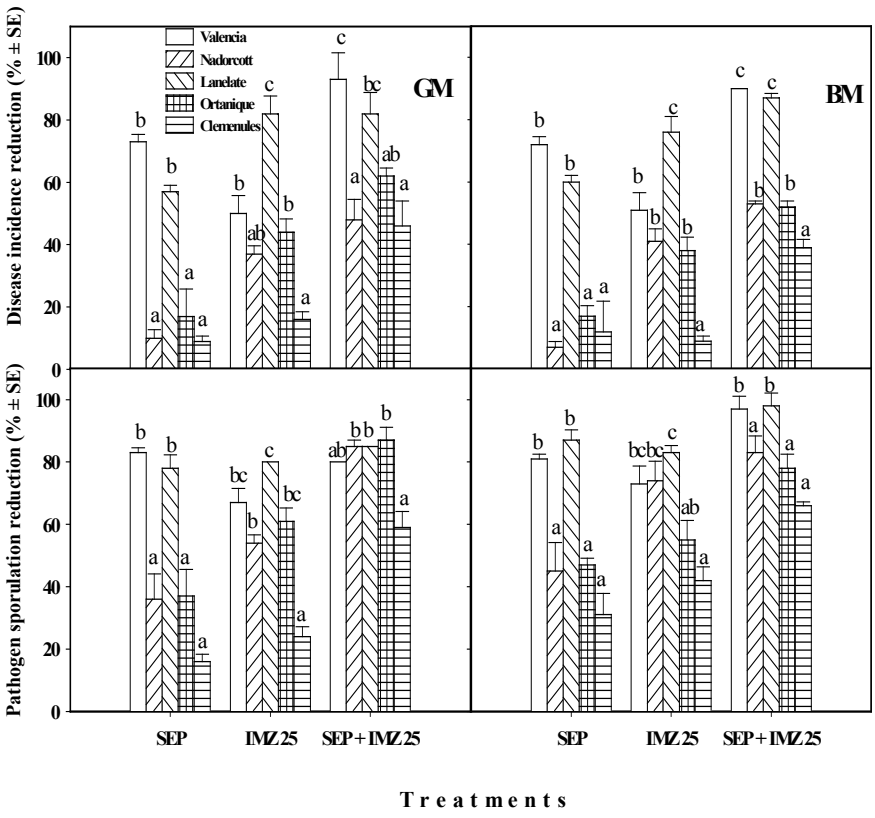

Figure 5. Incidence and sporulation of green (GM) and blue (BM) molds on citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, dipped 24 h later in water (control), 80 mM sodium ethylparaben alone (SEP), 25 µL L^{-1} imazalil (IMZ 25), or 80 mM SEP combined with 25 µL L^{-1} imazalil (SEP + IMZ 25) for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 93-100 and 95-100%, and pathogen sporulation of 80-100 and 65-100% for GM and BM, respectively, for all cultivars). For each mold and dip treatment, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown.

3.1.5. Effectiveness on long-term cold-stored fruit

The incidence of both GM and BM on 'Valencia' oranges stored for up to 8 weeks at 5°C and 90% RH followed by 7 d of shelf-life at 20°C were effectively reduced by the application of SEP, IMZ 25 and SEP + IMZ 25 as 60 s dips at 20°C (Fig. 6). The combined treatment was significantly more effective than the single treatments (reductions of GM and BM incidence after 8 weeks at 5°C of about 45-86% and 94-98%, respectively). In the case of GM, but not of BM, SEP was superior to IMZ 25 during the entire refrigeration period. After 8 weeks at 5°C plus 7 d of shelf-life at 20°C, the incidence of GM and BM on fruit treated with SEP + IMZ 25, SEP, and IMZ 25 was 3, 43 and 44%, and 7, 60 and 53%, respectively, while decay was 98% on control fruit (Fig. 6).

All three treatments significantly reduced the severity of GM and BM during the entire cold storage period at 5°C. Severity on control fruit was annotated until maximum values of 130 and 115 mm for GM and BM, respectively (Fig. 6). After 4 weeks at 5°C, lesion diameter on fruit treated with IMZ 25, SEP and the combination were about 20, 5, and 0 mm and 15, 10 and 0 mm for GM and BM, respectively (Fig. 6). Similarly, all three treatments significantly prevented pathogen sporulation on infected 'Valencia' oranges stored at 5°C, being the combined treatment more effective than SEP or IMZ 25 alone (Fig. 6). Pathogen sporulation on fruit treated with these treatments was 5, 14 and 36%, and 5, 54 and 51%, for GM and BM, respectively, after 8 weeks of cold storage at 5°C.

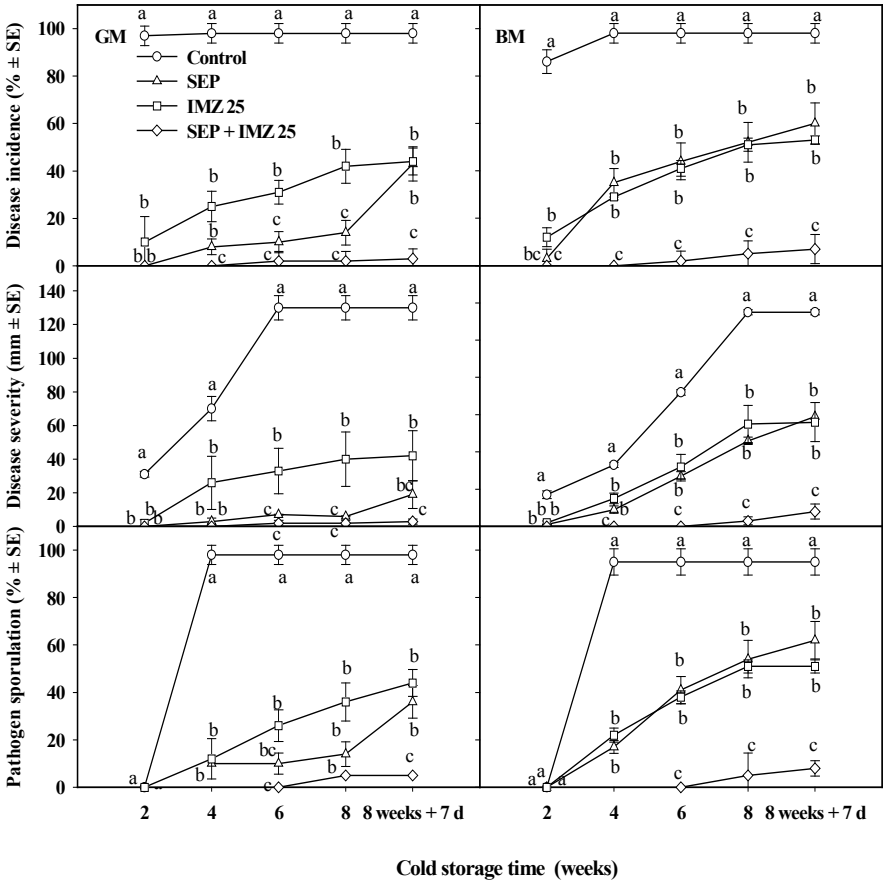

Figure 6. Incidence, severity and sporulation of green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum*, dipped 24 h later in water (control), 80 mM sodium ethylparaben alone (SEP), 25 µL L^{-1} imazalil (IMZ 25), or 80 mM SEP combined with 25 µL L^{-1} IMZ (SEP + IMZ 25) for 60 s at 20°C, and cold stored at 5°C and 90% RH for 8 weeks followed by 7 d of shelf-life at 20°C. For each mold and evaluation date, means with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means are shown.

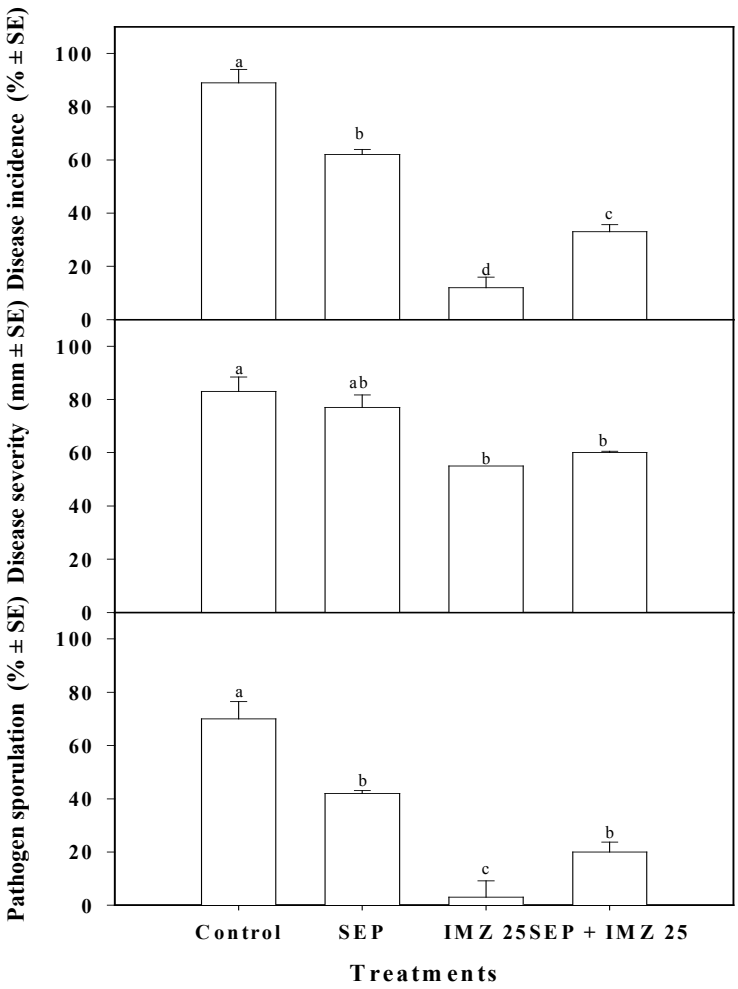

Figure 7. Preventive activity of sodium ethylparaben (SEP) at 80 mM, 25 µL L^{-1} fungicide imazalil (IMZ 25), or 80 mM SEP combined with 25 µL L^{-1} IMZ (SEP + IMZ 25) against green mold on 'Valencia' oranges treated, artificially inoculated 24 h later with *Penicillium digitatum*, and incubated for 7 d at 20°C and 90% RH. Control fruit were treated with water and inoculated. Columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means are shown.

3.2.Preventive activity

All three treatments effectively prevented GM on 'Valencia' oranges incubated at 20°C for 7 d, if compared to the control fruit (disease incidence of 89%), but IMZ 25 and SEP + IMZ 25 were superior to SEP alone (Fig. 7). The treatments IMZ 25 and SEP + IMZ 25 also significantly reduced disease severity. Likewise, pathogen sporulation followed a similar trend to disease incidence, being IMZ 25 the most effective treatment (Fig. 7).

4. DISCUSSION

In this work, the antifungal activity of postharvest SEP treatments against citrus GM and BM was determined. Firstly, we conducted an *in vivo* preliminary study in order to select the most effective concentration of SEP to inhibit the development of GM and BM on previously inoculated oranges (curative activity). Secondly, a set of experiments was designed to determine the most appropriate treatment conditions for dips in 80 mM SEP aqueous solutions, and dips at 20°C for 60 s were selected. Apparently, the combined effect of heat (water heated at non-phytotoxic temperatures) and SEP for GM and BM control is lower than that of heat and other food additives also tested as alternative control means (Palou et al., 2001; Montesinos-Herrero et al., 2009). SEP dips at room temperature showed a high control level that was not improved by heating to 50ºC. This may be due to the stability of SEP at high temperature, without significant changes in antimicrobial activity (Soni et al., 2005). Furthermore, slight phytotoxicities were observed at 62ºC. In any case, this is a factor that may facilitate the commercial adoption of postharvest SEP treatments in citrus packinghouses, since implementation and application costs of non-heated solutions would be considerably lower.

Potential antifungal activity of SEP against postharvest pathogens causing citrus fruit decay has been observed in a previous *in vitro* study, in which films containing SEP significantly inhibited the growth of *P. digitatum* and *P. italicum* in dichloran rose-bengal chloramphenicol agar (DRBC) (Valencia-Chamorro et al., 2008). Furthermore, edible coatings containing SEP as an ingredient also

reduced the incidence of both GM and BM on citrus fruits inoculated with the pathogens 24 h before treatment (Valencia-Chamorro et al., 2009).

