Desarrollo de métodos de predicción de la incidencia de 'bitter pit' en plantaciones de manzanas ‘Golden Smoothee’ (Malus domestica, L. Borkh.)

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TESIS DOCTORAL

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Memoria presentada para optar al grado de Doctor por la Universidad de Lleida
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A mi padre, de quién aprendí las
primeras lecciones
de agronomía

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RESUMEN

El *bitter pit* es la fisiopaía más importante en muchos cultivares de manzana. Sin embargo, no existe una estrategia de control completamente efectiva, por lo que un método de predicción que identifique años y plantaciones con alto potencial de desarrollar la fisiopatía permitirá evitar pérdidas económicas, especialmente durante la conservación y confección.

El objetivo principal de la presente tesis doctoral fue la puesta a punto de un sistema de predicción de la incidencia de *bitter pit* en plantaciones de manzanas 'Golden Smoothee'. Para ello, se investigaron diferentes métodos de predicción basados en tres tecnologías distintas: i) el análisis mineralógico de fruto (en estadios tempranos y en recolección), ii) la inducción de síntomas (infiltración de Mg, baños con etefón, embolsado de frutos y método pasivo) y iii) la espectroscopía VIS/NIR. Los distintos métodos se evaluaron en diferentes períodos de crecimiento del fruto. Paralelamente, se evaluó y cuantificó la eficacia de distintas estrategias para la mitigación del *bitter pit* basadas en aportaciones de $CaCl_2$ en pre y poscosecha (aplicaciones radiculares, foliares y baños en poscosecha).

El análisis temprano de Ca en fruto a 60 días después de plena floración (DDPF) mostró una precisión de predicción similar o mejor que el análisis de Ca en recolección. Se definió un umbral de referencia a 60 DDPF de 11 mg Ca 100 g^{-1} de peso fresco, por encima del cual se minimizó el riesgo de aparición del *bitter pit*. La mayoría de métodos basados en inducir síntomas, a excepción del embolsado de frutos, mostraron eficacia a partir de los 40 días antes de recolección (DAR), con una correlación con el *bitter pit* de poscosecha del 70-80%. La espectroscopía VIS/NIR mostró resultados poco satisfactorios para la predicción del *bitter pit*, sin embargo, sí fue capaz de discriminar frutos afectados cuando los síntomas eran visibles en poscosecha. Finalmente, se diseñó un modelo de predicción del *bitter pit* basado en el análisis de Ca en fruto a 60 DDPF y el método pasivo a partir de 40 DAR. Respecto la mitigación del *bitter pit*, los resultados obtenidos en años con alta incidencia mostraron una reducción de un 20% a un 12%, 8% o 3% mediante aplicaciones foliares, baños en poscosecha o la combinación de ambas, respectivamente, por lo que tanto las aplicaciones foliares de $CaCl_2$ como los baños poscosecha serían prácticas a recomendar en el caso de riesgo de *bitter pit*.

RESUM

El *bitter pit* és la fisiopatia més important en molts cultivars de pomes. No obstant, no existeix una estratègia completament efectiva per al seu control. Per aquest motiu, un mètode de predicció que identifiqui anys i plantacions amb un alt potencial de desenvolupar la fisiopatia permetrà evitar pèrdues econòmiques, especialment durant la conservació i confecció.

L'objectiu principal de la present tesi doctoral va ser la posada a punt d'un sistema de predicció de la incidència del *bitter pit* en plantacions de poma 'Golden Smoothee'. Per això, es van investigar diferents mètodes basats en tres tecnologies diferents: i) l'anàlisi mineralògic de fruits en diferent època (en estadis primerencs i a recol·lecció), ii) la inducció de símptomes (infiltració de Mg, banys amb etefón, embossat de fruits i mètode passiu) i iii) l'espectroscopía VIS/NIR. Els diferents mètodes es van avaluar en diferents períodes de creixement del fruit. Paral·lelament, es va avaluar i quantificar l'eficàcia de diferents estratègies per mitigar l'aparició de *bitter pit* basades en aplicacions de $CaCl_2$ a pre i postcollita (aplicacions radiculares, foliars i banys en poscosecha).

L'anàlisi primerenc de Ca en fruit a 60 dies després de la floració (DDPF) va mostrar una precisió en la predicció similar o millor que l'anàlisi a recol·lecció. Es va definir un valor de referència de 60 DDPF de 11 mg de Ca 100 g^{-1} de pes fresc, per sobre del qual es minimitza la incidència de *bitter pit*. La majoria de mètodes basats en induir símptomes, a excepció de l'embossat, van mostrar eficàcia a partir dels 40 dies abans de la recol·lecció (DAR), amb una correlació amb el *bitter pit* de postcollita del 70-80%. La espectroscopía VIS/NIR va mostrar resultats poc satisfactoris per a la predicció del *bitter pit*, però va ser capaç de discriminar fruits afectats quan els símptomes eren visibles a postcollita. Finalment, es va disenyar un model de predicció per al *bitter pit* basat en l'anàlisi de Ca en fruit a 60 DDPF i el mètode passiu a partir de 40 DAR. Respecte la mitigació del *bitter pit*, els resultats obtinguts en els anys amb alta incidència van mostrar una reducció del 20% a un 12%, 8% o 3% mitjançant aplicacions foliars, banys de postcollita o la combinació d'ambdues, respectivament, per tant, les aplicacions foliars de $CaCl_2$ així com els banys postcollita serien pràctiques a recomanar en els casos d'alt risc de *bitter pit*.

ABSTRACT

Bitter pit is the most important physiological disorder in many apple cultivars. However, there is no a completely effective control strategy. Therefore, a prediction method that identifies years and orchards with high potential to develop bitter pit will allow reducing economic losses, especially during storage and fruit packing.

The main objective of this PhD thesis was the development of a system to predict the incidence of bitter pit for 'Golden Smoothee' apple orchards. For this, different methods to predict bitter pit based on three different technologies were investigated: i) mineral analysis (at early stages and at harvest period, ii) induction of symptoms (Mg infiltration, dips with etephon solution, bagging of fruit and passive method) and iii) VIS/NIR spectrophotometry. The different methods were tested in different fruit growth stages. At the same time, the efficacy of different strategies based on $CaCl_2$ applications at pre- and postharvest (fertigation, foliar and postharvest dips) to mitigate bitter pit incidence, were evaluated and quantified.

The accuracy of mineral analysis at early development fruit after 60 days after full bloom (DAFB) was better or equal than Ca analysis at harvest. A reference threshold at 60 DAFB of 11 mg Ca 100 g^{-1} fresh weight was defined. Values equal or higher indicated a low risk of bitter pit. Most methods based on inducing symptoms, with the exception of bagging fruit, showed efficacy from 40 days before harvest (DBH), with a correlation with bitter pit at postharvest of 70-80%. VIS/NIR spectrophotometry showed unsatisfactory results for bitter pit prediction, however, it was able to discriminate affected apples when the symptoms were visible at postharvest. Finally, a bitter pit prediction model based on the analysis of Ca in fruitlet at 60 DAFB and the passive method from 40 DBH was designed. Regarding bitter pit mitigation, the results obtained in seasons with a high incidence showed a reduction from 20% to 12%, 8% or 3% using Ca sprays, postharvest dips or the combination of both, respectively. Therefore, Ca sprays and postharvest dips in $CaCl_2$ solutions are recommended practices when there is a diagnostic of high risk of bitter pit.

ABREVIATURAS

AECID	Agencia Española de Cooperación Internacional para el Desarrollo
AIA	Ácido indol-3-acético
ANOVA	Analysis of variance
B	Boro
BG	Bagging of fruit
BP	Bitter pit
BPAS	Bitter pit after storage
BPLS	Bitter pit-like symptoms
Ca	Calcio
CERCA	Centres de Recerca de Catalunya
DAFB	Days after full bloom
DAR	Días antes de recolección
DBH	Days before harvest
DDPF	Días después de plena floración
ET	Ethephon dips
EUA	Estados Unidos de America
FW	Fresh weight
GLM	Generalized linear model
HBP	Hight bitter pit
INIA	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
IRTA	Institut de Recerca i Tecnologia Agroalimentàries
K	Potasio
LBP	Low bitter pit
LDA	Linear discriminant analysis

LOX	Lisil oxidasa
Mg	Magnesio
MG	Infiltration with $MgCl_2$
N	Nitrógeno
NS	Non-significant
P	Probability value
PC	Principal components
PCA	Principal component analysis
PF	Peso fresco
PLS	Partial least squares
PPO	polyphenol oxidase
PS	Passive method
QDA	Quadratic discriminant analysis
RH	Relative humidity
ROS	Reactive oxygen species
SVM	Support vector machines
UdL	Universitat de Lleida
VIS/NIR	Visible and near infra-red radiation

ÍNDICE

INTRODUCCIÓN

1. INTRODUCCIÓN

En un mercado globalizado y exigente como el actual, uno de los mayores retos al que deben enfrentarse los fruticultores es la capacidad de poder mantener altos rendimientos productivos manteniendo una alta calidad hasta que los frutos llegan al consumidor. Sin embargo, durante el proceso de producción y conservación, las características del fruto pueden verse alteradas dando lugar a la aparición de alteraciones fisiológicas y a la disminución de los parámetros de calidad del fruto. En el cultivo del manzano, el *bitter pit* constituye el principal desorden fisiológico durante el desarrollo y conservación del fruto, ocasionando grandes pérdidas en cualquiera de los eslabones de producción y comercialización. A pesar de los esfuerzos llevados a cabo en los últimos cien años para determinar los principales factores implicados en su desarrollo y poder controlar su aparición, todavía hoy se desconocen con certeza los mecanismos que lo desencadenan. A todo ello, hay que añadir las altas exigencias de calidad en la fruta destinada a exportación, donde se exige que la presencia de frutos sin alteraciones sea inferior al 2%.

El *bitter pit* puede aparecer tanto en el campo como tras un periodo de conservación, y su incidencia puede variar entre años y plantaciones (Le Grange et al., 1998; Yuri, 2002). Es considerado un desorden fisiológico, lo que significa que no es causado por ningún agente patógeno. Aunque el consumo de frutos con *bitter pit* no supone un riesgo para la salud humana, las manzanas afectadas no suelen ser aptas para la comercialización debido a su apariencia, sabor amargo del tejido afectado y un mayor riesgo a sufrir podredumbres.