It was demonstrated in this work that SEP and the fungicide IMZ are compatible treatments that might be used in combination to control the development of GM and BM on citrus fruit. SEP, therefore, may be useful to reduce fungicide residues on fruit surface and also to establish a management program to diminish the risks of proliferation of resistant strains of *Penicillium* spp. in citrus packinghouses. An efficacy improvement was observed when SEP was combined with the fungicide IMZ at low dosages and in general the control of both GM and BM was improved in comparison with that provided by SEP and IMZ applied alone. The effectiveness of the combination (SEP + IMZ 25) was comparable to that of potassium sorbate combined with 25 $\mu L\ L^{-1}$ IMZ applied to oranges or mandarins in previous studies (Smilanick et al., 2008; Montesinos-Herrero et al., 2009).

Regarding the effectiveness of SEP to control GM and BM on major citrus species and cultivars, we have generally observed that the treatments were more effective on oranges than on mandarins. These differences on treatment effectiveness clearly show the strong influence that intrinsic fruit characteristics may have on either fruit susceptibility to infection by *P. digitatum* and *P. italicum* or on fruit response to SEP application. In fact, the influence of the type of fruit on the performance of other food additives or GRAS substances was also reported in previous studies. Palou et al. (2001, 2002a) found that treatments with aqueous solutions of sodium bicarbonate or sodium carbonate for 150 s were significantly less effective against GM and BM on mandarins than on oranges. Similar observations were reported by Montesinos-Herrero et al. (2009) regarding potassium sorbate treatments. In general, the inhibitory ability of low toxicity antifungal compounds such as SEP or other food additives depends on the presence of residues of the compound within the wound infection courts occupied by the fungus and on interactions between this residue and constituents of the rind (Smilanick et al., 1999; Palou et al., 2001, 2002b). Apparently, the nature of such interactions would be different according to the citrus species and cultivar as a consequence of different flavedo and albedo characteristics or presence of different

constituents (preformed antifungal compounds) in the rind. Additionally, such constituents and their concentration in the rind would be determined by not only the genotype but also the fruit physical and physiological condition. On the one hand, these factors determine the natural fruit susceptibility to decay; mature citrus fruits are typically more susceptible to decay than immature ones because, among other possible causes, their level of preformed antifungal compounds is lower. On the other hand, the biosynthesis and/or accumulation of antifungal compounds as a response to different postharvest treatments is also lower in mature fruit (del Río and Ortuño, 2004; Ben-Yehoshua and Porat, 2005). It is known that an indirect mechanism of action of certain postharvest treatments such as heat (Ben-Yehoshua and Porat, 2005) or solutions of some GRAS compounds (Venditti et al., 2005) is the induction in the treated fruit tissues of disease resistance. According to these considerations, the relatively poor performance of SEP and the other tested treatments on 'Clemenules', 'Nadorcott' and 'Ortanique' mandarins could be explained by the weaker physical condition of their rind and perhaps a reduced ability to synthesize antifungal compounds.

It is generally believed that SEP has an inhibitory effect on membrane transport and mitochondrial function processes, and its antimicrobial activity is higher against fungi than bacteria (Soni et al., 2005). SEP might interfere on both the germinative and vegetative phases of microbial development, but spore germination is much more susceptible to SEP than fungal vegetative growth (Watanabe and Takesue, 1976). Paraben salts like SEP are more soluble in water than their correspondent parabens. In aqueous solutions, they act as phenolic weak acids. When a weak acid is dissolved in water, equilibrium is established between undissociated acid molecules and charged ions, the proportion of undissociated acid increasing with lower pH. Currently, the most accepted theory for preservative action suggests inhibition via depression of internal pH. Undissociated acid molecules are lipophilic and pass readily through the plasma membrane by diffusion. In the cytoplasm (approximately pH 7.0), acid molecules dissociate into charged anions and protons. These cannot pass across the lipidic bilayer and accumulate in the cytoplasm, thus lowering pH and inhibiting metabolism (Krebs et al., 1983). Since the carboxylic acid ester is neutral and not acidic, parabens do not have

the same pH dependence than benzoic acid or other organic acids. In general, SEP is effective in acid, neutral and slightly alkaline solutions (pH 4.5-7.5) (Rosen and Berke, 1973; Soni et al., 2005), therefore, toxicity of SEP aqueous solutions at its natural pH of 9.9 is low. However, when applied to citrus fruit they become more active within the wounds in the albedo tissue because of the relatively low pH in these wounds. Rind pH of citrus fruit ranges from 4 to 6 depending on the species and cultivar (Smilanick et al., 1999, 2005; Prusky et al., 2004), and according to this, SEP would be more effective against *P. digitatum* or *P. italicum* on fruit with lower rind pH. However, such rind pH differences cannot explain alone the different effectiveness of the treatments among different citrus species and cultivars.

Overall, we found that dip treatments with SEP alone, IMZ 25, and SEP + IMZ 25 all were very effective to reduce GM and BM on inoculated and long-term cold-stored 'Valencia' oranges (Fig. 6). Among these treatments, the combination was significantly superior to the single treatments after 8 weeks at 5°C plus 7 d at 20°C. During cold storage, however, SEP and SEP + IMZ 25 controlled GM, but not BM, more effectively than IMZ 25 alone. In general, disease control was lower for BM than GM, which might be explained because *P. italicum* is better adapted to grow at temperatures below 10°C than *P. digitatum* (Brown and Eckert, 2000). In any case, citrus fruit treated with SEP or combinations could be used for long-term storage at commercial refrigeration temperatures.

In this study, SEP applied as dips at room temperature for 60 s showed a limited preventive activity against *P. digitatum* on oranges. Although this effect was considerably lower than that of IMZ 25 and SEP + IMZ 25, it was higher than that of other food additives or GRAS substances tested as postharvest antifungal treatments such as sodium carbonate, sodium bicarbonate or potassium sorbate (Usall et al., 2008; Montesinos-Herrero et al., 2009). In contrast to IMZ and other conventional postharvest fungicides, most of the chemicals recognized as food additives have a limited ability to spread through the epidermis to inner layers in the rind of citrus fruits.

Results from this research provide an important basis for further work into the uses of SEP for the control of citrus postharvest

penicillium molds. SEP treatments may be new tools to include in integrated disease management programs, especially in the case of citrus cultivars destined to markets with low or zero pesticide residue tolerances.

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CAPÍTULO 6

Antifungal activity of sodium propylparaben alone or in combination with low doses of imazalil against Penicillium digitatum *and* Penicillium italicum *on citrus fruit*

PEDRO A. MOSCOSO-RAMÍREZ,[1,2] CLARA MONTESINOS-HERRERO[1] AND LLUÍS PALOU[1]

[1]Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d´Investigacions Agràries (IVIA), 46113 Montcada, Valencia, Spain.

[2]Campus Tabasco, Colegio de Postgraduados, 86500 H. Cárdenas, Tabasco, México.

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Abstract

BACKGROUND: The performance of postharvest treatments with the GRAS compound sodium propylparaben (SPP), alone or combined with low doses of the fungicide imazalil (IMZ), against citrus green (GM) and blue (BM) molds was evaluated on several citrus species and cultivars artificially inoculated with *Penicillium digitatum* and *Penicillium italicum*, respectively, and incubated at 20°C or cold-stored at 5°C.

RESULTS: Effectiveness of 100 mM SPP dips at 20°C for 60 s was higher on oranges than on mandarins, with GM and BM incidence reductions of up to 60-90% after 7 d at 20°C. Irrespective of citrus cultivar and storage condition, SPP generally improved the curative action of 25 μL L^{-1} IMZ to control penicillium molds. In additional tests, 100 mM SPP dips at 20°C for 60 s only prevented GM on 'Valencia' oranges inoculated 24 h after treatment when combined with IMZ.

CONCLUSION: It can be concluded that postharvest SPP treatments might be a nonpolluting alternative to be included in citrus postharvest disease control programs in the future.

Key words: citrus, food additives, green mold, blue mold, alternative postharvest disease control, imazalil

INTRODUCTION

Significant losses can occur after harvest during storage and marketing of citrus fruit primarily due to green mold (GM), caused by the pathogen *Penicillium digitatum* (Pers.:Fr.) Sacc. and secondarily by blue mold (BM) caused by *P. italicum* Wehmer.[1] Currently, these diseases are primarily controlled by application of conventional synthetic fungicides such as imazalil or thiabendazole.[2,3] However, resistance development to fungicides by plant pathogens is a factor that restricts the fruit production worldwide due to the decrease in efficacy of fungicides.[4] Also, in the development and use of chemical fungicides for postharvest decay control, considerable attention must be given to the preservation of the global environment. Thus, alternative methods are needed for the control of postharvest diseases, such as biological (antagonistic microorganisms), physical (i. e. heat or radiations) or safe low-toxicity chemical methods, like the use of food additives.[5-9]

Parabens, the alkyl esters of *p*-hydroxybenzoic acid, and their sodium salts are a class of antimicrobial agents particularly useful against molds and yeasts, with a broad-spectrum antimicrobial activity, and commonly used as food preservatives.[10,11] These compounds are classified as "generally regarded as safe" (GRAS) and approved for use in foods by the US Food and Drug Administration (FDA) and European Union (EU) regulations.[12] The main advantages of using GRAS compounds as antifungal chemicals include their relatively low mammalian toxicity, broad spectrum of modes of action, safety for usage (i.e. relatively non-irritating, non-sensitizing and non-poisonous), stability over the pH range, and high solubility in water to produce the effective concentration in aqueous phase.[11,12] Propylparaben and the salt sodium propylparaben (SPP) are among the most commonly used antimicrobial preservatives and have been added to foods, drugs and cosmetics for over 50 years.[11]

Previous studies with SPP have demonstrated potential to control postharvest pathogens on some fruit species like strawberry or citrus.[9,13] However, in these research works, SPP was tested in *in vitro* conditions or incorporated to edible coatings as additional ingredients. Recently, the GRAS salts sodium ethylparaben (SMP)[14] and sodium

methylparaben (SEP)[15], applied *in vivo* to citrus fruits as postharvest aqueous dips, have demonstrated significant curative antifungal activity against citrus GM and BM. Since scarce information is available about the use of aqueous solutions of SPP as potential postharvest antifungal treatments, particularly against penicillium molds of citrus fruit, the objectives of this study were: (i) to preliminarily evaluate the curative activity of SPP at different concentrations against GM and BM, (ii) to optimize the SPP dip treatment conditions, (iii) to determine the SPP compatibility with low doses of the conventional postharvest fungicide imazalil under selected dip treatment conditions, (iv) to determine the effectiveness of SPP treatments on economically important citrus species and cultivars, (v) to evaluate the curative activity of SPP treatments on long-term cold-stored citrus fruit, and (vi) to evaluate the preventive activity of SPP treatments against citrus postharvest GM and BM.

MATERIALS AND METHODS

Fruit

The experiments were conducted with 'Valencia' and 'Lanelate' oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' (synonyms: 'Nules', 'Clementina de Nules') clementine mandarins (*Citrus reticulata* Blanco), and 'Nadorcott' (*C. reticulata* x *C. sinensis*; synonyms: 'Afourer', 'W. Murcott') and 'Ortanique' [*C. reticulata* x (*Citrus sinensis* x *C. reticulata*); synonym: 'Topaz'] hybrid mandarins. Fruit were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored for up to 1 week at 5°C and 90% relative humidity (RH) before use. Before each experiment, fruit were selected, randomized, washed with tap water and allowed to air dry at room temperature.

Fungal inoculation

P. digitatum and *P. italicum*, isolates NAV-7 and MAV-1, respectively, from the fungal culture collection of the IVIA CTP, were cultured on potato dextrose agar (PDA, Sigma-Aldrich Chemical Co., St. Louis, MA, USA) plates at 25ºC. Conidia of each fungus from 7 to

14-days-old cultures were taken from the agar surface with a sterile glass rod and transferred to a sterile aqueous solution of 0.5g kg^{-1} Tween® 80 (Panreac, S.A.U., Barcelona, Spain). Conidial suspensions were filtered through two layers of cheesecloth to separate hyphal fragments and adjusted to a concentration of 10^5 or 10^6 spores mL^{-1} using a haemocytometer. Unless otherwise stated, for fruit inoculation, the tip of a stainless steel rod, 1 mm wide and 2 mm in length, was immersed in the conidial suspension and inserted in the fruit rind afterwards. Fruit were inoculated at two opposite points in the equatorial zone, one with *P. digitatum* and the opposite with *P. italicum.* Inoculated fruit were kept in a temperature-controlled room at 20ºC for 24 h, until treatment.

Antifungal curative action

In vivo primary screenings

SPP (Propyl 4-hydroxybenzoate sodium salt; Merck KgaA, Darmstadt, Germany; Table 1; Fig. 1) was tested at nine concentrations to control citrus postharvest GM and BM on fruit previously inoculated with the pathogens. A sterile mother solution of SPP was prepared at a concentration of 250 mM. Sterile solutions at concentrations of 0.1, 1, 4, 7, 10 and 100 mM SPP were prepared by diluting with sterile water. Inoculum preparation was performed following the procedure described above. For fruit inoculation, 30 µL of conidial suspension of *P. digitatum* or *P. italicum* were placed, using a micropipette, in rind wounds made with the stainless steel rod previously described. About 24 h after the inoculation of the pathogen, 30 µL of SPP solution at the above mentioned concentrations were placed, using a micropipette, in the same inoculation rind wound. Control fruit were treated with 30 µL of sterile distilled water. For each combination of concentration of SPP and pathogen, 4 replicates of 5 'Valencia' oranges each were used. Treated fruit were incubated at 20ºC and 90% RH for 3 and 6 d, at which time disease incidence (% of infected wounds) and severity (lesion diameter) were determined. Severity was assessed over the entire fruit sample, not only over infected and symptomatic fruit.

Table 1. Physicochemical properties of sodium propylparaben

Characteristics	Description
Formula	$C_{10}H_{11}NaO_3$ (Fig. 1)
Molecular weight	202.18
Synonyms	Sodium propyl *p*-hydroxybenzoate Sodium 4-propoxycarbonylphenolate Sodium 4-propoxycarbonylphenoxide 4-hydroxybenzoic acid propyl ester sodium salt
E-number	E-216
Physical state	White crystalline powder
Melting point (°C)	105
Boiling point (°C)	294.3
Solubility in water	Soluble
pH	9.5-10.5 (concentration w/w: 0.1%)
Stability	Stable

Source: Merck-Millipore, MA, USA, and EMD Chemicals Inc., NJ, USA.

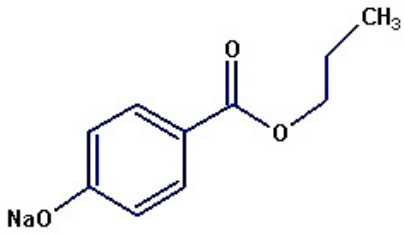

Figure 1. Chemical structure of sodium propylparaben

Determination of dip treatment conditions

Small-scale laboratory trials were conducted using 'Valencia' oranges to establish the best dip treatment conditions. Fungal inoculation with a concentration of 10^6 spores mL^{-1} was carried out following the procedure mentioned above.

Stainless steel buckets containing 10 L aqueous solution of 100 mM (18.02 g L^{-1}; 1.8% (w/v)) SPP were used. This concentration of SPP was selected according to previous results obtained in the *in vivo* primary screenings. When needed, solutions were heated by placing the buckets in a 250-L stainless steel water tank fitted with two electrical resistances (4.5 kW each), a thermostat, and an automatic water-recirculating system. Fruit were placed into 18 L multi-perforated wall stainless steel containers, exactly fitting in the above mentioned buckets, and completely immersed in the treatment solution for 30, 60 or 150 s at 20, 50 or 62°C. After treatment, fruit were rinsed for 5 s with tap water at low pressure in order to eliminate paraben salt residues. Control fruit were dipped in water alone at 20°C. Sixty fruit per treatment (3 replicates of 20 fruit each) were arranged in plastic cavity sockets on cardboard trays. Treated fruit were incubated at 20°C and 90% RH for 7 d. Disease incidence was assessed after 7 d of incubation. Potential fruit phytotoxicity caused by SPP or heat was visually assessed after 3 d at 20°C. For this purpose, fruit were classified into one of four categories, depending on rind appearance: 0 = no rind damage; 1 = slight brownish blemishes present (<10% fruit surface); 2 = moderate brownish blemishes present (>10% and <25% fruit surface) and 3 = severe rind injury (>25% fruit surface). A ponderate rind pitting index (0–3 scale) was calculated for each treatment.

Combination with low doses of imazalil

To determine the effect of the combination of the paraben salt with low doses of the chemical fungicide imazalil (IMZ; (±)-1-(2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl)-1H-imidazole; Fecundal-S 7.5% EC; Fomesa Fruitech S.L., Valencia, Spain) to control GM and BM, the following treatments were considered: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µL L^{-1} IMZ (IMZ 25), (4) 50 µL L^{-1} IMZ (IMZ 50), and (5) combination of 100 mM SPP with 25 µL L^{-1} IMZ (SPP + IMZ 25). Aqueous solutions of both chemicals were mixed into 10 L buckets and manually stirred with a clean plastic rod. IMZ was used at two doses considerably lower than those recommended for commercial applications. This experiment was conducted using 'Valencia' oranges. Fungal inoculation and dip treatments were

performed following the procedure mentioned above. Dip conditions were temperature of 20ºC and immersion time of 60 s. After treatment, only fruit treated with SPP were rinsed with tap water for 5 s. Each treatment was applied to 3 replicates of 20 fruit each. Disease incidence and pathogen sporulation (% of lesions showing spores) were determined after 7 d of incubation at 20ºC and 90% RH. Pathogen sporulation was assessed over the entire fruit sample, not only over infected and symptomatic fruit. Fruit phytotoxicities were assessed after 3 d at 20ºC. The experiment was conducted twice.

Effectiveness on major citrus species and cultivars

To assess whether the effectiveness of curative treatments was dependent on the host fruit, different citrus species and cultivars were subjected to the following treatments: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µL L^{-1} IMZ (IMZ 25), or (4) 100 mM SPP + 25 µL L^{-1} IMZ (SPP + IMZ 25). All treatments were applied as dips at 20ºC for 60 s. The experimental design, fungal inoculation and dip treatments followed the same procedures previously described. Treated fruit were not rinsed with tap water, with the exception of fruit treated with SPP alone. Treated fruit were incubated at 20ºC and 90% RH for 7 d, at which time disease incidence and pathogen sporulation were assessed.

Effectiveness on long-term cold-stored citrus fruit

To evaluate the curative capability of SPP against GM and BM on 'Valencia' oranges subjected to long-term cold storage, an experiment was conducted using inoculated fruit treated with: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 100 mM SPP + 25 µL L^{-1} IMZ (SPP + IMZ 25). All treatments were applied as dips at 20ºC for 60 s. Treated fruit were not rinsed with tap water, with exception of fruit treated only with SPP. Treated fruit were stored up to 8 weeks at 5ºC and 90% RH. Following the refrigeration period, the fruit were subjected to 7 d of shelf-life at 20ºC and 70-80% RH. Disease incidence and severity and pathogen sporulation were assessed after 2, 4, 6, and 8 weeks at 5ºC and 8 weeks at 5ºC plus 7 d at 20ºC.

Antifungal preventive action

To evaluate whether SPP treatments or combinations showed a preventive effect on the control of GM and BM, an experiment was conducted with 'Valencia' oranges in which a 1 mm wide, 2 mm deep wound was made with a stainless steel rod on the equatorial region of each fruit to simulate natural wounds. Then, the following treatments were applied by dipping the fruit in aqueous solutions at 20ºC for 60 s: (1) water (control), (2) 100 mM SPP (SPP), (3) 25 µL L^{-1} IMZ (IMZ 25) and (4) 100 mM SPP + 25 µL L^{-1} IMZ (SPP + IMZ 25). About 24 h later, fruit were inoculated with the tip of a stainless steel rod, 1 mm wide and 2 mm in length that had been immersed in a 10^5 spores mL^{-1} conidial suspension of *P. digitatum* and inserted in a new adjacent wound (about 2 mm of separation between wounds). Each treatment consisted of 3 replicates of 20 fruit each. After inoculation, treated fruit were incubated at 20ºC and 90% RH for 7 d, at which time disease incidence and severity and pathogen sporulation were assessed. The experiment was conducted twice.

Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) with Statgraphics software (Statgraphics Plus version 4.1; Manugistics Inc., Rockville, Maryland, USA). Data on disease incidence and pathogen sporulation were transformed to the arcsine of the square root of the proportion of infected or sporulated fruit to assure the homogeneity of variances. In some cases, reductions with respect to the controls were calculated as percentages. Statistical significance was judged at the level $P = 0.05$. When appropriated, the Fisher's Protected Least Significant Difference (LSD) test was used to separate means. Since experiment was a non-significant factor in the ANOVA, shown values are non-transformed means from repeated experiments.

RESULTS

Antifungal curative action

In vivo primary screenings

Among the concentrations of SPP evaluated in this set of experiments, the concentration of 100 mM completely inhibited the development of BM and reduced the incidence of GM by 94% on 'Valencia' oranges after incubation at 20ºC for 6 d (Fig. 2). Moreover, the concentrations of 7 and 10 mM SPP significantly reduced the incidence of GM and BM by 94 and 89%, and 70 and 88%, respectively, after 6 d of incubation at 20°C. In contrast, the concentrations of 0.1 and 1 mM SPP were not effective to reduce the incidence of the molds on 'Valencia' oranges (Fig. 2). Treatments with 100 mM SPP effectively reduced the severity of GM and BM by 38 and 100%, respectively, but concentrations of 0.1, 1, 4 and 7 mM SPP did not significantly reduce the severity of both molds (Fig. 2).

Determination of dip treatment conditions

A concentration of 100 mM SPP was selected in the previous *in vivo* primary screenings as the most appropriate for use in subsequent trials. SPP at 100 mM applied at 50°C for 60 or 150 s significantly reduced the incidence of GM in comparison with fruit dipped for 30 s at the same temperature (Fig. 3). On fruit dipped at 20 or 62°C for 30, 60 or 150 s, GM incidence reductions did not differ from each other after 7 d at 20°C. On the other hand, SPP at 100 mM applied at 50°C for 150 s, significantly reduced the incidence of BM when compared with fruit dipped for 30 or 60 s at the same temperature, with incidence reductions of 97, 81 and 78%, respectively. Likewise, on fruit dipped at 62°C for 30, 60 or 150 s, SPP at 100 mM significantly reduced the incidence of BM, with reductions of 83, 93 and 100%, respectively (Fig. 3). Irrespective of treatment duration, temperatures of 62°C improved the effectiveness of SPP dips with respect to that obtained at 20°C, but temperatures of 50°C did not improve the performance of dips at 20°C (Fig. 3). However, up to 60% of oranges dipped at 62°C showed slight phytotoxic injuries on the rind (superficial brownish blemishes of irregular size). Thus, according to

overall results from this set of trials, SPP dips at 20°C for 60 s were selected for use in further experiments.

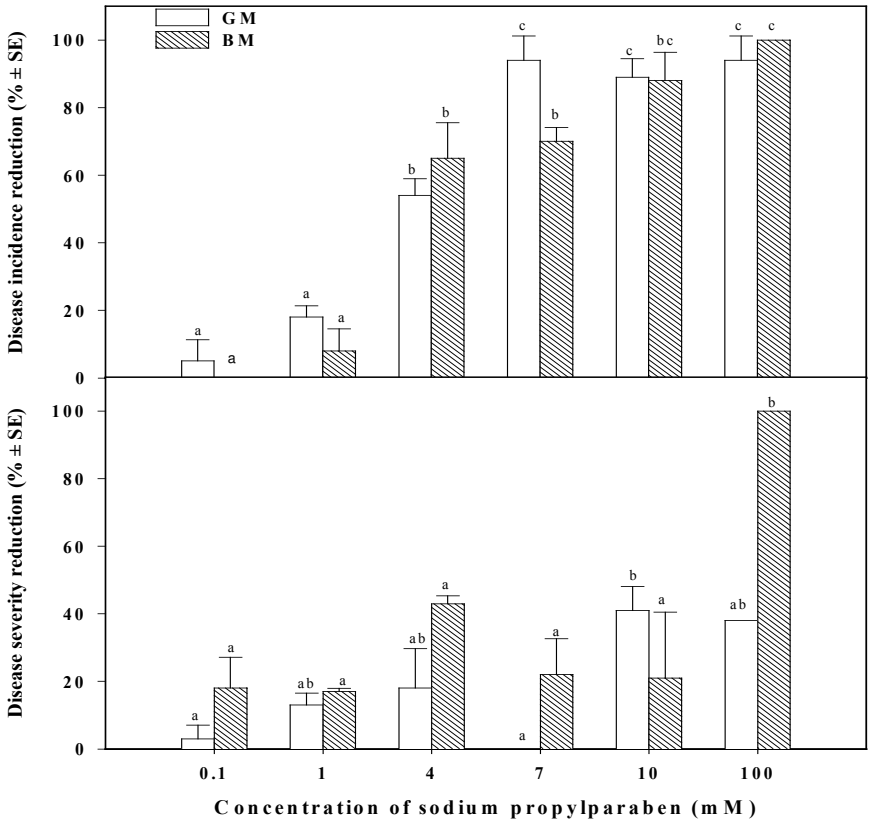

Figure 2. Curative activity of sodium propylparaben at different concentrations against green (GM) and blue (BM) molds in *in vivo* primary screenings with 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later, and incubated for 6 d at 20°C and 90% RH. Reductions of disease incidence and severity were determined with respect to control fruit treated with water (incidence of 95 and 75-85% for GM and BM, respectively, and severity of 104-112 mm and 39-52 mm for GM and BM, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Incidence values were arcsine-transformed. Non-transformed means are shown.