Los síntomas se manifiestan en el fruto, especialmente en la mitad calicina, como manchas de color amarillento en la periferia y de un verde intenso en el interior. Su localización no va más allá del exterior del mesocarpio, a 1 ó 2 mm por debajo de la piel del fruto, donde adquiere una textura acorchada de sabor amargo que da nombre a la enfermedad (Ferguson y Watkins, 1989; Reid y Padfield, 1975). Las manchas del *bitter pit* están constituidas por células muertas y deshidratadas que conservan intactas sus paredes celulares. Smock y Van Doren (1937) realizaron los primeros estudios sobre *bitter pit* a nivel histológico, deduciendo que el desorden comienza con un colapso de la pared celular ocasionando una plasmólisis del citoplasma cuando los síntomas todavía no son visibles.

La aparición del *bitter pit* se ha atribuido tradicionalmente a una deficiencia de calcio (Ca). De forma empírica se ha demostrado que todos aquellos aspectos y prácticas culturales que influyen en la nutrición del Ca se convierten en puntos críticos en su control. En este sentido, la transpiración (Kirkby, 1993; Monge et al., 1995), el tipo de suelo (Sio et al., 1999), el porta-injerto (Fallahi, 2012; Fazio et al., 2013), el cultivar (Kirkby, 1993), la estrategia de poda (Terblanche et al., 1974), el control de la carga del árbol (Ferguson y Watkins, 1992), la fertilización (Terblanche et al., 1975) o el tipo de riego (Sio et al., 1999) pueden influenciar en la incidencia del *bitter pit*, por lo que debería tratarse como un fenómeno multifactorial con implicaciones tanto a nivel de campo como de conservación.

Desde que Garman y Mathis propusieron en 1956 (Val et al., 2002) la aplicación foliar de sales de Ca para reducir la incidencia del *bitter pit*, se han realizado numerosos trabajos con el objetivo de evaluar y mejorar su eficacia, aunque con resultados irregulares y, en la mayoría de casos, con una eficacia insuficiente cuando las incidencias son elevadas (Fernandez et al., 2009; Manganaris et al., 2006; Val et al., 2008). Aún así, las aplicaciones de Ca siguen siendo, a día de hoy, la herramienta más utilizada para el control del *bitter pit*, con una eficacia estimada del 50% (Casero et al., 2010; Ferguson et al., 1999; Lotze y Theron, 2007; Peryea et al., 2007).

La recomendación generalizada es realizar más de 10 tratamientos durante todo el desarrollo del fruto, lo que supone un gran coste económico y de tiempo para los agricultores, con eficacias insuficientes y sin que permita erradicar totalmente la alteración. La puesta a punto de un método fiable de predicción del *bitter pit* permitiría implementar una estrategia integral de control en campo en los años y plantaciones de riesgo, lo que implicaría una mejora en la gestión de los recursos destinados para su control (aplicaciones foliares de Ca, reducción de abonados nitrogenados y potásicos, control del crecimiento vegetativo, etc.). Además, en la entrada en las centrales de confección y conservación, permitiría clasificar las partidas de frutos en función de su potencial a desarrollar síntomas durante la conservación, escogiendo para cada caso la estrategia de poscosecha y comercialización más idónea. Todo ello se traduciría en un mayor beneficio económico para el sector ya que, por un lado, se reducirían las pérdidas y reclamaciones debidas a frutos afectados y, por otra parte, se optimizarían los inputs utilizados durante el cultivo y la poscosecha. Por todo ello, la predicción del *bitter pit* se considera de especial interés y absolutamente necesaria para el sector productor de manzana.

Un método o modelo de predicción óptimo debe proporcionar resultados veraces e independiente de los factores de variabilidad del modelo. Además, para que un método predictivo del *bitter pit* pueda ser implementado en el proceso productivo, la metodología debe ser i) suficientemente sencilla como para poder ser abordada a pie de campo o a nivel de central, ii) los resultados deben estar disponibles en el momento adecuado para la toma de decisiones y iii) el beneficio obtenido debe ser claramente superior al coste que supone la predicción.

Para que el segundo punto sea posible, la predicción se debe realizar con la suficiente antelación para poder efectuar las actuaciones de mitigación necesarias. Sin embargo, sólo con que el método fuera fiable para épocas cercanas a la recolección permitiría a las centrales frutícolas la oportunidad de tomar decisiones relevantes en la gestión de los lotes de entrada, como decidir si aplican baños con sales de Ca o no, la decisión de aplicar 1-MCP o no, así como manejar el destino y régimen de almacenamiento o venta más adecuado en función del riesgo. Esto redundaría, sin lugar a duda, en una optimización de los recursos y en una reducción de los costes de conservación, de las pérdidas de producto y de las reclamaciones posventa. Por eso, los métodos de predicción se consideran de gran interés y absolutamente necesarios para una mejora de la calidad de la fruta resultante.

En las últimas décadas han aparecido distintas propuestas para la predicción del *bitter pit*, sin embargo, debido al incumplimiento de alguno de los puntos mencionados anteriormente (fiable, sencillo, precoz y de bajo coste) no han sido implantadas a nivel comercial, aunque sí se han utilizado a modo de investigación y experimentación para un mayor conocimiento del problema. El primero de los métodos propuestos y el más utilizado en todo el mundo ha sido el análisis mineral del fruto justo antes de la recolección (Autio et al., 1986; Wolk et al., 1998). Sin embargo, dichos análisis no dejan margen de maniobra a los productores para tomar medidas de mitigación y, con bastante frecuencia, las predicciones suelen ser poco fiables. Otros investigadores han propuesto sistemas de predicción basados en la anticipación de los síntomas, con un mayor grado de fiabilidad pero con una implementación más dificultosa. Este tipo de sistemas incluye a los baños con etefón y la infiltración con sales de Mg (Bangerth, 1974; Retamales et al., 2000). Además, en los últimos años, se han estudiado métodos no destructivos como la espectroscopía VIS/NIR para detectar frutos con *bitter pit* (Kafle et al., 2016; Nicolai et al., 2006). Mediante la presente tesis doctoral se pretende encontrar un método o una estrategia de predicción del *bitter pit* fiable para plantaciones de manzanas 'Golden' en condiciones del Valle del Ebro. Para ello se han evaluado en condiciones locales los métodos mencionados anteriormente y se han desarrollado nuevos métodos con la intención de mejorar los aspectos citados (fiabilidad, sencillez, precocidad y coste). Además se ha cuantificado la eficacia de distintas estrategias de mitigación del *bitter pit* basadas en aplicaciones de Ca, con la finalidad de poder dar una recomendación contrastada según el riesgo de *bitter pit*.

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ANTECEDENTES

2. ANTECEDENTES

2.1. Posibles causas del bitter pit

A pesar de los numerosos trabajos de investigación para determinar las causas del *bitter pit*, a día de hoy todavía se desconoce con certeza los motivos que desencadenan su aparición. Aznar (2001), en su tesis doctoral sobre la caracterización fisiológica del *bitter pit*, cita la existencia de cuatro posibles teorías que intentan explicar el origen de la enfermedad. En cada una de estas teorías, los principales factores implicados serían: i) la deficiencia de Ca, ii) las giberelinas, iii) las auxinas, y iv) la metabolización de sustancias generadas durante la fotosíntesis. A estas cuatro teorías habría que añadir una quinta, formulada recientemente por Saure (2014), basada en los fenómenos de oxidación que se producen en situaciones de estrés abiótico.

Desde la identificación del desorden, se han realizado numerosos esfuerzos para intentar explicar las causas que provocan el *bitter pit*. Tradicionalmente se ha asociado con desequilibrios nutricionales en el fruto y, en particular, con la deficiencia de Ca desde que DeLong (1936) observó por primera vez que frutos con *bitter pit* tenían concentraciones de Ca inferiores a los frutos no afectados. Según Ferguson y Triggs (1990), la escasez de Ca en las membranas incrementaría la permeabilidad de ácidos y fenoles y, consecuentemente, éstos penetrarían con más facilidad en el citoplasma, destruyendo o coagulando enzimas de las mitocondrias o de otras partículas subcelulares. Sin embargo, existen varios estudios que citan una falta de correlación entre el contenido de Ca y la incidencia del *bitter pit* (Le Grange et al., 1998; Lotze et al., 2008;

Terblanche et al., 1980). Val et al. (1999) desarrollaron un método de detección específica de Ca en los tejidos del fruto, con el que mostraron que, dentro de un mismo fruto, los tejidos afectados por *bitter pit* muestran una mayor acumulación de Ca, por lo que se replantearon que el *bitter pit* se deba a una deficiencia de Ca.

En las últimas décadas han aparecido nuevas teorías distintas a la deficiencia del Ca. Saure (1996) propuso el aumento de giberelinas en el fruto como causa principal del *bitter pit.* Esta teoría se fundamenta en que aplicando fitorreguladores de crecimiento que inhiben la formación de giberelinas se consiguen reducciones de la incidencia de *bitter pit*, independientemente de la concentración de Ca en el fruto. Un incremento de esta hormona provocaría un incremento de la permeabilidad celular y, consecuentemente, un aumento del riesgo de deshidratación. Según esta teoría, un alto contenido de giberelinas en el fruto impediría la movilidad de Ca hacia el fruto, mientras que frutos con alto contenido de Ca podrían estabilizar las membranas, mitigando el problema, por lo que el Ca jugaría un papel secundario. Los resultados obtenidos por Silveira et al. (2012) respaldan dicha teoría al lograr una reducción y un incremento del *bitter pit* aplicando prohexadiona de Ca (un inhibidor de la síntesis de giberelinas) y giberelina GA_3, respectivamente.