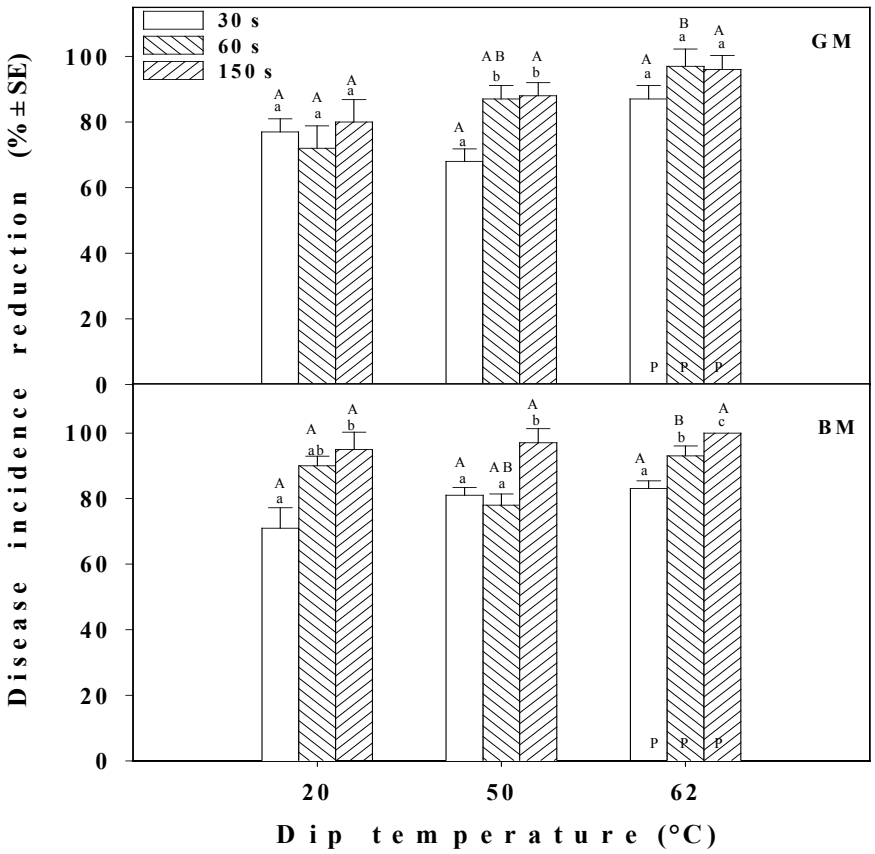

Figure 3. Effect of dip temperature and length on the effectiveness of 100 mM sodium propylparaben to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence were determined with respect to control fruit treated with water (incidence of 100 and 98% for GM and BM, respectively, for all temperatures and times). For each mold, columns with different lowercase and capital letters indicate significantly different dip length and dip temperature, respectively, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown. "P" indicates appearance of slight phytotoxicities on the fruit rind.

Combination with low doses of imazalil

Dips of 'Valencia' oranges at 20°C for 60 s with the combination SPP + IMZ 25 significantly enhanced the control of GM when compared to the rest of the treatments, with a GM incidence reduction after 7 d at 20°C of 96%. SPP, IMZ 25 and IMZ 50 reduced GM incidence by 72, 50 and 68%, respectively, and did not significantly differ from each other (Fig. 4). In contrast, SPP + IMZ 25 treatment did not improve the control of BM in comparison to SPP and IMZ 50 treatments. IMZ 25 was the least effective treatment against BM, and reduced its incidence by 51%. On the other hand, all treatments exerted a high anti-sporulant activity against both *P. digitatum* and *P. italicum*, with sporulation reductions with respect to the control treatments ranging from 70 to 95% (Fig. 4).

Effectiveness on major citrus species and cultivars

Overall, the effectiveness of 100 mM SPP aqueous treatment applied alone at 20°C for 60 s to control GM and BM was significantly higher on oranges than on mandarins. After incubation at 20°C for 7 d, SPP applied alone significantly reduced the incidence of GM and BM by 72 and 58% and 90 and 54% on 'Valencia' and 'Lanelate' oranges, respectively (Fig. 5). Conversely, SPP reduced the incidence of GM and BM by less than 36% on mandarins. Similarly, IMZ 25 and the combined treatments were more effective to reduce the incidence of GM and BM on oranges than on mandarins. IMZ 25 and combined treatments reduced the incidence of the molds, especially on 'Lanelate' and 'Valencia' oranges, respectively, and were much less effective on 'Clemenules' mandarins (Fig. 5). On the other hand, all three treatments presented an important anti-sporulant activity on most of the cultivars after 7 d at 20°C. Irrespective of the dip treatment, sporulation of both *P. digitatum* and *P. italicum* was higher on 'Clemenules' mandarins than on the rest of citrus cultivars (Fig. 5).

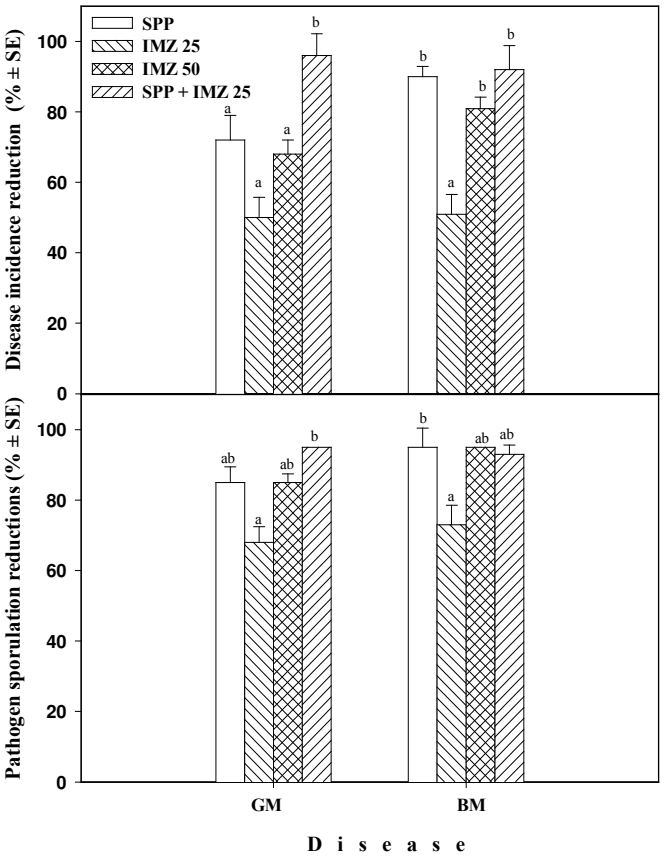

Figure 4. Effectiveness of 100 mM sodium propylparaben alone (SPP), 25 µL L^{-1} imazalil (IMZ 25), 50 µL L^{-1} imazalil (IMZ 50) and combination of 100 mM SPP and 25 µL L^{-1} imazalil (SPP + IMZ 25) to control green (GM) and blue (BM) molds on 'Valencia' oranges artificially inoculated with *Penicillium digitatum* or *P. italicum*, treated 24 h later for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 100 and 98% for GM and BM, respectively, and pathogen sporulation of 100 and 97-98% for *P. digitatum* and *P. italicum*, respectively). For each mold, columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means from two experiments are shown.

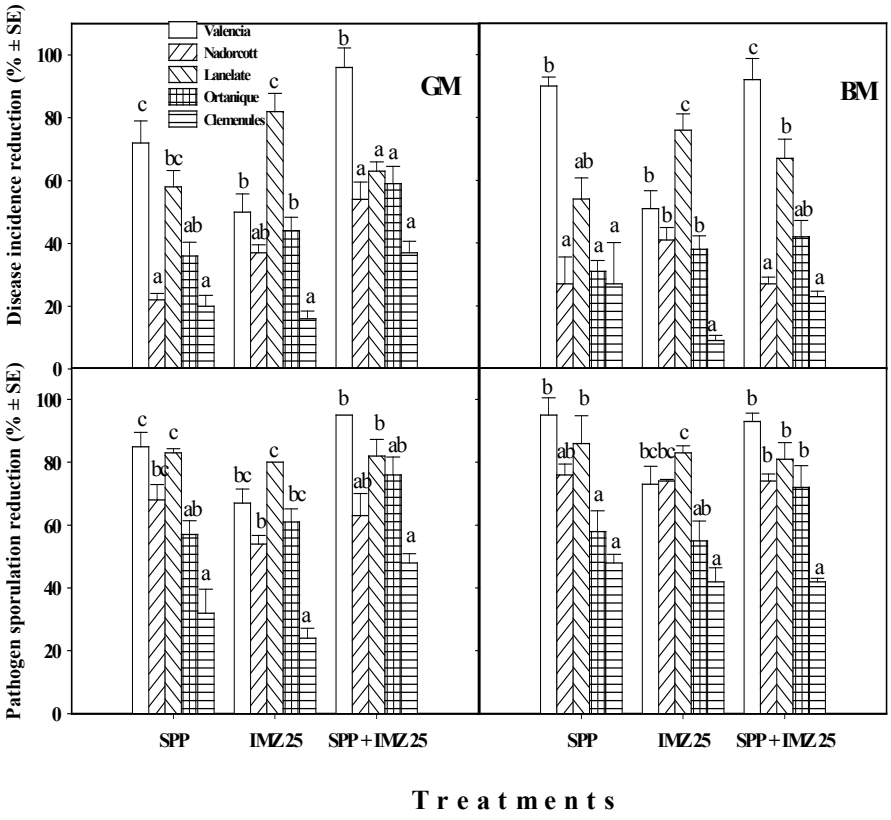

Figure 5. Incidence and sporulation of green (GM) and blue (BM) molds on citrus species and cultivars artificially inoculated with *Penicillium digitatum* or *P. italicum*, dipped 24 h later in water (control), 100 mM sodium propylparaben alone (SPP), 25 µL L^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µL L^{-1} imazalil (SPP + IMZ 25) for 60 s at 20°C, and incubated for 7 d at 20°C and 90% RH. Reductions of disease incidence and pathogen sporulation were determined with respect to control fruit treated with water (incidence of 93-100 and 95-100% for GM and BM, respectively, and pathogen sporulation of 80-100 and 65-100% for *P. digitatum* and *P. italicum*, respectively, for all cultivars). For each mold and dip treatment, columns with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA to the arcsine-transformed values. Non-transformed means are shown.

Effectiveness on long-term cold-stored fruit

The incidence of both GM and BM on 'Valencia' oranges stored up to 8 weeks at 5°C and 90% RH followed by 7 d of shelf-life at 20°C were effectively reduced by the application of SPP, IMZ 25 and SPP + IMZ 25 as 60 s dips at 20°C (Fig. 6). The SPP and combined treatments were significantly more effective than the IMZ 25 treatment (reductions of GM and BM incidence after 8 weeks at 5°C of about 80-97% and 48-57%, respectively). After 8 weeks at 5°C plus 7 d of shelf-life at 20°C, the incidence of GM and BM on fruit treated with SPP + IMZ 25, SPP, and IMZ 25 was 9, 17 and 44%, and 14, 20 and 53%, respectively, while decay was 98% on control fruit (Fig. 6).

In general, all three treatments significantly reduced the severity of GM and BM during the entire cold storage period at 5°C, but the SPP and combined treatments were more effective than IMZ 25. While GM- and BM-infected control oranges were completely rotten, with maximum lesion diameters of 130 and 72 mm after 4 weeks at 5°C, respectively, GM and BM lesion diameters on treated fruit at this time were 0-26 and 2-15 mm, respectively (Fig. 6). Similarly, all three treatments significantly prevented pathogen sporulation on decayed 'Valencia' oranges stored for 8 weeks at 5°C plus 7 d of shelf-life at 20°C, being the SPP and combined treatments superior to IMZ 25 alone (Fig. 6).