Hertel (1983) y Banuelos (1987) relacionaron la aparición del *bitter pit* con la presencia de auxinas en el fruto. Hertel (1983) propuso que el transporte de ácido indolil-3-acético (AIA) puede promover la liberación de cationes de Ca desde las vacuolas o de las paredes celulares al citoplasma con la intención de incrementar el contenido de Ca citoplasmático. Este aumento de Ca citoplasmático, estaría asociado con una estimulación de la síntesis o renovación de las paredes celulares, reduciendo los riesgos de degradación y, por tanto, de aparición de desórdenes fisiológicos. Banuelos et al. (1987) describieron los mecanismos de transporte del Ca controlado por el AIA endógeno en los propios frutos e independiente del flujo de transpiración. En este sentido, las auxinas producen un efecto sumidero en los tejidos de las plantas, por lo que los tejidos con mayor contenido de auxinas adquieren cierta prioridad en el suministro de nutrientes y agua respecto a los tejidos con menor contenido de auxinas. Además, en distancias muy cortas, de célula a célula, se produce una movilización de Ca mediante el mecanismo denominado *auxina pump* (bomba de auxina). Según Banuelos et al. (1987), una cantidad insuficiente de auxinas impediría el transporte del Ca por estas dos vías, provocando la aparición del *bitter pit* en frutos con deficiencia de Ca. Las auxinas son producidas en las flores, semillas y en hojas jóvenes de las puntas de los brotes o cerca de los frutos, por lo que los árboles con una buena floración, un buen cuajado, un buen desarrollo foliar y frutos con un mayor número de semillas, tienen mayor capacidad de absorber Ca que aquellos árboles con una pobre floración, una baja producción y un follaje de mala calidad. Por lo tanto, el número de semillas en el fruto podría tener un efecto en los movimientos del Ca dentro del fruto. En esta línea, Brookfield et al. (1996) y Broom et al. (1998), señalaron una correlación negativa entre el número de semillas viables en los frutos y la incidencia de *bitter pit*, así como el contenido de Ca. El número de semillas en el fruto también afecta a los movimientos del Ca en la pulpa durante la conservación del fruto, promovidos por las hormonas producidas por las propias semillas, de forma que al final del periodo de conservación se observa una disminución de la concentración de Ca en la zona más cortical de la pulpa, justo debajo de la piel (Perring y Pearson, 1986).

Otro de los postulados en la aparición del *bitter pit* apunta a los mecanismos de metabolización de algunas de las sustancias tóxicas que se generan durante la fotosíntesis, como el ácido oxálico y el ácido cítrico (Steenkamp et al., 1983). Debido a que las plantas no tienen mecanismos para eliminar este tipo de sustancias, intentarían metabolizarse por medio de la formación de sales insolubles con el Ca. En el caso de que el contenido de Ca no fuera suficiente para metabolizar estos ácidos se produciría la muerte celular, lo que daría lugar a los síntomas del *bitter pit*. Más recientemente, Saure (2014) indicó el estrés abiótico como principal causa de los desórdenes fisiológicos que sufren diversos cultivos hortofrutícolas relacionados con el Ca. Según el autor, el estrés aumenta la producción de especies reactivas de oxigeno (ROS, por sus siglas en inglés) lo cual causaría la peroxidación de lípidos y, en consecuencia, una falta de estabilidad de las membranas, provocando una rápida vacuolización de las células parenquimáticas y la pérdida de iones como Ca apoplástico. Por lo tanto, la deficiencia de Ca relacionada con la aparición del desorden sería como resultado del estrés y no la causa de la enfermedad. Con anterioridad al postulado de Saure (2014), Wińska-Krysiak y Łata (2010) investigaron en diferentes cultivares la relación entre el desarrollo del *bitter pit* y la actividad de la enzima lipoxygenasa (LOX), enzima involucrada en la respiración del fruto, concluyendo que aquellos cultivares con mayor actividad de la LOX y, consecuentemente más estresados, son más susceptibles a padecer *bitter pit* y a contener una menor concentración de Ca. Recientemente, Krawitzky et al. (2016) identificaron que varios tipos de proteínas relacionadas con la patogénesis del estrés oxidativo se sobreexpresan en frutos afectados por *bitter pit*.

La mayoría de estas teorías mantienen la deficiencia de Ca como denominador común en la aparición del *bitter pit*, ya sea de una forma más o menos directa y, de hecho, no se contradicen las unas con las otras por lo que, tal como apunta Aznar (2001) en su tesis 'Caracterización fisiológica del *bitter pit*: aspectos nutricionales, fenológicos y de diagnóstico', es muy probable que las verdaderas causas se encuentren en la integración de todas ellas.

2.2. Métodos de predicción

La aparición de *bitter pit* puede variar entre cultivares, años, plantaciones e incluso entre frutos de un mismo árbol (Ferguson y Triggs, 1990; Le Grange et al., 1998; Yuri, 1995). Dada la irregularidad con la que aparece, es vital para el sector frutícola un método fiable de predicción que permita la identificación de partidas con alto potencial de desarrollarlo, especialmente antes de la exportación, y consecuentemente reducir las pérdidas económicas durante la comercialización debidas a las reclamaciones por la aparición de frutos con síntomas. No obstante, el número de trabajos basados en su predicción son escasos y la mayoría de ellos están centrados en el análisis mineralógico.

Una primera aproximación para conocer el riesgo de *bitter pit* es conocer el historial de la plantación. Aunque para una misma finca el *bitter pit* puede manifestarse de manera irregular, existen plantaciones que todos los años presentan un porcentaje de *bitter pit* residual, viéndose incrementado considerablemente en años en que la incidencia se generaliza a un mayor número de plantaciones en el territorio. De manera contraria, existen plantaciones en las que prácticamente no se observan síntomas de *bitter pit* independientemente de los años.

Aun así, la irregularidad con la que aparece el *bitter pit* en la mayoría de plantaciones, especialmente durante la conservación de la fruta, hace necesaria un modelo de predicción de mayor precisión que ayude a optimizar la toma de decisiones. Hasta la fecha, los métodos para predecir el *bitter pit* citados en la literatura pueden englobarse en cuatro grupos: i) los basados en el análisis mineralógico de hoja o fruto, ii) los basados en la anticipación de síntomas mediante tratamientos químicos iii) modelos multifactoriales basados en parámetros agronómicos, edafológicos y/o meteorológicos, y iv) métodos no destructivos mediante espectroscopía del infrarrojo cercano (NIR por sus siglas en inglés), fluorescencia o rayos X.

Cabe matizar que los resultados de los distintos trabajos que se recogen en cada uno de estos grupos fueron obtenidos y, en el mejor de los casos, validados bajo condiciones locales, por lo que son dependientes a la localización del ensayo y al cultivar utilizado, tal como puntualizan la mayoría de sus respectivos autores. En España, únicamente se han realizado pruebas piloto a muy pequeña escala de alguno de los métodos mencionados, sin que ninguno de ellos haya sido implementado a nivel comercial, por lo que se desconoce su precisión y viabilidad en nuestras condiciones. A continuación, se detallan los antecedentes y estado del arte de los distintos métodos mencionados para predecir el *bitter pit.*

2.2.1. Análisis mineralógico

Los análisis de fruto realizados entre 2 y 4 semanas antes de la recolección ha sido la forma más utilizada para conocer el potencial de una plantación a desarrollar *bitter pit*. No obstante, dichos análisis, por el momento en el que se realizan, no dejan margen de maniobra a los productores para tomar medidas correctoras que permitan mitigar el *bitter pit*. Aún con todo, si los resultados se obtienen con cierta rapidez, pueden proporcionar información valiosa para las centrales de conservación, permitiéndolas segregar partidas según los resultados de los análisis. Sin embargo, debido al alto coste de los mismos y a la complejidad en la interpretación de los resultados, no suele ser una práctica habitual.

Con anterioridad al análisis de fruto, se estudió la posibilidad de predecir el *bitter pit* mediante el análisis de hoja. Shear (1972) obtuvo correlaciones similares utilizando el análisis de hoja dos semanas antes de recolección y el análisis de fruto en recolección, para la predicción del *cork spot* (alteración similar al *bitter pit*) en manzanos 'York Imperial'. Según Shear (1972), en los años con periodos de sequía el análisis de fruto sería más sensible a la predicción del *bitter pit*, mientras que el análisis de hoja sería más errático. Drake et al. (1974) correlacionaron el contenido de Ca en hoja con el contenido de Ca en frutos de manzanas 'Baldwin', pero los resultados parecían tener una fuerte dependencia con la carga de frutos, con correlaciones que iban del 6,69% al 53,3% en años consecutivos. Sharples (1979) y Holland (1980), confirmaron una mayor precisión mediante el análisis de frutos que mediante el análisis de hojas en el cultivar 'Cox's Orange Pippin', proponiendo un umbral de referencia de Ca en el fruto por debajo del cual se incrementa significativamente el riesgo de *bitter pit*, siendo todavía hoy una referencia en la mayoría de laboratorios de análisis de material vegetal de todo el mundo. Van der Boon (1980a) obtuvo mejores predicciones, mediante la concentración de Ca en frutos que en hojas. Val et al. (1999), tras investigar el comportamiento de los nutrientes a lo largo del ciclo vegetativo, concluyeron que el riesgo de *bitter pit* aumenta considerablemente cuando entre los 80-100 días después de plena floración (DDPF) el valor de la relación foliar K/Ca desciende por debajo de 2 y en fruto aumenta por encima de 25.

Con el tiempo, el análisis de fruto se ha impuesto al análisis de hoja al considerarse de mayor precisión. Sharples (1979) posicionó el umbral de bajo riesgo a desarrollar *bitter pit* cuando la concentración de Ca en el fruto se encuentra por encima de los 5-6 mg 100 g^{-1} de peso fresco (PF), considerando a los frutos con valores por debajo de dicho umbral de alto riesgo. Hoy día continúa siendo una referencia para cualquier cultivar en cualquier parte del mundo (Lotze et al., 2008; von Bennewitz et al., 2015). Sin embargo, con bastante frecuencia, las predicciones basadas en dichos ratios pueden ser erráticas, habiendo partidas con una relativa alta concentración de Ca (superior a 5 mg Ca 100 g^{-1} PF) que acaban desarrollando *bitter pit*, y viceversa (Le Grange et al., 1998; Terblanche et al., 1980).