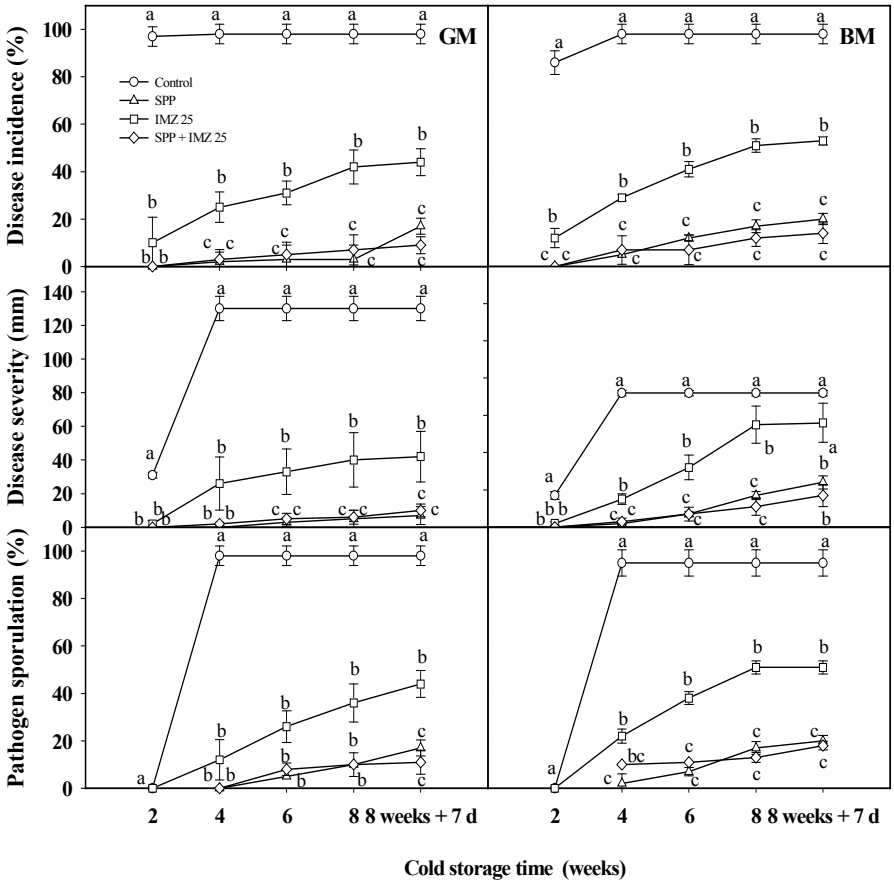

Figure 6. Incidence and severity of green (GM) and blue (BM) molds, and sporulation of *Penicillium digitatum* and *P. italicum* on 'Valencia' oranges artificially inoculated with the pathogens, dipped 24 h later in water (control), 100 mM sodium propylparaben alone (SPP), 25 µL L^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µL L^{-1} imazalil (SPP + IMZ 25) for 60 s at 20°C, and cold stored at 5°C and 90% RH for 8 weeks followed by 7 d of shelf-life at 20°C. For each mold and evaluation date, means with different letters are significantly different, according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means are shown.

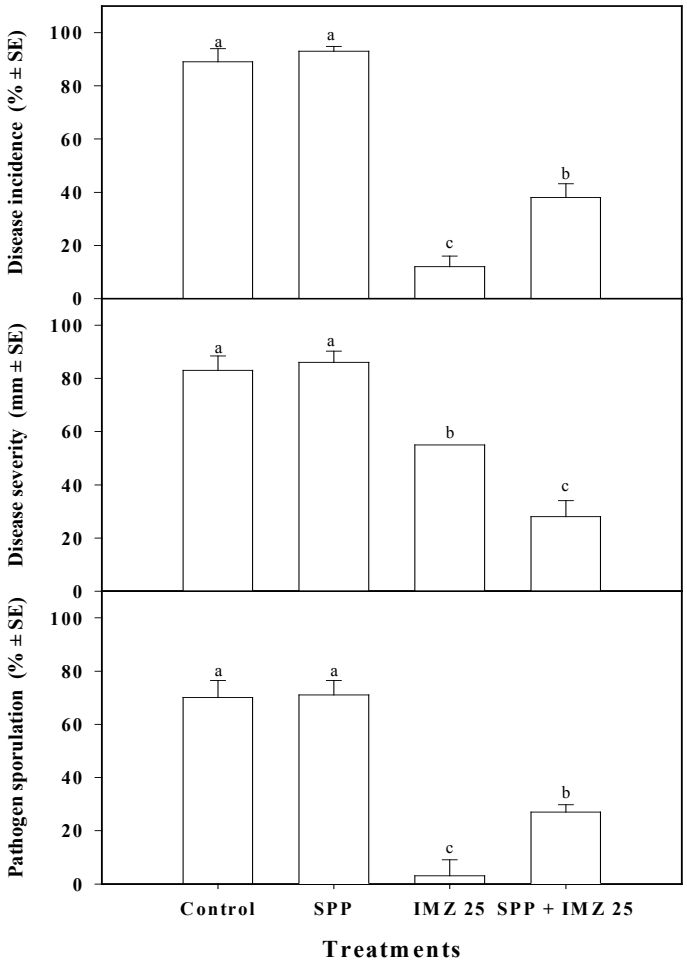

Figure 7. Preventive activity of sodium propylparaben (SPP) at 100 mM, 25 µL L^{-1} imazalil (IMZ 25), or 100 mM SPP combined with 25 µL L^{-1} imazalil (SPP + IMZ 25) against green mold on 'Valencia' oranges treated, artificially inoculated 24 h later with *Penicillium digitatum*, and incubated for 7 d at 20°C and 90% RH. Control fruit were treated with water and inoculated. Columns with different letters are significantly different according to Fisher's protected LSD test ($P \leq 0.05$) applied after an ANOVA. Disease incidence and pathogen sporulation were arcsine transformed. Non-transformed means from two experiments are shown.

Antifungal preventive action

The treatments IMZ 25 and SPP + IMZ 25 effectively prevented GM on 'Valencia' oranges inoculated with *P. digitatum* after treatment and incubated at 20°C for 7 d. In contrast, SPP treatment showed no preventive activity and did not reduce the incidence of GM with respect to the control fruit (Fig. 7). Likewise, similar results were obtained for disease severity and pathogen sporulation (Fig. 7).

DISCUSSION

The antifungal action of postharvest SPP treatments against citrus GM and BM was evaluated in this research work in order to set the basis for potential nonpolluting commercial treatments alternative or complementary to conventional chemical fungicides. *In vivo* preliminary tests to select the most effective concentration of SPP to control GM and BM on previously inoculated oranges (curative action) were first conducted. Among the wide range of concentrations tested, a concentration of 100 mM SPP was selected to be used in small-scale trials to determine the most appropriate dip treatment conditions. Since dips in SPP aqueous solutions at 20°C for 60 s were selected instead of dips in hot solutions, it seems that the synergy between heat (water heated at non-phytotoxic temperatures) and SPP for disease control is lower than that between heat and other food additives also tested as alternative control means.[8,16] Similar lack of complementary activity was observed when aqueous solutions of the paraben salts SEP[14] and SMP[15] were heated to 50ºC. As in the case of SPP, heating these solutions to 62ºC also resulted in production of slight superficial browning on the citrus fruit rind. The elevated disease control level obtained with SPP dips at room temperature is a factor that might facilitate the commercial adoption of postharvest SPP treatments in citrus packinghouses, since implementation and application costs of non-heated solutions would be considerably lower. Nevertheless, the effectiveness of 100 mM SPP dips at 20ºC for 1 min was lower than that obtained in *in vivo* primary screenings, possibly because of the longer contact time of the SPP drop with the rind wound inoculated with *P. digitatum* or *P. italicum* compared with the dip contact time.

Potential antifungal activity of SPP against postharvest pathogens causing fruit decay has been demonstrated in some previous studies *in vitro*. For instance, mycelial growth and spore germination of several postharvest potato pathogens were significantly inhibited by SPP.[12] In other works, SPP showed a strong inhibitory effect on mycelial growth and conidia germination of *Botrytis cinerea* at the high dose of 1,000 μg mL^{-1},[13] and films containing SPP inhibited the growth of *P. digitatum* and *P. italicum* in dichloran rose-bengal chloramphenicol agar (DRBC).[17] Furthermore, in *in vivo* research, edible fruit coatings containing SPP as an ingredient significantly reduced the incidence of GM on 'Clemenules' mandarins artificially inoculated with *P. digitatum* 24 h before treatment and incubated at 20°C for 7 d.[9] In this work, the antifungal curative action against GM and BM of SPP applied alone at the same dip and incubation conditions to 'Valencia' oranges or 'Clemenules' mandarins was equiparable or even higher than that of sodium carbonate, potassium benzoate, sodium bicarbonate (disease incidence reductions of about 50%), sodium benzoate or potassium sorbate (reductions of up to 70%).[3,5,16] Moreover, when compared to similar postharvest treatments with other sodium paraben salts to 'Valencia' and Lanelate' oranges, the performance of SPP dips at 1.8% (w/v)was superior to that of SEP dips at 1.3%[14] and equiparable to that of SMP dips at 3%.[15] As it has been reported[18], the general antimicrobial activity of parabens increases as the chain length of the alkyl group increases.

Overall, our results suggest that SPP applications were compatible with the fungicide IMZ at low doses, and consistently improved its performance for the control of GM. Nevertheless, the control of BM on 'Valencia' oranges incubated at 20°C for 7 d was not improved by the combination of both active ingredients. Furthermore, the combined treatment (SPP + IMZ 25) did not improve the performance of SPP alone for the control of both GM and BM on 'Valencia' oranges stored at 5°C for 8 weeks plus 7 d of shelf-life at 20°C. From this point of view, the behavior of SPP clearly differed from that of other paraben salts like SEP or SMP, which not only were compatible with the fungicide IMZ, but also significantly improved their performance against GM and BM when combined with the treatment IMZ 25 under similar experimental conditions.[14,15] In terms of disease reduction, the effectiveness of the combination SPP + IMZ 25 and SPP applied alone

to oranges were comparable to that obtained in previous research[7,8,14,15] with potassium sorbate, SMP and SEP, all combined with 25 μL L^{-1} IMZ.

Regarding the effectiveness of SPP to control GM and BM on major citrus species and cultivars, we have generally observed that the treatments were more effective on oranges than on mandarins. These differences on treatment effectiveness clearly show the strong influence that intrinsic fruit characteristics may have on either fruit susceptibility to infection by *P. digitatum* and *P. italicum* or on fruit response to SPP application. In fact, the influence of the type of fruit on the performance of other food additives or GRAS substances was also reported in previous studies. Palou *et al.*[5,16] found that treatments with aqueous solutions of sodium bicarbonate or sodium carbonate for 150 s were significantly less effective against GM and BM on mandarins than on oranges. Similar observations were reported by Montesinos-Herrero *et al.*[8] and Moscoso-Ramírez *et al*.[14,15] regarding potassium sorbate, SEP and SMP treatments. In general, the inhibitory ability of low toxicity antifungal compounds such as SPP or other food additives depends on the presence of residues of the compound within the wound infection courts occupied by the fungus and on interactions between this residue and constituents of the rind.[5,16,19] Apparently, the nature of such interactions would be different according to the citrus species and cultivars as a consequence of different flavedo and albedo characteristics or presence of different constitutive antifungal compounds in the rind. Additionally, such constituents and their concentration in the rind would be determined by not only the genotype but also the fruit physical and physiological condition. On the one hand, these factors determine the natural fruit susceptibility to decay; mature citrus fruits are typically more susceptible to decay than immature ones because, among other possible causes, their level of preformed antifungal compounds is lower. On the other hand, the biosynthesis and/or accumulation of antifungal compounds as a response to different postharvest treatments is also lower in mature fruit.[20,21] It is known that an indirect mechanism of action of certain postharvest treatments such as heat[21] or solutions of some GRAS compounds[22] is the induction in the treated fruit tissues of disease resistance. According to these considerations, the relatively poor performance of SPP and the other

tested treatments on 'Clemenules', and in some cases on 'Nadorcott' mandarins, could be explained by the weak physical condition of their rind and perhaps a reduced ability to synthesize antifungal compounds. At commercial maturity, the rind of these mandarin cultivars is typically soft and thin and after harvest they can evolve to senescence or overmature stages easier and faster than other citrus species and cultivars such as 'Valencia' or 'Lanelate' oranges.