Para mejorar la precisión de predicción, además del Ca, algunos autores han propuesto considerar otros minerales que pueden influir en la asimilación y transporte del Ca dentro del árbol, como son el potasio (K), el magnesio (Mg), el nitrógeno (N) o el boro (B) (Casero et al., 2004; Ferguson et al., 1999). En general, altos contenidos de K, Mg y N se relacionan con una mayor sensibilidad al *bitter pit* (Alonso et al., 2003; Ben, 1997; de Freitas et al., 2010;

Marcelle, 1993; Tomala, 1997b). El K y Mg, a diferencia del Ca, son fácilmente transportados por el floema, por lo que, si la concentración de Ca en la membrana celular es insuficiente, los iones de Mg y K podrían reemplazarlo, ocasionando una peor estabilidad y permeabilidad en la membrana (Tagliavini et al., 2000).

Mientras que en el caso del Mg y K es conocido su efecto antagonista respecto al Ca, no ocurre lo mismo con el N. Altos contenidos de N en fruto pueden provocar una rápida expansión de las células y una mayor velocidad de crecimiento del fruto, lo que provocaría una disolución del contenido de Ca y, en consecuencia, una mayor susceptibilidad al *bitter pit* (de Freitas et al., 2015; Ferguson y Watkins, 1989; Saure, 2005). Además, aportaciones de N en exceso se relacionan con una mayor tasa de crecimiento vegetativo lo que provocaría una mayor competencia en el suministro de Ca entre hojas y frutos. Sin embargo, Val et al. (2000), a diferencia de lo que cabría esperar, observaron una relación positiva entre el contenido de Ca y N, aunque en un nuevo ensayo obtuvieron una mayor incidencia de *bitter pit* al aumentar la fertilización de N (Alonso et al., 2003). La forma en la que se aporta el N podría tener un efecto sobre el *bitter pit*; en este sentido, aportaciones de N en forma de sales amoniacales podrían incrementar la sensibilidad de los frutos al *bitter pit* en comparación a los nitratos (Fukumoto y Nagai, 1983; Ludders, 1979).

El contenido de B en el fruto también se ha relacionado con la incidencia del *bitter pit*. Este es un microelemento esencial para las plantas con un rol muy importante en un gran número de reacciones enzimáticas como el transporte de azúcares, la división y crecimiento celular, la respiración, la fotosíntesis y la síntesis de la pared celular. En general, altas concentraciones de B se relacionan con una mejor absorción y transporte del Ca dentro del árbol y, consecuentemente, con períodos de conservación más largos y menor incidencia de *bitter pit* (Wojcik et al., 1999b; Wojcik y Cieslinski, 2000). No obstante, existen resultados contradictorios en este sentido. Al igual que el Ca, el B es transportado principalmente por el xilema y su deficiencia puede provocar sintomatologías muy parecidas al *bitter pit*, más conocidas como *cork spot*. Wooldridge et al. (1974) señalaron una relación positiva entre la absorción de Ca y el contenido de B en el suelo. Wojcik y Cieslinski (2000) lograron incrementar la concentración final de B y Ca en fruto mediante aplicaciones de B por vía foliar y radicular. Por contra, Lidster et al. (1975) observaron una correlación negativa entre la incidencia del *bitter pit* y el contenido de B en el fruto, y Benavides et al. (2002) obtuvieron una correlación negativa entre el contenido de Ca y B en fruto. Por lo tanto, se desconoce la influencia del B sobre la incidencia del *bitter pit*, por lo que son necesarios más estudios antes de utilizarlo para la predicción del *bitter pit*.

La mayoría de investigadores están de acuerdo en que es tanto o más importante conocer las proporciones de Ca respecto al K, Mg o N, que los niveles minerales de cada uno por sí solos. Es por ello que el cociente entre la concentración de dichos elementos con la concentración de Ca se suele utilizar para determinar el potencial de *bitter pit* (Casero et al., 2010; de Freitas et al., 2015; Drahorad y Aichner, 2003; Marcelle, 1993; Miqueloto et al., 2014b; Val et al., 1999; Van der Boon, 1980b; Waller, 1980; Wills et al., 1976). El antagonismo del Ca con el K es más alto en comparación con el del Mg (Vaysse et al., 1993), por lo que el ratio K/Ca suele ser el más utilizado. Wills et al. (1976) obtuvieron mejores predicciones utilizando el ratio K/Ca que el Ca por sí sólo. Cooper y Bangerth (1976) mejoraron sus resultados utilizando el ratio Mg/Ca. Waller (1980) propuso que valores del cociente K/Ca inferiores a 30 se relacionaban con un bajo riesgo de *bitter pit*, mientras que un ratio superior a 35 suponía un alto riesgo de *bitter pit*. Terblanche et al. (1980) propusieron que valores de (K + Mg)/Ca inferiores a 30 no suponían riesgo de *bitter pit*. Holland (1980) combinó la relación de K y Mg con el Ca mejorando las correlaciones obtenidas con el Ca por sí sólo (56% vs. 49%).

Cabe matizar que la utilización de dichos ratios han sido puestos en entredicho por algunos autores, quienes no encontraron una mejoría significativa en las predicciones al incorporar nutrientes distintos al Ca. Ferguson et al. (1979) encontraron una asociación aceptable entre el *bitter pit* predicho y el real clasificando los frutos en cinco categorías basadas exclusivamente en la concentración de Ca. En trabajos posteriores, sugirieron que añadir la concentración de K y Mg no mejoraba la precisión de predicción en muestreos a gran escala (Ferguson y Triggs, 1990). Bramlage et al. (1985) adaptaron un método de puntuación del riesgo de *bitter pit* en manzanas 'McIntosh' a partir de la suma de los valores de Ca, N, P/Ca, K/Ca y Mg/Ca en fruto y observó que únicamente con el contenido el Ca en fruto era capaz de obtener predicciones fiables sobre su potencial de conservación, aunque respecto al *bitter pit* obtuvo resultados irregulares, supuestamente, debido a una baja frecuencia. Autio et al. (1986), en manzanas 'Cox's Orange Pippin', obtuvieron un coeficiente de predicción del 49% basándose en la concentración de Ca y K, mientras que utilizando únicamente la concentración de Ca la precisión aumentó hasta el 55%. Del mismo modo, Ben (2006) obtuvo mejores correlaciones utilizando sólo la concentración de Ca en fruto que con la relación K/Ca.

El análisis mineral en etapas tempranas de desarrollo podría ofrecer a los productores un mayor tiempo de reacción para tomar las medidas correctoras necesarias con el objetivo de mitigar la aparición del *bitter pit*. Sanz y Machín (1999) estudiaron la posibilidad de aplicar el análisis mineralógico en flor para predecir el *bitter pit*, pero obteniendo resultados poco concluyentes. Algunos autores han propuesto el análisis de frutos en estadios de desarrollo temprano. Dicha propuesta se basa, en primer lugar, en la correlación significativa entre la concentración de Ca en recolección y la incidencia del *bitter pit* y, en segundo lugar, en la correlación significativa entre la concentración de Ca en estadios de desarrollo temprano y su concentración en recolección, por lo que si es posible predecir el contenido de Ca en fruto cerca de recolección también podría ser posible predecir el riesgo de *bitter pit* (Conway et al., 1994; Ferguson y Triggs, 1990). Weibel (1997) confirmó el potencial de predecir el riesgo de *bitter pit*, así como el ratio K/Ca en recolección, mediante el ratio K/Ca en frutos en 'estadio T' (aproximadamente, entre 30 y 50 DDPF) en los cultivares 'Boskoop' y 'Maigold', indicando un riesgo considerable de desarrollar *bitter pit* cuando la relación K/Ca es por encima de 5,8. Lotze et al. (2008) encontraron mejores correlaciones entre el *bitter pit* y el contenido de Ca cuando éste se analizaba a media estación, entre 41 y 82 DDPF, en comparación al final de estación, entre 83 y 118 DDPF. Sin embargo, la época propuesta por la mayoría de autores para realizar los análisis tempranos de frutos es a partir de 80 DDPF (Drahorad y Aichner, 2001; Ferguson et al., 1979; Martin et al., 1975; Terblanche et al., 1980; Val et al., 1999; Waller, 1980; Wills et al., 1976).

Además del momento en que se realizan los análisis, otro factor a considerar es el tejido del fruto a analizar. Algunos autores han encontrado mejores correlaciones entre el *bitter pit* y el contenido de Ca analizando el epicarpio o piel, que analizando el mesocarpio o pulpa (do Amarante et al., 2006; do Amarante et al., 2009; do Amarante et al., 2013a; do Amarante et al., 2013b; Peryea et al., 2007; Val et al., 2008). Recientemente, Baugher et al. (2017) propusieron un modelo multifactorial para la predicción de la incidencia de *bitter pit* en el cultivar 'Honeycrisp' basado, en parte, en el ratio N/Ca analizado en la piel.

En los últimos años, algunos estudios han correlacionado la aparición de *bitter pit* con las distintas fracciones en las que el Ca puede encontrarse en los tejidos del fruto. Se considera que el Ca puede encontrarse en los tejidos en 4 formas distintas: i) el Ca ligado que forma parte de la estructura de las paredes celulares en forma de pectatos cálcicos; ii) el Ca soluble en el apoplasto de las células y en el simplasto, en forma de nitratos, cloruros y aminoácidos; y por último iii) el Ca insoluble y residual, presente en las vacuolas en forma de precipitados de fosfatos, carbonatos y oxalatos, mayoritariamente, y en formas muy insolubles como silicatos. Según Pavicic et al. (2004) y de Freitas et al. (2010), la fracción de Ca soluble junto con la fracción ligada en las membranas mantienen una mayor correlación con el *bitter pit*, en comparación al análisis tradicional de Ca total. No obstante, son pocos los laboratorios de análisis que disponen de la tecnología y experiencia para proporcionar el contenido de Ca de forma fraccionada (AGQLabs, 2014). No hay que obviar, que la realización de análisis mineralógicos requiere de equipos sofisticados y de personal especializado, lo que suele incrementar el coste de los modelos de predicción en los que se basan.