It is accepted that SPP, as other paraben salts, has an inhibitory effect on membrane transport and mitochondrial function processes, and its antimicrobial activity is higher against fungi than bacteria.[23] SPP might interfere on both the germinative and vegetative phases of microbial development, but spore germination is much more susceptible to SPP than fungal vegetative growth.[24] Paraben salts like SPP are more soluble in water than their correspondent parabens and they act as phenolic weak acids in aqueous solutions. When a weak acid is dissolved in water, equilibrium is established between undissociated acid molecules and charged ions, the proportion of undissociated acid increasing with lower pH. It is believed that the mode of action of SPP consists of the passage by diffusion of the undissociated form through the plasma membrane, then, once inside the cell in a higher pH environment, the acid dissociates causing an accumulation of protons and anions which cannot pass back across the plasma membrane and cause the pH to low with a consequent inhibition of cell metabolism.[25] Since the carboxylic acid ester is neutral and not acidic, parabens do not have the same pH dependence than benzoic acid or other organic acids. In general, SPP is effective over a wide pH range of 4-8.[26,27] Thus, the toxicity of SPP aqueous solutions at its natural pH of 10.0 is low. However, when applied to citrus fruit they become more active within the wounds in the albedo tissue because of the relatively low pH in these wounds. Rind pH of citrus fruit ranges from 4 to 6 depending on the species and cultivars,[19,28] and according to this, SPP would be more effective against *P. digitatum* or *P. italicum* on fruit with lower rind pH. However, such rind pH differences cannot explain alone the different effectiveness of the treatments among different citrus species and cultivars.

In this research, the three treatments SPP applied alone, IMZ 25, and SPP + IMZ 25 were all effective to reduce the incidence of GM and BM on inoculated and long-time cold-stored 'Valencia' oranges, although SPP alone and the combination were significantly superior to IMZ 25 at the end of the shelf-life period at 20°C. The effect of these treatments on disease severity and pathogen sporulation showed a very similar trend. It can be concluded that, in general, citrus fruit treated with SPP alone or combined with IMZ can be exposed to commercial cold storage temperatures for relatively long periods of time.

It is clear from this study that SPP, applied alone at 100 mM in dips at room temperature for 60 s about 24 h prior to fungal inoculation, showed no preventive effect to protect 'Valencia' oranges from infections by *P. digitatum*. In contrast, in these experimental conditions, preventive treatments with IMZ (alone or combined with SPP) greatly reduced GM after 7 d of incubation at 20°C. This result indicates that SPP, unlike IMZ, is not able to spread itself through flavedo and albedo tissues, hence providing protective effect against further wound fungal infections. Similar results have been reported for other food additives or GRAS substances tested as postharvest antifungal treatments such as sodium carbonate, sodium bicarbonate or potassium sorbate.[8,29] This is a clear disadvantage of these alternative control means with respect to conventional fungicides. Although less cost-effective, an option to overcome this weakness might be the integration of alternative treatments with different modes of action.

CONCLUSIONS

It can be concluded from this research work that postharvest SPP treatments may be effective enough to be potentially included in integrated disease management programs as novel tools for a nonpolluting control of citrus GM and BM. This is especially important in citrus growing areas with high levels of fungicide resistant strains of *Penicillium* spp. In addition, they could be used in combination with the conventional fungicide IMZ to effectively reduce its doses, which is currently an important issue for citrus

exporters to EU markets. Obviously, large-scale trials in citrus packinghouses with naturally infected fruit are needed before pursuing a registration of this GRAS compound for postharvest commercial use.

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DISCUSIÓN GENERAL

Efecto de la desverdización en la susceptibilidad y calidad de frutos cítricos y en el desarrollo de las podredumbres verde y azul

La desverdización comercial con etileno exógeno, en las condiciones más comunes que se aplican en España, realizada antes de la inoculación con *P. digitatum* y *P. italicum*, no afectó la susceptibilidad de frutos cítricos a las podredumbres verde y azul, causadas por estos hongos patógenos. Resultados equivalentes se obtuvieron en distintas variedades de importancia comercial como son mandarinas clementinas 'Clemenules' y 'Clemenpons', mandarinas híbridas 'Nova' y naranjas 'Navelina' de recolección precoz. Estos resultados no coinciden con los reportados por Brown (1973), quien encontró un aumento de la resistencia del fruto a la podredumbre verde cuando naranjas se desverdizaron a 30°C y 90-96% HR. Estas diferencias pueden ser explicadas por el hecho de que la desverdización a temperaturas y humedades tan altas como 30°C y 95% HR, puede inducir la formación de lignina en las heridas de los frutos evitando el desarrollo del hongo, y por tanto disminuyendo la susceptibilidad a la enfermedad (Brown, 1973). Por otro lado, estos resultados son favorables para la industria citrícola española, porque si la fruta desverdizada fuera más susceptible a las podredumbres podría requerir un tratamiento antifúngico de poscosecha preventivo para protegerla de las infecciones de *P. digitatum* o *P. italicum* en las centrales citrícolas, lo cual podría implicar gastos económicos adicionales.

El estudio del efecto de la desverdización en el desarrollo de las podredumbres se realizó desverdizando frutos previamente inoculados con *P. digitatum* y *P. italicum*. No se encontró efecto significativo en la incidencia de las podredumbres verde y azul en frutos de mandarinas y naranjas con diferentes índices de color (CI) inicial de la piel incubados a 20°C durante 7 días. Estos resultados son similares a los reportados en mandarinas 'Clemenules' para las mismas podredumbres evaluadas después de la desverdización (Plaza et al., 2004). Contrariamente, la desverdización afectó la severidad de las podredumbres verde y azul, independientemente de las condiciones de almacenamiento de los frutos (incubación de 7 días a 20°C o conservación de 14 días a 5°C), pero el efecto sobre la severidad dependió en primera instancia de la resistencia natural de la fruta

hospedera de los diferentes cultivares de cítricos probados, determinados por el genotipo y la condición fisiológica del fruto (Palou et al., 2007), y en segunda instancia del CI inicial de la piel del fruto. En este sentido, la desverdización comercial no afectó la severidad de las podredumbres en frutos de mandarinas y naranjas con CI inicial de la piel más bajo (frutos más verdes), pero significativamente incrementó la severidad de las podredumbres en frutos con CI inicial más alto (frutos más maduros). Así, en frutos de mandarinas 'Clemenules' con CI inicial de la piel de -0,07 y 0,9, la desverdización comercial aumentó significativamente la severidad de las podredumbres verde y azul, mientras que no le afectó en mandarinas con CI inicial de -6,5. Resultados equivalentes se obtuvieron con naranjas 'Navelina' con CI inicial de 1,1 o 1,7 (severidad incrementada) y -5,3 (severidad no afectada). Teniendo en cuenta que el etileno no presenta capacidad antifúngica per se, la no influencia de la desverdización a 21°C sobre la incidencia de las podredumbres era esperable, puesto que habría significado un efecto antifúngico directo sobre las esporas sin germinar o recién germinadas. En este sentido, Chalutz (1979) reportó que el etileno no juega ningún papel en la patogenicidad de *P. digitatum*. En cambio, el incremento de la severidad de las podredumbres en frutos desverdizados puede estar asociado a una aceleración de la degradación de compuestos constituyentes de la piel del fruto (Porat, 2008). Se sabe que el contenido de compuestos con actividad antifúngica presentes naturalmente en la piel del fruto (en su mayoría compuestos fenólicos tales como flavonas, flavanonas, etc) es más alto en frutos inmaduros, y disminuye a medida que los frutos maduran o senescen (Ortuño et al., 2006; Palou et al., 2007). Así, la desverdización como factor inductor de senescencia en el fruto, habría podido contribuir a la reducción de los niveles de estos compuestos antifúngicos, y más especialmente en frutos ya más maduros, como indica el mayor CI. Estos resultados contrastan con los obtenidos en naranjas 'Navel' desverdizadas a 1, 10, 100 o 1000 μL L^{-1} durante 6 días y previamente inoculadas con *P. italicum*, donde la desverdización no afectó la severidad de la podredumbre azul (El-kazzaz et al., 1983). En frutos de mandarinas ' Clemenules' con dos diferentes CI inicial de la piel (CI= -0,07 y 0,9), la desverdización comercial no afectó la severidad de las podredumbres verde y azul. En general, el genotipo, la condición de la fruta y las condiciones del

tratamiento, tales como la concentración de etileno, la temperatura, la HR y el tiempo de exposición usadas durante la desverdización, pueden explicar los resultados contradictorios reportados en la literatura sobre el efecto de la desverdización con etileno exógeno en la incidencia y severidad de las podredumbres de poscosecha de los cítricos.

Respecto al efecto de la desverdización comercial en los atributos de calidad de los frutos cítricos, en general, no se encontró ningún efecto práctico ni en la calidad externa de frutos de mandarinas 'Clemenpons', 'Clemenules' y 'Nova', y naranjas 'Navelina', ni en la calidad interna de frutos de mandarinas 'Clemenpons' y 'Clemenules'. Estos hallazgos son apoyados por otros trabajos de investigación relacionados con el efecto de la desverdización en la calidad externa (Carvalho et al., 2006; Sdiri et al., 2012) e interna (Tietel et al., 2010; Sdiri et al., 2012) de los frutos cítricos.

Evaluación de tratamientos poscosecha con inductores químicos de resistencia para controlar las podredumbres verde y azul en frutos de naranja

Inductores químicos de resistencia

Ensayos primarios de actividad preventiva *in vivo*, mostraron que los primeros cuatro de los siete inductores químicos probados (silicato de sodio (SSi), ácido 2,6-dicloroisonicotínico (INA), ácido *β*-aminobutírico (BABA), benzotiadiazol (BTH), ácido salicílico (SA), Harpin y el ácido acetil salicílico (ASA)) indujeron resistencia en el fruto, al menos en una de las concentraciones ensayadas, contra las podredumbres verde y azul en naranjas 'Valencia' o 'Lanelate', con reducciones de incidencia del 20 al 89%. Los inductores químicos de resistencia actuaron como un regulador de crecimiento debido a que fueron más efectivos a una concentración óptima.

El tratamiento con SSi a 1000 mM fue más eficaz para prevenir las podredumbres verde y azul (reducciones de incidencia de 89 y 71%, respectivamente) que el resto de tratamientos con inductores químicos e incluso tuvo un mejor desempeño que tratamientos reportados

anteriormente por otros investigadores para controlar la podredumbre verde en frutos cítricos (Liu et al., 2010; Youssef et al., 2012). Sin embargo, SSi a esta concentración no pudo ser recomendado para ensayos posteriores, por la presencia de residuos muy adherentes sobre la piel del fruto alrededor del punto de tratamiento. Reportes previos indicaron que SSi causó fitotoxicidad en frutos de melón 'Hami' a la concentración de 200 mM (Bi et al., 2006).

INA indujo resistencia en partes vegetativas contra varias enfermedades en el cultivo de algodón en condiciones de campo (Colson-Hanks et al., 2012). Nosotros encontramos que INA puede inducir resistencia a las podredumbres verde y azul en órganos reproductivos, tales como los frutos de cítricos, a una concentración óptima de 0,03 mM (reducciones de incidencia de 25 y 60%, respectivamente).

BABA redujo la incidencia de la podredumbre azul (39%) en naranjas 'Valencia' a una concentración óptima de 0,3 mM, pero no la de la podredumbre verde. Esta reducción fue inferior a las reportadas en trabajos previos, por ejemplo en naranja dulce 'Darabi' (60% de reducción) a una concentración de 40 mM (Tavallali et al., 2008) o en frutos de pomelo con un 65% de reducción a la podredumbre verde tras la aplicación de BABA (Porat et al., 2003). Estas diferencias en la concentración óptima sugieren que los niveles de inducción de BABA pueden variar dependiendo del cultivar, lo que implica diferentes interacciones en función del huésped y el patógeno. Por su parte, con tratamientos con BTH (0,9 mM) se obtuvo el nivel más bajo de inducción de resistencia en frutos de naranjas 'Lanelate' (20% de reducción de la podredumbre verde).

En general, y excluyendo el tratamiento de SSi a 1000 mM por problemas de residuos en la piel del fruto, en términos de capacidad de control, INA fue el mejor inductor químico de resistencia para prevenir la infección de *P. digitatum* y *P. italicum*, con reducciones de las podredumbres del 25% y el 60%, respectivamente, en frutos inoculados después del tratamiento. También con la excepción de SSi, en este trabajo no se encontraron tratamientos con actividad curativa de poscosecha contra las podredumbres verde y azul cuando los inductores químicos de resistencia se aplicaron en pruebas primarias

de efectividad sobre naranjas ‘Valencia’ o ‘Lanelate’ previamente inoculadas con los patógenos.

En general, los tratamientos con inductores químicos de resistencia aquí probados no tuvieron o tuvieron un muy ligero efecto preventivo o curativo aplicados en soluciones acuosas. Estos resultados, en el caso de la actividad preventiva, pudieron haberse debido a varias razones: primeramente a que el tiempo de contacto entre la solución acuosa de los inductores químicos depositada con micropipeta en las pruebas primarias de selección en la herida de la piel de la fruta fue considerablemente mayor que los 60 s de contacto que hubo en el caso de los tratamientos por inmersión en la solución acuosa. En segundo lugar, los tratamientos de los inductores químicos de resistencia en soluciones acuosas fueron aplicados 2-3 h antes de la inoculación fúngica, mientras que los tratamientos en las pruebas de efectividad preliminar fueron aplicados 24 h antes de dicha inoculación. Esta diferencia es importante porque se ha observado que los diferentes inductores químicos pueden inducir resistencia sistémica o local en lapsos de tiempos diferentes (Vallad y Goodman, 2004). En tercer lugar, algunos inductores químicos de resistencia, concretamente el INA y el BABA fueron aplicados en soluciones acuosas sobre naranjas ‘Lanelate’, mientras que los tratamientos en las pruebas primarias de efectividad fueron aplicados sobre naranjas ‘Valencia’. Puesto que se han descrito diferentes interacciones en función del huésped y el patógeno (Sticher, 1997), podría ser que las diferencias en los niveles de inducción de algunos compuestos pudieron haber variado dependiendo del cultivar.

El único tratamiento con actividad curativa significativa entre los inductores químicos de resistencia aquí probados en soluciones acuosas fue el de BTH a 0,9 mM a 50°C durante 150 s, particularmente sobre la podredumbre azul en naranjas ‘Lanelate’. No obstante, este tratamiento careció de acción curativa en las pruebas primarias. Por tanto, consideramos que las reducciones de incidencia y severidad que alcanzaron hasta un 50% obtenidas con este tratamiento fueron debidas mayoritariamente a la temperatura del baño y a la duración del mismo, y no al efecto en sí del ingrediente activo del BTH.

Una posible explicación a la escasa capacidad de inducción de resistencia con tratamientos poscosecha con los inductores ensayados aquí, si se compara con la reportada en la literatura, podría ser que en general, la resistencia contra patógenos de plantas se induce más fácilmente en partes vegetativas de las plantas que en partes reproductivas, como serían los frutos cítricos en nuestro caso (Porat et al., 2003). Además, en un gran número de investigaciones realizadas en diferentes cultivos con inductores químicos de resistencia se ha visto que los mejores resultados, en términos de reducción de enfermedades, se han obtenido con tratamientos en precosecha (Buonaurio et al., 2009; Iqbal et al., 2012; Youssef et al., 2012; Feliziani et al., 2013). Por tanto, se sugiere abrir una investigación sobre el uso de estos inductores químicos aplicados en precosecha evaluando las podredumbres verde y azul en poscosecha. Se puede concluir que, como tratamiento de poscosecha, estos compuestos no pueden ser recomendados para su inclusión en programas de CINCEP.

Actividad preventiva y curativa de tratamientos de poscosecha con silicato de potasio contra las podredumbres verde y azul

En pruebas de efectividad preliminar, tratamientos poscosecha con silicio en la forma de silicato de potasio (PSi), mostraron actividad preventiva (tratamiento aplicado 24 h antes de la inoculación fúngica) y curativa (tratamiento aplicado 24 horas después de la inoculación fúngica) significativas contra las podredumbres verde y azul, en naranjas 'Valencia' almacenadas a 20°C durante 6 días. Entre el rango de concentraciones ensayadas (0,9, 9, 30, 90 y 150 mM), el PSi fue más efectivo a una concentración óptima de 90 mM para controlar las podredumbres verde y azul en naranjas 'Valencia' o 'Lanelate'. PSi a 90 mM controló la podredumbre verde de forma similar en aplicaciones preventivas y curativas (reducción de incidencia de un 25% en ambos casos). Por el contrario, el control de la podredumbre azul fue ligeramente superior en aplicaciones preventivas (reducción de incidencia del 50%) que en aplicaciones curativas (reducción de incidencia del 40%). Estos resultados en reducción de incidencia fueron similares o ligeramente inferiores a los reportados con otros tratamientos de PSi contra la podredumbre verde en frutos cítricos (25-55% de reducción) (Liu et al., 2010), o contra la podredumbre

azul causada por *P. expansum* en frutos de jojoba (Tian et al., 2005). Nuestros resultados sugieren que se podría mejorar la efectividad del tratamiento con PSi en combinación con otros métodos alternativos no contaminantes, buscando un efecto sinérgico. Dado que los resultados mostraron que PSi tiene una actividad tanto preventiva como curativa; suponemos que el modo de acción de PSi fue tanto por un efecto indirecto sobre la fruta hospedera como por un efecto directo sobre el patógeno, tal como se ha sugerido en otros trabajos de investigación con otros cultivos (Epstein, 1999; Buonaurio et al., 2009; Liu et al., 2010).

Los ensayos de influencia temporal en la actividad preventiva del tratamiento con PSi a 90 mM, revelaron un control satisfactorio en la incidencia y severidad de la podredumbre verde, sólo cuando el lapso de tiempo entre el tratamiento y la inoculación fue de 2 h, con respecto al resto de los tratamientos (24, 48, 72 y 96 h), los cuales no causaron ningún efecto en la efectividad de PSi a 90 mM. Estos resultados sugieren que residuos de ingrediente activo de PSi podían encontrarse en cantidades suficientes en el interior de la herida para afectar la presencia de conidios y el crecimiento de *P. digitatum*, mientras que para el resto de los tiempos esta cantidad de residuos de PSi habría disminuido considerablemente pudiendo haberse perdido por difusión. Además, estos resultados indican que el PSi no fue capaz de inducir mecanismo alguno de defensa a los frutos, durante esos lapsos de tiempo. Este tipo de estudios son importantes porque se ha observado que los diferentes inductores químicos pueden inducir resistencia sistémica o local en lapsos de tiempos diferentes, lo cual condiciona en gran medida el tipo de tratamiento más efectivo que debería utilizarse para cada caso concreto (Vallad y Goodman, 2004).

El tratamiento de PSi a 90 mM en solución acuosa a 20°C durante 60 s mostró mayor actividad preventiva contra la podredumbre verde (37% de reducción) que la observada en las pruebas de efectividad preliminar (23% de reducción). Estos resultados pueden ser debidos a que el tratamiento en solución acuosa fue aplicado 2 h antes de la inoculación fúngica, mientras que los tratamientos en las pruebas de efectividad preliminar fueron aplicados 24 h antes de dicha inoculación, y de acuerdo con nuestros resultados en el ensayo de influencia temporal, el PSi protegió más efectivamente cuando fue

aplicado 2 h antes de la inoculación fúngica que a las 24 h. Nuestros resultados de influencia espacial indicaron que el PSi a 90 mM no mostró un efecto sistémico a través de la corteza de los frutos cítricos porque las heridas inoculadas con *P. digitatum* localizadas a las distancias de 10, 20 y 30 mm del punto de tratamiento no registraron reducciones de incidencia y severidad de la podredumbre verde, la cual fue similar a la de la fruta control. En cambio, PSi tuvo un efecto preventivo cuando la inoculación de *P. digitatum* se realizó en el mismo sitio de tratamiento o a una distancia de 2 mm de éste, como se ha mostrado en otros ensayos de este estudio. Estos resultados y observaciones significan que PSi puede inducir resistencia local al fruto, limitado al punto de inoculación o tener un efecto directo sobre el patógeno, tal como se ha señalado anteriormente. En este sentido, ya se ha comentado que la resistencia sistémica a patógenos de plantas se induce más fácilmente en partes vegetativas que en partes reproductivas como sería el fruto ya recolectado (Porat et al., 2003).

El PSi a 90 mM aplicado en solución acuosa a temperatura ambiente durante 60 s, no tuvo acción protectiva contra las podredumbres verde y azul en frutos almacenados a 5°C durante 6 semanas, aunque redujo significativamente la severidad de las podredumbres verde y azul partir de la segunda semana de almacenamiento frigorífico. Por el contrario, este mismo tratamiento tuvo una acción curativa significativa, reduciendo tanto la incidencia como la severidad de las podredumbres verde y azul después de la segunda semana de almacenamiento. De acuerdo con estos resultados, PSi podría ser recomendado para aplicaciones a frutos cítricos que serán almacenados por largos periodos en frío.

En general, nuestros resultados con tratamientos de poscosecha de PSi fueron prometedores, porque PSi mostró actividad preventiva y curativa a una dosis óptima de 90 mM contra las podredumbres verde y azul en frutos cítricos. Considerando que las fuentes de obtención de silicio en la naturaleza son muy abundantes, que este elemento está fácilmente disponible, es relativamente barato y, además que ha sido incluido en la lista de sustancias sintéticas aprobadas para el uso en producción orgánica (USDA AMS NOP, 2010) en EE UU, podemos concluir que el PSi tiene un potencial importante para el control de las podredumbres verde y azul de frutos cítricos y podría ser incluido

como tratamiento alternativo en programas de manejo integrado de enfermedades de poscosecha de cítricos.

Caracterización de tratamientos poscosecha con sales de parabenos sódicos para el control de las podredumbres verde y azul de los cítricos

Este apartado se centra en la discusión de los resultados obtenidos en los ensayos de la tesis realizados con metil parabeno sódico (SMP) (capítulo 4), etil parabeno sódico (SEP) (capítulo 5) y propil parabeno sódico (SPP) (capitulo 6), debido a que están relacionados.

Se obtuvieron excelentes resultados de efectividad con las sales de parabenos sódicos (SMP a 200 mM, SEP a 80 mM y SPP a 100 mM) cuando se aplicaron en baños a 20°C durante 60 s sobre naranjas previamente inoculadas artificialmente con *P. digitatum* y *P. italicum*. Sin embargo, esta actividad curativa fue algo más baja que la obtenida en las pruebas *in vivo* de selección primaria de la mejor concentración, en las que la reducción de la incidencia de las podredumbres a estas concentraciones fue prácticamente del 100%. Esta diferencia posiblemente fue debida a que el tiempo de contacto entre la solución acuosa de los parabenos sódicos depositada con micropipeta en las pruebas de selección en la herida inoculada de la piel de la fruta fue mayor que los 60 s de contacto que hubo en el caso de los tratamientos por inmersión en la solución acuosa. Estas concentraciones fueron seleccionadas como las más efectivas entre más de 23 concentraciones probadas en el rango de 0,1 a 250 mM.

Se ha reportado que, en general, la toxicidad y la actividad antimicrobiana de los parabenos y sus sales sódicas son directamente proporcionales a la longitud del radical alquilo y viceversa (Giordano et al., 1999). Nuestros resultados verifican esta tendencia porque en las pruebas primarias de efectividad, la concentración óptima requerida fue casi el doble para el SMP (radical alquilo más corto) que para el SEP o el SPP. También se corroboró con las reducciones de incidencia de las podredumbres obtenidas con los parabenos sódicos aplicados en baños en naranjas (SMP a 200 mM, SEP a 80 mM y SPP a 100 mM).