2.2.2. Métodos por anticipación de síntomas

a) Adelanto de la maduración (tratamientos con etefón)

El etileno es la hormona responsable de la maduración de los frutos, por lo que tratamientos con etileno antes de la cosecha comercial tienen la capacidad de adelantar la maduración del fruto. Según varios estudios, los frutos tratados con etileno son capaces de expresar síntomas de *bitter pit* antes de que estos se desarrollen de manera natural (Ginsburg et al., 1976; Lojko y Krivorot, 1996; Lyford y Mulligan, 1976; Sestari et al., 2009; Taylor et al., 1978). No obstante, aún no está claro el verdadero papel que juega el etileno en la aparición del *bitter pit*, ya que *a priori* cabría esperar un efecto opuesto tal como muestran algunos estudios. El estado de madurez en el que se recogen los frutos tiene una influencia directa sobre la susceptibilidad al *bitter pit*, agravándose el problema cuando éstos se recolectan inmaduros en estadios cercanos a la recolección (Ferguson et al., 1993; Oud, 1974; Prange et al., 2011; Watkins et al., 2005). A partir de esta observación, Prinja (1990) y Watkins et al (1989) consiguieron reducciones del *bitter pit* mediante la aplicación foliar de etefón antes de la recolección. Sin embargo, la utilización de etefón para predecir el *bitter pit* antes de la maduración comercial es una recomendación que puede encontrarse en diversos manuales de fruticultura (Hanrahan y Schuhmann, 2018; Ohlendorf, 1991). Pouwer (1974) utilizó el etefón para predecir el *bitter pit*, con una correlación del 70% entre el *bitter pit* inducido y el *bitter pit* real, pero indicando que el método podía sobre-expresar los síntomas

(Lotze y Theron, 2006). A finales de los 70s, en Sudáfrica se desarrollaron dos métodos, denominados método Ginsburg y método Bangerth, basados en la aplicación de gas acetileno y etefón, respectivamente (Eksteen et al., 1977; Lotze, 2005). El método Ginsburg consistía en adelantar la maduración de los frutos recolectados 14 días antes de la recolección (DAR) comercial con 1% de gas acetileno. El método Bangerth consistía en sumergir una muestra de frutos en una solución de agua con un 0,2% de etefón. Después de cada tratamiento, los frutos se mantenían a 20 °C y una humedad relativa del 90%. El método Ginsburg determinó el potencial de *bitter pit* a los 14 días, coincidiendo con el momento de la recolección comercial, mientras que el Bangerth era capaz de mostrar resultados a los 10 días, adelantándose por lo tanto a la recolección comercial. Ambos métodos proporcionaron una precisión media del 50% en los cultivares 'Golden Delicious' y 'Cox's Orange Pippin', pero sin resultados en el cultivar 'White Winter Pearmain' (Eksteen et al., 1977). Lotze et al. (2010) reevaluaron el método en Sudáfrica para manzanas 'Braeburn' y 'Golden Delicious' con una correlación del 13%.

b) Infiltración con sales de magnesio

Tal como se ha comentado anteriormente, un mayor contenido de Mg en fruto, a diferencia del Ca, se relaciona positivamente con una mayor incidencia de *bitter pit*. Hopfinger y Poovaiah (1979) observaron la aparición de síntomas tipo *bitter pit* una semana después de que los frutos fueran infiltrados con un 2% de $MgCl_2$. La aparición de síntomas después de la infiltración se debería a un desplazamiento del Ca por el Mg en los tejidos generando procesos degradativos que se traducirían en la expresión de manchas tipo *bitter pit* (Burmeister y Dilley, 1993; Burmeister y Dilley, 1994; Retamales et al., 2000; Tomala et al., 1993). Burmeister y Dilley (1991) utilizaron dicha metodología para estudiar el desarrollo del *bitter pit* en poscosecha realizando distintos tratamientos de infiltración y se percataron de que el número de manchas inducidas era inversamente proporcional al nivel de Ca en los tejidos. Esto proporcionó la base para desarrollar un método para predecir la incidencia de *bitter pit* a partir de la infiltración de Mg en fruto.

A finales de la década de los 90s, investigadores chilenos perfeccionaron la técnica y empezaron a utilizar la infiltración de Mg para predecir el *bitter pit* (Retamales y Valdes, 2001). Según Retamales y Valdes (1996), a diferencia de la aceleración de la madurez mediante etileno, la infiltración puede ser utilizada con anterioridad, a partir de 60 DAR, aunque suele ser más eficiente a partir de 40 DAR y mejora cuanto más cerca de la recolección comercial. Aun así, en comparación al método por inmersión en etefón y al análisis mineralógico, la infiltración de Mg permitiría una mayor precocidad en la predicción y un mayor tiempo de reacción para realizar actuaciones en campo para mitigarlo.

Según los mismos autores propulsores del método, el procedimiento de muestreo es una fase de vital importancia, por lo que si los frutos son tomados incorrectamente se pueden alterar los resultados (Retamales y Valdes, 1996; Retamales y Valdes, 2001), siendo necesario tomar un mínimo de 40 frutos por parcela, cada uno procedente de un árbol distinto, del calibre más frecuente y ubicados a una altura de 120-140 cm y a 40-80 cm desde la periferia del sector sur-poniente de la copa. Los árboles deben ser uniformes en tamaño y vigor, condición de suelo y carga frutal. No deben usarse árboles enfermos ni con problemas nutricionales. La infiltración debe realizarse dentro de 24 horas de ser recolectados, sumergiendo los frutos en una solución de agua destilada con $MgCl_2$ (10,2 g L^{-1}), Sorbitol (86 cc L^{-1}) y Tween 20 (0,1 cc L^{-1}), sometiéndolos al vacío durante 2 minutos, más 15 minutos en reposo con los frutos sumergidos, lo que permite que los espacios intercelulares constreñidos por la acción del vacío, se rellenen de la solución de infiltración y no de aire (Retamales et al., 2001). Posteriormente, los frutos se secan y se dejan a 20 °C. Tras 8-10 días empiezan a aparecer las manchas, aunque la propuesta comercial para evaluar los síntomas es esperar 18 días cuando los frutos ya han desarrollado todo su potencial (Retamales y Valdes, 2001).

Los resultados obtenidos suelen ser mejores que con el análisis mineralógico o los baños con etefón. En Chile, en manzanas 'Braeburn', lograron correlaciones del 90% entre la incidencia de síntomas inducidos por la infiltración de Mg respecto la incidencia de *bitter pit* después en poscosecha (Retamales y Valdes, 1996; Retamales et al., 2000). En Michigan (EUA), Burmeister y Dilley (1994) obtuvieron correlaciones del 73% al 86% para el cultivar 'Northern Spy'. Do Amarante et al. (2009) reportaron que el método puede ser fiable para predecir *bitter pit* en manzanas 'Gala' del sur de Brasil, pero no proporcionaron niveles significativos de correlación. En España, Díaz et al. (2006) utilizaron la infiltración en poscosecha para provocar la aparición de síntomas en manzanas 'Golden' mediante 15 minutos de vacío y 24 horas de inmersión, consiguiendo que los síntomas aparecieran a las 48 horas, pero no existen resultados publicados en España para predecir el *bitter pit* en precosecha.

La infiltración de Mg requiere de una tecnología más compleja que la inmersión en etefón (recipiente preparado para el vacío, bomba de vacío, manómetro, reactivos, agua destilada) y, siguiendo las recomendaciones de Retamales et al. (2001), un mayor tiempo de tratamiento por muestra (2 minutos de vacío + 15 minutos de inmersión). A todo ello hay que añadir que los niveles de vacío a los que deben someterse los frutos pueden variar en función del cultivar (Retamales y Valdes, 1996; Retamales et al., 2000), lo que complica todavía más su implementación a nivel comercial. Por otro lado, se ha observado que otros factores metodológicos, como el uso repetido de la solución de $MgCl_2$, no afectarían a la precisión del método. Aún con todo, las altas correlaciones obtenidas en comparación a los otros métodos mencionados anteriormente, hacen de la infiltración de Mg el método de predicción de *bitter pit* preferido por varios investigadores (do Amarante et al., 2012; Manning, 2011; Piestrzeniewicz et al., 2000; Retamales y Valdes, 2001).

c) Desarrollo natural del bitter pit en precosecha

La aparición del *bitter pit* en frutos se ha estudiado, en mayor medida, durante su proceso de conservación. No obstante, su desarrollo puede desencadenarse en campo, pudiéndose observar síntomas entre 20 y 15 días previos a la recolección (Ferguson y Watkins, 1989). Cada año, a partir de 3 semanas antes de la recolección, aproximadamente, en las plantaciones de manzanas 'Golden' del Valle del Ebro pueden verse frutos en el suelo con síntomas visibles de *bitter pit*. Dicho fenómeno apenas se ha estudiado científicamente y, hasta la fecha, se desconoce si los frutos afectados presentes en el suelo desarrollan los síntomas antes o después de desprenderse del árbol.

La única referencia encontrada que advierte de dicho acontecimiento corresponde a England y Larsen (1973). En dicho trabajo observaron que frutos sanos de manzanas de los cultivares 'Goldspur' y 'Wellspur' desarrollaban *bitter pit* cuando éstos eran desprendidos del árbol entre 1 y 3 semanas antes de la recolección y dejándolos entre 7 y 14 días a 20-22 °C. Los mismos autores propusieron que dicha incidencia de *bitter pit* podría mantener una relación con el *bitter pit* desarrollado durante la poscosecha. No obstante, no se conocen otros trabajos que hayan evaluado o investigado dicha propuesta para predecir el *bitter pit*. Dicho método, de ser viable, sería un gran avance ya que, gracias a su sencillez y bajo coste al no requerir ni reactivos, ni equipos, ni personal especializados, se facilitaría su implementación a escala comercial.

2.2.3. Modelos multi-factoriales

Son numerosos los estudios que combinan diferentes parámetros de carácter agronómico y/o meteorológico para predecir la aparición de desórdenes fisiológicos (Baugher et al., 2017; Ferguson y Watkins, 1992; Jemric et al., 2016; Sharples et al., 1979; Sió et al., 1999; Tomala, 1997a, b; Weibel et al., 2000). De forma empírica se ha demostrado que todos aquellos aspectos y prácticas culturales que influyen en la nutrición del Ca se convierten en puntos críticos para el control del *bitter pit* (Jemric et al., 2016). En este sentido, tanto el tipo de suelo (Sió, 2010; Weibel et al., 2000), como el portainjerto (Ben, 1999; Biskup et al., 2003; Fallahi, 2012), el cultivar (Miqueloto et al., 2014a;

Volz et al., 2006), la poda (Guerra y Casquero, 2010; Preston y Perring, 1974), la fertilización (Alonso et al., 2003; Casero et al., 2017; Recasens et al., 2004; Wojcik et al., 1999a), el tipo de riego (Fallahi, 2012; Lopez-Cuevas y Robinson, 2006; Silva y Rodriguez, 1996; Sió et al., 1999; Tukey et al., 1978) y el aclareo o carga de frutos (Seo et al., 2007; Telias et al., 2006; Tough et al., 1998; Volz y Ferguson, 1999; Volz et al., 1993) pueden influenciar en la incidencia del *bitter pit*.

Naumann (1974) apuntó como factores influyentes en la aparición del *bitter pit* la fertilización, el manejo del suelo, el riego, el tamaño del fruto, el portainjerto, la aplicación de reguladores de crecimiento, la poda, el momento de cosecha y la conservación. Tomala (1997a), además, expuso otros factores como el número de semillas en el fruto y la posición del fruto en el árbol. A partir de todas estas premisas, en trabajos posteriores, Piestrzeniewicz y Tomala (2001) diseñaron un modelo de predicción que abordaba hasta 40 variables distintas relacionadas con las mencionadas anteriormente (tipo de suelo, análisis de hojas y análisis de frutos en distintos estadios de desarrollo), así como parámetros de madurez como la concentración interna de etileno, la firmeza, sólidos solubles, índice de almidón y peso del fruto. Posteriormente, para reducir el número de variables, combinaron el ratio K/Ca y Mg/Ca en hoja con el número de manchas aparecidas en frutos infiltrados con Mg, obteniendo una precisión de la predicción del *bitter pit* mayor que la obtenida mediante la infiltración por sí sola (79% vs. 61%), aunque los resultados parecieron depender del tipo de cultivar.

Van der Boon (1980a) utilizó un modelo de regresión lineal múltiple que incluía el ratio (K+Mg)/Ca del fruto, el ratio nº hojas/nº frutos, y la uniformidad de la carga de frutos respecto años anteriores para predecir el *bitter pit* en el cultivar 'Cox's Orange Pippin', mejorando la exactitud de los resultados en comparación al análisis de Ca en fruto por sí solo (correlaciones del 60% al 75% durante tres campañas). No obstante, la precisión de la predicción disminuía en aquellos años con una baja incidencia de *bitter pit*, por lo que propuso la inclusión de la temperatura diaria a partir de mediados de agosto para mejorar la predicción, aunque sin materializar la propuesta. Previamente, Shear (1972), al obtener frecuentemente resultados erróneos mediante modelos basados únicamente en el análisis mineral, manifestó la dependencia de otros factores distintos además del estado nutricional del fruto y dependientes del año. Johnson y Ridout (1998) introdujeron en un modelo de predicción para 'Cox Orange Pippin' variables meteorológicas (precipitación y temperatura diaria a partir de julio) además del contenido de Ca en fruto y hoja (en fruto a mediados de julio y cosecha y en hoja a mediados de agosto) logrando una exactitud de predicción del 67%. Brooks (2001) desarrolló una plataforma informática (Megalab) para predecir la incidencia de *bitter pit* a partir de la combinación del análisis mineral de frutos con otros factores como el cultivar, la región, el año y las prácticas culturales. La plataforma fue presentada como un sistema dinámico con la posibilidad de actualizarse y adaptarse a cualquier región. No obstante, se desconoce su precisión a nivel comercial (Lotze y Theron, 2006).

No hay que obviar que las predicciones a partir de los modelos multifactoriales suponen un mayor esfuerzo por el gran número de datos a recopilar y, aunque en general mejoran la precisión de predicción en comparación al análisis mineralógico, los resultados no difieren sustancialmente en comparación a otros métodos como la inmersión en etefón o la infiltración de Mg.

2.2.4. Métodos no destructivos

Dos de los principales inconvenientes de las técnicas mencionadas anteriormente para predecir el *bitter pit* son: i) que son destructivas y ii) la existencia de un retraso, de 7 a 18 días, desde la realización de los análisis o muestreos a la obtención de resultados. Las distintas metodologías englobadas dentro del campo de la espectroscopía podrían superar dichos inconvenientes. Diferentes técnicas de espectroscopía (VIS/NIR, NIR, rayos X) se han estudiado en diferentes tipos de frutas y algunas incluso se utilizan a nivel comercial para determinar atributos de calidad, como el contenido de azúcares, la detección de defectos o el contenido de materia seca. Dichas técnicas tienen como principales ventajas la rapidez en la medición, la posibilidad de repetitividad de las mediciones, la no utilización de químicos y la capacidad de medir múltiples atributos simultáneamente.

De todas estas técnicas, la más utilizada es la espectroscopía VIS/NIR, o la espectroscopía NIR dependiendo la longitud de onda que abarque. Dicha técnica mide la cantidad de energía radiante por un sistema químico en las longitudes de onda comprendidas de los 700 nm hasta los 3000 nm, aproximadamente, dependiendo del espectrómetro utilizado. Cuando las radiaciones VIS/NIR penetran en los productos, sus características espectrales cambian debido a la dispersión y absorción que depende de la composición química y/o física de los productos. Estos cambios espectrales dan información sobre la microestructura del producto, como su rigidez, la presencia de daños internos o composición química.

Muchos estudios han explorado la posibilidad de usar la espectroscopía VIS/NIR en manzanas para estudiar la calidad y la presencia de transtornos o defectos (Ariana et al., 2006; Mehl et al., 2004; Menesatti et al., 2009; S. Kim et al., 2002; Xing et al., 2006; Xing y De Baerdemaeker, 2007; Xing et al., 2003; Zhang et al., 2017; Zhang et al., 2012). En los últimos años, con el rápido desarrollo de la tecnología, se han diseñado espectrofotómetros VIS/NIR compactos y portátiles específicos para el control de parámetros de calidad y madurez de diferentes tipos de frutas. Además, hoy en día, podemos encontrar líneas de clasificación de frutas y verduras que incorporan dicha tecnología para medir el contenido de sólidos solubles y/o de agua.

La mayoría de los espectrofotómetros comerciales, generalmente, ofrecen mediciones en rangos de longitud de onda en el rango del VIS/NIR, por debajo de los 1100 nm. Aún así, existen numerosos trabajos con resultados interesantes en la detección de defectos superficiales en frutos dentro de estos rangos. A modo de ejemplo, ElMasry et al. (2008) desarrollaron un sistema de imágenes hiperespectrales basado en una región espectral entre 400 y 1000 nm para la detección temprana de golpes en manzanas. Kleynen et al. (2005) observaron que los 750 y 800 nm ofrecía un buen contraste para detectar daños en el tejido interno de las manzanas, como daños por granizo, golpes o machucones. Este sistema fue utilizado más tarde por Unay y Gosselin (2006, 2007) y Unay et al. (2011) para la detección de defectos superficiales y la posterior clasificación de frutos en distintas categorías.

Respecto a la identificación de frutos con *bitter pit*, son escasos los trabajos publicados hasta el momento. Lotze (2005), utilizando imágenes de fluorescencia, logró una clasificación de manzanas 'Braeburn' con y sin *bitter pit* con una precisión del 75-100%. Nicolai et al (2006), para identificar lesiones de *bitter pit* visibles y no visibles, utilizaron un escáner en línea que incorporaba un espectrógrafo VIS/NIR y una cámara infrarroja para capturar la imagen espectral. Ariana et al. (2006) presentaron un modelo de reflectancia y fluorescencia para diferenciar manzanas con *bitter pit* con una precisión de hasta el 87%. Recientemente, Kafle et al. (2016) y Jarolmasjed et al. (2017) lograron clasificar manzanas con y sin *bitter pit* por medio de espectroscopía NIR con una precisión del 70-100%. En todos estos casos, el *bitter pit* fue detectado en poscosecha en frutos sintomáticos, una vez aparecidos los síntomas, ya fueran internos, y por lo tanto no visibles, o externos, y por lo tanto visibles, pero en ningún caso se exploró la predicción del *bitter pit* con anticipación a la aparición de los síntomas en precosecha.

Cabe remarcar, que en los últimos años se han publicado trabajos que exploran la tomografía computarizada (Jarolmasjed et al., 2016; Si y Sankaran, 2016), la fluorescencia de la clorofila (Mirzaee, 2015; Mirzaee et al., 2015) o la fluorescencia de rayos X (Kalcsits, 2016; Zúñiga et al., 2017) para identificar el *bitter pit* o medir el contenido de Ca en los frutos. El avance y la innovación en estas tecnologías y de todas sus posibilidades en la clasificación y determinación de la calidad en frutas y verduras, es clave para conseguir avances en la predicción del *bitter pit* mediante esta metodología.

2.3. Estrategias de mitigación

2.3.1. Aplicaciones de calcio en precosecha

a) Aplicaciones foliares

La fertilización foliar se fundamenta en la premisa de que las hojas de las plantas están capacitadas para poder absorber nutrientes, aunque ésta no sea su principal función, por lo que sólo un pequeño porcentaje del fertilizante se incorpora en los tejidos de forma efectiva (Fernandez et al., 2009). Aún así, desde que Garman y Mathis (1956) propusieran los tratamientos con sales de Ca para la reducción del *bitter pit*, las aplicaciones de Ca foliar se han convertido en el método de referencia para mitigar el *bitter pit*. Ello se debe a la limitada capacidad de las plantas por regular la distribución interna del Ca en relación a las necesidades de los órganos con baja transpiración.

El Ca es absorbido por la planta como ion libre o como Ca complejado por ácidos orgánicos como el ácido cítrico o málico (Vang-Petersen, 1981). Una vez absorbido por la planta, la principal vía de translocación del Ca es el xilema. Esta translocación en el interior de la planta es mediante un proceso, esencialmente, pasivo, con sentido ascendente e impulsado por la corriente de transpiración, reteniéndose en tejidos en crecimiento, como frutos, hojas y ápices de los brotes, que requieren de dicho elemento para abastecer los nuevos centros de conexión que se forman mediante la multiplicación celular. Es por ello que en hojas, con una gran tasa de transpiración, y en brotes en crecimiento, con una gran actividad meristemática, el contenido de Ca es proporcionalmente mayor que en otros órganos con menor tasa de transpiración como frutos y semillas (Alarcón et al., 1998; Kirkby, 1993). Una vez retenido, el Ca difícilmente vuelve a translocarse por el interior de la planta, es por ello que las aplicaciones foliares pueden ser de gran utilidad para aplicar Ca a órganos deficitarios con baja tasa de transpiración y multiplicación celular, como son los frutos en fase de elongación (Casero et al., 2002; Casero et al., 2017).