Aunque Valencia-Chamorro et al. (2009) ya habían demostrado el potencial de las tres sales de parabenos sódicos incorporadas a recubrimientos comestibles en frutos de naranja 'Valencia'para el control de las podredumbres verde y azul, con reducciones de menos del 50%, los resultados obtenidos en esta tesis con tratamientos con estas mismas sales aplicadas como soluciones acuosas fueron considerablemente mejores, con reducciones de incidencia del 60-90% en naranja 'Valencia'. Estas diferencias indicaron que la efectividad de las sales de parabenos puede variar también dependiendo del método de aplicación. Es posible que la liberación del ingrediente activo antifúngico incorporado a los recubrimientos comestibles localizados sobre la piel del fruto se vea dificultada por las propias características de los componentes de la matriz del recubrimiento (polisacáridos y lípidos), mientras que en las soluciones acuosas no existe esta barrera. De hecho, esta dificultad se ha puesto de manifiesto en algunos estudios sobre la liberación de aditivos alimentarios o agentes antimicrobianos presentes en films o recubrimientos comestibles (Limm y Holifield, 1995; Ponce et al., 2008)

En general, el control de las podredumbres no mejoró significativamente cuando se incrementó la temperatura de baño en los tratamientos con los tres parabenos sódicos, lo que parece indicar que la sinergia entre el calor (agua calentada a temperaturas no fitotóxicas) y los parabenos sódicos para el control de las podredumbres verde y azul es más baja que la sinergia observada entre el calor y otros aditivos alimentarios probados también como tratamientos alternativos al control químico convencional (Palou et al., 2001; Montesinos-Herrero et al., 2009). Esto podría deberse a que en general, los parabenos sódicos son estables a temperaturas altas, y en estas condiciones experimentan cambios importantes en su actividad antimicrobiana (Soni et al., 2005). Este hallazgo podría facilitar la adopción comercial de tratamientos poscosecha con parabenos sódicos en las centrales citrícolas, desde el punto de vista del ahorro de costos que supondría la no necesidad de instalación de sistemas de calentamiento de los caldos.

Los parabenos sódicos y el fungicida convencional imazalil a una dosis tan baja como 25 $\mu L\ L^{-1}$ fueron compatibles para su aplicación

en naranjas 'Valencia' y la combinación mejoró la efectividad de cada uno de los tratamientos por separado en el control de las podredumbres verde y azul, independientemente de las dos condiciones de almacenamiento estudiadas (7 días a 20°C y 8 semanas a 5°C y 90% HR, más 7 días a 20°C de vida de anaquel). La única excepción fue la del SPP empleado con imazalil para controlar la podredumbre azul. Resultados similares se obtuvieron con tratamientos combinados de sorbato de potasio e imazalil a una dosis de 25 μL L^{-1} en naranjas y mandarinas (Smilanick et al., 2008; Montesinos-Herrero et al., 2009). Esto puede ser importante porque actualmente el sector de la exportación requiere de tratamientos alternativos que les permitan reducir significativamente los niveles de residuos de fungicidas en los frutos, para satisfacer así las demandas particulares de muchos importadores y cadenas de distribución europeas. Además, una reducción sustancial en la presión de selección sobre los patógenos puede retrasar a largo plazo la proliferación de cepas resistentes de *P. digitatum* y *P. italicum*, lo cual puede ser muy útil en los programas de manejo de las centrales citrícolas que aun no presenten problemas importantes de proliferación de cepas resistentes a *Penicillium*.

En los ensayos de efectividad a pequeña escala realizados con distintos cultivares de cítricos de importancia económica, se encontró que los tratamientos con soluciones acuosas de los tres parabenos sódicos controlaron consistentemente las podredumbres en naranjas, mientras que el control fue mucho más restringido en mandarinas. Estas diferencias muestran la fuerte influencia que las características intrínsecas de los frutos (genéticas y también el estado físico y fisiológico en el momento del tratamiento de poscosecha) tienen tanto en la susceptibilidad de los frutos a la infección por *P. digitatum* o *P. italicum* como en la respuesta de los frutos a la actividad de los parabenos sódicos. Resultados similares se han observado con otros aditivos alimentarios como carbonatos o sorbatos que se han evaluado como tratamientos alternativos antifúngicos de poscosecha a los fungicidas convencionales (Palou et al., 2001, 2002a; Montesinos-Herrero et al., 2009). Se ha comprobado en esta tesis que el modo de acción de productos alternativos de baja toxicidad como son las sales de parabenos sódicos es, al igual que el de otros aditivos alimentarios, más fungistático que fungicida, y que depende en gran medida de la

presencia de residuos del compuesto dentro de la herida infectada ocupada por el hongo y de las interacciones que se establecen entre este residuo, el fruto huésped y el hongo patógeno (Smilanick et al., 1999; Palou et al., 2002b). Además, la actividad curativa de estos productos alternativos es en general poco persistente, a diferencia de la de los fungicidas sintéticos, que son capaces de matar directamente el hongo fitopatógeno.

Este trabajo demuestra que los distintos parabenos sódicos, aplicados como tratamientos de poscosecha en solución acuosa de forma adecuada, pueden controlar satisfactoriamente las podredumbres causadas por *Penicillium* spp. en naranjas, por lo cual se pone a disposición del sector una nueva herramienta potencial para aquellos casos en que el mercado de destino o el tipo de producción no admitan fungicidas convencionales o restrinjan la cantidad de sus residuos a niveles muy bajos. Puesto que los resultados indicaron que el uso del SPP no implica un aumento significativo de efectividad respecto al del SMP y el SEP, se recomienda la utilización de estos dos últimos por su actual inclusión en la lista de aditivos alimentarios autorizados en la UE.

La naturaleza poco tóxica de estos productos, tal como se ha demostrado en estudios toxicológicos experimentales (Soni et al., 2005), que precisamente posibilita su clasificación como aditivos alimentarios o sustancias GRAS, implica limitaciones importantes en su capacidad de control, como por ejemplo su efectividad restringida en mandarinas o en partidas de fruta altamente susceptibles a las podredumbres. La posible utilización comercial de estos aditivos alimentarios en programas de control integrado de enfermedades de poscosecha de cítricos dependerá de si los productores y/o empresas del sector consideran necesario realizar estudios económicos y de viabilidad para su posible registro para este uso.

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CONCLUSIONES

1. La desverdización comercial con 2 μL L^{-1} de etileno exógeno a 21ºC y 95-100% HR durante 3 días no afectó a la susceptibilidad del fruto a las podredumbres verde y azul en mandarinas 'Clemenules', 'Clemenpons' y 'Nova,' y naranjas 'Navelina' inoculadas 2 h después con *Penicillium digitatum* o *P. italicum*, respectivamente.

2. La desverdización comercial 2 h después de la inoculación fúngica incrementó significativamente la severidad de las podredumbres verde y azul sólo en frutos cítricos con un índice de color inicial de la piel alto.

3. La desverdización comercial no tuvo un impacto práctico sobre los principales atributos de calidad externa e interna de los frutos cítricos.

4. Se evaluaron en pruebas primarias de efectividad *in vivo* tratamientos de poscosecha con los siguientes inductores químicos: silicato de sodio (SSi), ácido 2,6-dicloroisonicotínico (INA), ácido *β*-aminobutírico (BABA), benzotiadiazol (BTH), ácido salicílico (SA), harpin y ácido acetil salicílico (ASA). Los siguientes compuestos y concentraciones se seleccionaron por su mayor actividad preventiva o curativa contra las podredumbres verde y azul: SSi a 1000 mM, INA a 0,03 mM, BABA a 0,3 mM, BTH a 0,9 mM y SA a 0,25 mM.

5. SSi a 1000 mM dejó residuos visibles en la piel y se descartó por riesgos de producción de fitotoxicidad. El resto de tratamientos seleccionados no mostraron actividad preventiva ni curativa aplicados en naranjas como soluciones acuosas a temperatura ambiente durante 60 s. Por tanto, estos tratamientos no pueden ser recomendados para ser incluidos en un programa de control integrado de las podredumbres verde y azul en centrales citrícolas.

6. Tratamientos de poscosecha con silicato de potasio (PSi) a 90 mM aplicado en baños de 60 s a 20°C mostraron tanto actividad preventiva como curativa significativas contra las

podredumbres verde y azul en naranjas. Estos tratamientos resultaron más efectivos aplicados 2 h antes de la inoculación y no mostraron un efecto sistémico a través de la corteza del fruto. Se pueden considerar como una nueva herramienta para programas de control integrado no contaminante de enfermedades de poscosecha (CINCEP) en centrales citrícolas.

7. Metil parabeno sódico (SMP) a 200 mM, etil parabeno sódico (SEP) a 80 mM y propil parabeno sódico (SPP) a 100 mM, aplicados en baños de 60 s a 20ºC mostraron la mayor actividad curativa en frutos cítricos inoculados 24 h antes con *P. digitatum* y *P. italicum*. Estos baños fueron compatibles y sinérgicos con dosis bajas de imazalil de 25 $\mu L\ L^{-1}$.

8. La actividad curativa de los tres parabenos sódicos en solución acuosa fue consistentemente mayor en naranjas que en mandarinas. En general la actividad fue más fungistática que fungicida y no muy persistente. Estos tratamientos pueden ser recomendados como una nueva alternativa no contaminante para el control de podredumbres de cítricos especialmente para aquellos mercados que demandan productos inocuos o con niveles de residuos muy bajos de fungicidas convencionales.

ANEXO

Anexo fotográfico

Foto 1. Cámara de desverdización comercial en una central citrícola (Moncada, Valencia)

Foto 2. Naranja 'Navelina' desverdizada con 2 µL L^{-1} de etileno a 21°C y 90-95% HR durante 3 días

Foto 3. Cámara frigorífica en la planta piloto del IVIA (Moncada, Valencia).

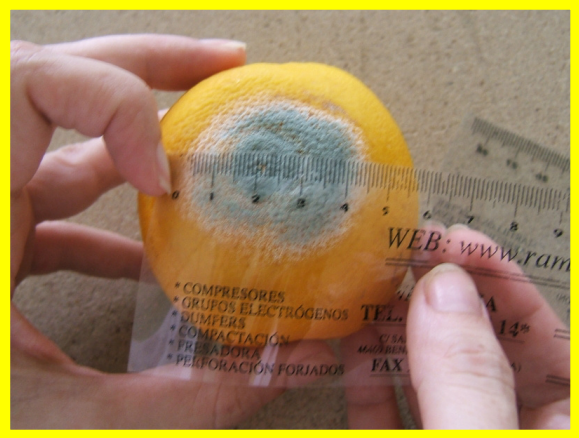

Foto 4. Medición del diámetro de lesión ocasionada por la podredumbre azul en una naranja 'Valencia'

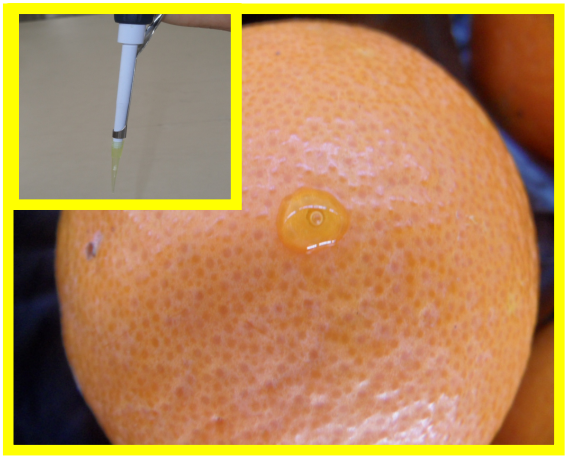

Foto 5. Aplicación de tratamientos de inductores químicos de resistencia con micropipeta en ensayos primarios

Foto 6. Sustancias químicas inductoras de resistencia.

Foto 7. Bañadora para el tratamiento en poscosecha de frutos en la planta piloto del IVIA (Moncada, Valencia)

Foto 8. Contenedores multiperforados para realizar los baños de frutos cítricos

Foto 9. Naranjas 'Valencia' inoculadas con *Penicillium digitatum* y *P. italicum* en caras opuestas de la región ecuatorial del fruto, bañadas 24 h después durante 60 s con agua a 20°C (control con tres repeticiones de arriba) o metil parabeno sódico a 200 mM combinado con imazalil a 25 µL L^{-1} (3 repeticiones de abajo) e incubadas durante 7 días a 20°C.

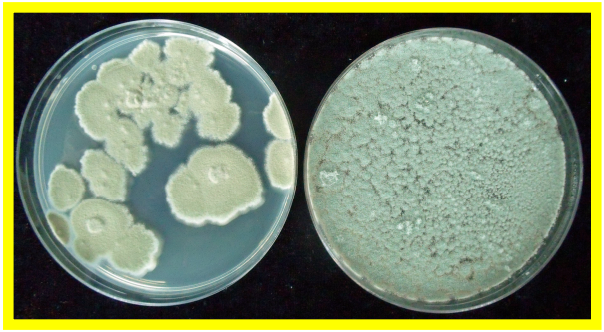

Foto 10. Aspecto de los hongos *Penicillium digitatum* (izquierda) y *P. italicum* (derecha), creciendo en placas de PDA durante 7 días a 25°C