En general, la recomendación para mitigar el *bitter pit* es realizar el mayor número posible de pulverizaciones con soluciones de sales de Ca. No obstante, su efectividad es irregular y puede depender del número de aplicaciones, tipo de sal (nitrato, cloruro, tiosulfato u otros) y período de suministro (Val et al., 2008; Wojcik y Borowik, 2013; Wooldridge et al., 1998). En general, y hasta el día de hoy, el $CaCl_2$ es la sal que ha demostrado una mayor eficacia y regularidad en la reducción de *bitter pit*, posiblemente gracias a poseer un punto de delicuescencia (capacidad de absorción de vapor de agua del aire) más bajo que otras sales, como nitratos o sales orgánicas (Schönherr, 2001). Sin embargo, no existe un consenso sobre cual debe de ser el momento para realizar o empezar el programa de aplicaciones.

Algunos autores posicionan el mejor momento para empezar con el programa de aplicaciones foliares justo después de la floración, durante la fase de división celular, cuando el tamaño de los frutos es todavía muy pequeño. Dichas aplicaciones podrían tener la ventaja de que el bajo desarrollo de la cutícula favoreciera la penetración de Ca en el fruto (Neilsen et al., 2005; Wilsdorf et al., 2012). Sin embargo, varios trabajos han mostrado mejores resultados concentrando las aplicaciones durante la segunda mitad de desarrollo del fruto, en la fase de expansión celular (Benavides et al., 2001; Johnson et al., 2001; Lotze et al., 2008; Peryea et al., 2007). Johnson et al (2001), en manzanas 'Cox's Orange Pippin', únicamente obtuvieron incrementos del contenido de Ca mediante aplicaciones tardías de nitrato de calcio. Peryea et al. (2007) demostró que iniciar el programa de aplicaciones de $CaCl_2$ a partir de junio es la medida más eficiente económicamente para aumentar el contenido de Ca en el fruto y reducir el riesgo de *bitter pit*. Casero et al. (2002) no obtuvo una mejora significativa por adelantar el inicio de las aplicaciones de 70 a 10 DDPF, lo que le supuso un incremento de 6 a 10 aplicaciones respectivamente. Su hipótesis es que la mayor acumulación de Ca en el fruto se efectúa durante el primer período de su crecimiento, mientras que en la segunda mitad se ralentiza debido a un mayor efecto sumidero por parte de las hojas, por lo que es en ese preciso momento cuando las aplicaciones foliares podrían ser más efectivas (Casero et al., 2017).

b) Aplicaciones radiculares

La vía natural de absorción y entrada de Ca a la planta, así como de cualquier otro nutriente mineral, son las raíces. Las raíces, a medida que se desarrollan, exploran nuevas capas de suelo quedando la masa radicular en contacto con la solución del suelo y con las superficies de los coloides que retienen a los iones, produciéndose así el intercambio iónico por contacto entre las raíces y los nutrientes presentes en la solución o adheridos a la superficie de los coloides. Este mecanismo por sí solo representa para la absorción de nutrientes una aportación relativamente baja ya que las raíces suelen ocupar en el suelo un volumen inferior al 5%. Es por ello que el contacto de las raíces con la solución no sería capaz de satisfacer las necesidades de Ca de las plantas, siendo la absorción hídrica por flujo de masa, debida al proceso de transpiración de las plantas, el principal mecanismo de transporte del Ca, así como de otros nutrientes como el Mg y el N, hasta la superficie de las raíces. Aún con todo, se estima que las raíces aprovechan menos del 3% del Ca disponible en la solución del suelo, cantidad que suele ser suficiente para satisfacer las demandas de los frutales (Barber et al., 1963).

La contribución del flujo de masa a la aproximación y contacto de los nutrientes a las raíces depende de la actividad del nutriente en la solución y de la tasa de transpiración de la planta, siendo ésta tanto mayor cuanto más rica la solución del suelo y mayor la tasa de transpiración. La transpiración, a su vez, depende de la especie y variedad, de su edad y de las condiciones exteriores como la humedad relativa del aire, la temperatura y el contenido de agua en el suelo. Por lo tanto, el mantenimiento de un contenido adecuado de agua en el suelo es de primordial importancia para que el flujo de masa ocurra sin limitaciones, de ahí la importancia del régimen hídrico en la nutrición del Ca (Kirkby y Pilbeam, 1984).

Cabe remarcar que el Ca presenta la particularidad, a diferencia de otros nutrientes, que el lugar de las raíces por donde es absorbido está restringido a la zona apical de la raíz, o sea, en las extremidades de las raíces jóvenes, o raicillas, donde aún no ha ocurrido la suberización de las paredes celulares de la endodermis. El desplazamiento del Ca por esta vía hacia el xilema se va bloqueando progresivamente a medida que la endodermis se suberiza y lignifica, debido a las bandas de Caspary, considerada una estructura impermeable al Ca. De aquí la importancia de una continua actividad radicular mediante un buen manejo hídrico y nutricional para facilitar la absorción de Ca, dado que su absorción está restringida a las raícillas jóvenes no suberizadas. Esto justifica el hecho de que el K, a pesar de encontrarse en la solución del suelo en concentraciones aproximadamente 10 veces más bajas que las del Ca, alcanza en el conjunto de la planta concentraciones similares ya que tiene la capacidad de poder ser absorbido a lo largo de toda la raíz sin tener que hacer frente a la barrera constituida por las bandas de Caspary (Jakobsen, 1993).

Otro de los factores que interfieren en la absorción radicular es la concentración salina de la solución, principalmente debido a antagonismos con el ion de sodio y al efecto competitivo de las altas concentraciones de otros cationes rápidamente absorbidos por la raíz, como el K^+, Mg^+, NH_4^+ (Bangerth, 1979; Cramer et al., 1989).

A pesar de todas las limitaciones que presenta la absorción de Ca radicular, las aportaciones mediante fertirrigación se han convertido en una práctica habitual generalizada con la finalidad de favorecer al máximo su vía natural de absorción e independientemente de que éstas estén o no justificadas en función de la disponibilidad de Ca en la solución del suelo. Mientras que la mayoría de trabajos sobre la fertilización del Ca en manzanos se han centrado en las aportaciones mediante aplicaciones foliares, apenas existen resultados sobre la eficacia del suministrado de Ca vía radicular, ya sea para el control del *bitter pit* o para mejorar la calidad del fruto. Wilsdorf et al. (2012) evaluaron distintas estrategias de aplicación de Ca radicular y foliar en la asimilación de Ca en el fruto, obteniendo los mejores resultados mediante las aplicaciones foliares, seguidas de las aplicaciones radiculares realizadas en la poscosecha de la campaña anterior, en comparación a aplicaciones radiculares realizadas durante el desarrollo del fruto. Esto estaría justificado al considerar que las primeras necesidades de Ca, cuando se inicia un nuevo ciclo vegetativo, se cubren a través del Ca translocado de las reservas del año anterior, situadas en la raíz y ramas inferiores (Terblanche et al., 1979). Wilsdorf et al. (2012) no observaron incidencias de *bitter pit*, por lo que no pudieron evaluar la eficacia de los tratamientos para su control. Cabe remarcar que no existen en la bibliografía otros estudios en los que se haya evaluado la eficacia de los tratamientos radiculares para el control del *bitter pit*.

2.3.2. Tratamientos en poscosecha

Otra práctica para la mitigación de la incidencia del *bitter pit* es la inmersión de los frutos en soluciones de Ca después de la recolección. Dicha técnica ha mostrado también una eficacia en aumentar el contenido de Ca de los frutos por lo que, además de disminuir el *bitter pit*, puede ayudar a mantener la calidad durante la conservación (Manganaris et al., 2007).

Sin embargo, son escasos los trabajos que presentan resultados contrastados científicamente sobre la efectividad de los baños de Ca en poscosecha. Guerra y Casquero (2010) observaron en el cultivar 'Reineta del Canadá' una disminución del 16,7% en la incidencia de *bitter pit* mediante un tratamiento de Ca en poscosecha, logrando incrementos en el contenido de Ca en la piel del 118% y en la pulpa del 44%. Mignani et al. (1983), en el cultivar 'Maygold', obtuvieron una reducción del 17% del *bitter pit* mediante la misma técnica. Mignani y Bassi (2005), mediante aplicaciones de Ca en poscosecha, lograron incrementos del contenido de Ca en 'Braeburn' de entre 10-60% al final del periodo de conservación. Dierend y Rieken (2007) estudiaron durante cuatro años la eficacia de los baños en poscosecha y observaron que la absorción de Ca estaba influenciada por: i) la concentración de $CaCl_2$ de la solución de inmersión (para 2 minutos de inmersión se requieren concentraciones de $CaCl_2$ de 7 o 7,5%), ii) la duración de la inmersión (una elongación del tiempo de inmersión o la duración de la humectación aumentaron la absorción de Ca), iii) el cultivar, iv) la adición de un agente humectante (necesario para una captación de Ca apreciable) y v) el estado de madurez del fruto. Aún con todo, el aumento del contenido de Ca pudo demostrarse sólo cerca de la piel. Estos resultados coinciden con los que previamente publicaron Ait-Oubahou et al. (1995) quienes observaron los mejores resultados sumergiendo los frutos durante 2 minutos a una concentración de $CaCl_2$ al 7%.

Además de los baños con sales de Ca, la conservación y envasado en atmósfera modificada han demostrado su eficacia en la preservación de la calidad y la reducción del *bitter pit* (Khan et al., 2006). Jankovic y Drobnjak (1994) presenciaron que manzanas de distintas variedades almacenadas en atmosfera controlada no mostraron ningún tipo de fisiopatía, mientras que algunas variedades almacenadas en condiciones de atmosfera normal presentaban problemas de *bitter pit.*

Otra alternativa para reducir la incidencia del *bitter pit* en poscosecha es el tratamiento de bajo oxígeno antes de la frigoconservación. Dicho tratamiento, propuesto por Val et al. (2010), consiste en almacenar los frutos en condiciones de bajo O_2 y a una temperatura de 20 °C durante los primeros 10 días después de su recolección para posteriormente pasar a una conservación convencional a largo plazo. Mediante esta práctica, además de reducir el *bitter pit*, se mantiene la firmeza, se aumenta el contenido de sólidos solubles y la acidez y se acelera la degradación de la clorofila, favoreciendo en manzanas 'Golden' el amarillamiento del fruto.

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OBJETIVOS

3. OBJETIVOS

El *bitter pit* es la fisiopatía que ocasiona mayores pérdidas durante la poscosecha en la producción de manzanas 'Golden', el grupo varietal más producido y consumido en Europa y España. A pesar de los numerosos estudios científicos realizados, todavía no se conocen con certeza las causas que desencadenan su aparición.

El objetivo general de este trabajo es encontrar un modelo de predicción del *bitter pit* para manzanas 'Golden Smoothee' cultivadas en el Valle del Ebro, principal zona productora de España, y que permita reducir los costes asociados a su incidencia.

Para alcanzar este fin se plantea la consecución de los siguientes objetivos específicos:

1. Evaluar en condiciones locales (Valle del Ebro) y para el cultivar 'Golden Smoothee' el uso del análisis mineralógico en época temprana de desarrollo del fruto como herramienta de predicción del *bitter pit* y determinar el mejor momento para realizar los análisis.

2. Conocer la interacción entre los principales macronutrientes relacionados con la aparición del *bitter pit*, y su relación con la aparición de la fisiopatía, durante el desarrollo del fruto, con el objetivo de determinar el mejor indicador mineralógico para predecir el *bitter pit* en época temprana.

3. Verificar en condiciones locales y para el cultivar 'Golden Smoothee', los métodos de predicción de referencia por

inducción de síntomas, utilizados en otras regiones productoras, como son los baños con etefón y la infiltración de magnesio, desarrollados en Sudáfrica y Chile, respectivamente, y determinar los mejores momentos para realizar los muestreos de cada método y su posible optimización.

4. Evaluar la espectrofotometría VIS/NIR como tecnología para la identificación de frutos afectados por *bitter pit* y determinar la precisión de clasificación, tanto antes como después de la aparición de síntomas.

5. Cuantificar la respuesta de los tratamientos radiculares y foliares de Ca en precosecha, y de los baños en poscosecha, así como sus combinaciones, en el contenido mineralógico del fruto, parámetros de calidad e incidencia del *bitter pit*.

6. Validar y comparar la precisión de los distintos métodos de predicción de la aparición de *bitter pit* propuestos en el transcurso de la presente tesis, y definir un proceso de predicción transversal en las diferentes etapas de desarrollo del fruto.

Los resultados que se deriven de este trabajo, además de contribuir a aumentar el cúmulo de conocimientos básicos sobre el *bitter pit*, ayudarán a establecer las bases para la implantación práctica de un sistema de predicción del *bitter pit* a nivel comercial, así como recomendaciones para su mitigación.

RESULTADOS

CAPÍTULO 1

Early stage fruit analysis to detect a high risk of bitter pit in 'Golden Smoothee' apples

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ABSTRACT

Fruit mineral analysis at harvest is recommended as a predictive method to assess the risk of bitter pit (BP) in apple orchards, although it only provides valuable information if conducted just before harvest. To gain more time to implement corrective action, some studies proposed early season analysis of fruitlets. However, neither result was reported for analysis accuracy, nor the best time to perform it. The objective of this study was to evaluate the accuracy of early season fruitlet analyses at different stages—40, 60 and 80 days after full bloom (DAFB)—to predict BP in 'Golden Smoothee' apples. Multivariate models for each early stage were developed and compared to a linear model using the calcium (Ca) content alone. Both the multivariate analyses and linear correlations suggested 60 DAFB as the best time to perform early mineral analysis. The Ca concentration in the fruit contributed greatly to BP incidence either at an early stage or at harvest. The boron concentration showed a negative correlation with Ca concentration and a positive correlation with BP incidence. The other tested nutrients (magnesium, nitrogen, potassium) showed little effect on the prediction models and/or an irregular pattern. The accuracy of the multivariate model ($R^2 = 0.580$) was not significantly better than the analysis of Ca alone ($R^2 = 0.504$) when the occurrence of BP was high. Finally, a Ca threshold at 60 DAFB equal to or greater than 11.0 mg 100 g^{-1} fresh weight (FW) indicated a low risk of BP (<10% of incidence). This early season threshold value was a better indicator of the BP risk than the traditional threshold value at harvest (5–6 mg Ca 100 g^{-1} FW).

1. INTRODUCCTION

Bitter pit (BP) is a disorder in apples (*Malus domestica* L. Borkh.) that is closely related to calcium (Ca) nutrition, as well as to other nutrients such as boron (B), magnesium (Mg), nitrogen (N), or potassium (K) (Casero et al., 2004; Ferguson et al., 1999). In general, high Ca and B contents are related to a low incidence of BP, while high incidence is positively associated with N, K and Mg. The incidence of BP can fluctuate seasonally in a same orchard (Lotze et al., 2006; Torres et al., 2015, 2017). Because of the absence of an effective control method for BP, some studies have focused on prediction. Effective BP prediction allows optimizing control measures (e.g., increasing Ca sprays, or decreasing N and K fertilization), or helping the packing house in choosing the right management approach (Manganaris et al., 2005; Torres and Alegre, 2012; Torres et al., 2015). Methods such as inducing symptoms before they naturally occur, like for instance 'Mg infiltration' (Burmeister and Dilley, 1993; Retamales et al., 2000), 'ethephon dips' (Eksteen et al., 1977) or the 'passive method' (Torres et al., 2015), can predict BP 30–10 days before harvest. However, these methods do not provide enough time for growers to take new actions to control BP.

Another predictive method that has been used for years to assess the risk of BP is based on fruit mineral analysis at harvest for macronutrients, such as Mg, K, N, and especially, Ca contents (Autio et al., 1986; Ferguson and Triggs, 1990; Wolk et al., 1998). Sharples et al. (1979) documented fruit mineral standards for 'Cox's Orange Pippin' apples, suggesting that the behaviour of fruit in storage can be predicted from an analysis at harvest. Terblanche et al. (1980) and

Waller (1980) reported that balanced levels of Ca with other minerals (N, Mg, K) ensured the absence of BP in fruit. Currently, most of these thresholds are still used for all varieties of apples (Lotze et al., 2008; von Bennewitz et al., 2015). However, these ratios can only provide valuable information just before harvest.

Some authors have suggested early season analysis of fruitlets in order to gain time and then implement corrective action if a BP risk is present (Brooks, 2001; Conway et al., 1994; Drahorad and Aichner, 2001; Ferguson and Triggs, 1990). The concept of early season fruitlet analysis has been previously investigated by other authors to predict post-harvest fruit quality attributes, but its relationship with BP has not been studied (Fallahi et al., 1985; Marcelle et al., 1989).

The hypothesis that early analysis can be a useful tool to predict BP is based on the strong relationship between the Ca concentration in fruitlets at the early stage and in fruit at harvest. Brooks (2001) reported that the Ca content in a 50 g fruitlet reflected the Ca content at harvest and, consequently, predicted BP, but he did not determine the statistical accuracy of the proposed approach. Drahorad and Aichner (2001) reported that the K/Ca ratio in fruit at harvest can be predicted at early stages and, consequently, could provide useful information about the risk of BP and other physiological disorders, but they did not report information about the precision of the model.

The results of Brooks (2001) and Drahorad and Aichner (2001) were obtained from fruitlets with an average weight of 50 g and 70 g, respectively, which are approximately equivalent to 80 and 90 days after full bloom (DAFB) under our growing conditions, respectively. Nevertheless, Lotze et al. (2008) found a better correlation using mid-season fruitlets (41–82 DAFB) than those of late season (83–118 DAFB). Those results were presented as preliminary, but such research has not been further reported. Hence, there are no current references that clearly indicate the best sampling date for BP prediction based on an early stage mineral analysis, which is a critical point related to method accuracy.

The objective of this study was to evaluate the accuracy of early season fruitlet analyses at different times (40, 60, and 80 DAFB, and at harvest) as a predictive tool for post storage BP in 'Golden Smoothee' apples from a semi-arid region (Ebro Valley, Spain).

2. MATERIAL AND METHODS

2.1. Plant material

Experiments were carried out in Lleida (NE Spain), over three consecutive seasons in 10 commercial apple orchards of 'Golden Smoothee' with different levels of BP susceptibility. All of the orchards studied were mature, and their trees were normally spaced (approximately 4 × 1.2 m), grafted onto M9 rootstock, and fully irrigated. They were all subjected to standard cultural practices of pruning, fertilization, irrigation and crop management.

2.2. Fruit sampling

For mineral analysis, 20-fruit samples were collected from each orchard at 40, 60, and 80 DAFB, and at commercial harvest when the starch index was between 7 and 8 (starch chart EC-Eurofru). In each orchard, 20 trees that showed equally vigorous growth were selected. An apple was taken from each tree at 130–170 cm height above the ground and from spurs on 2-year-old shoots. Another 80- fruit sample was collected from each orchard at harvest to evaluate the incidence of post-harvest BP.

2.3. Mineral analysis

Fruit for mineral analyses were carefully washed, and two longitudinal slices were cut from the opposite sides of each fruit, excluding the core and seeds. The complete sample from each group was weighed, dried at 75 °C to constant weight, and then re-weighed to determine the dry mass percentage. The dried tissue was ground, and a sub-sample was wet-digested with concentrated nitric acid and hydrogen peroxide in a microwave oven (Milestone MCR 6E, Bergamo, Italy). The nutrient concentrations were determined according to standard procedures (AOAC, 2006). We focused on nutrients related to BP according to literature review: B, Ca, Mg, N and K. The B, Ca, Mg and K concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES). The N concentration was determined via Kjeldahl analysis.

2.4. Bitter pit evaluations

The 80-fruit sample collected from each orchard at harvest were placed in cold storage at 0 °C and at 90% relative humidity (RH). After four months in cold storage, samples were transferred at 20 °C and 45% RH for 7 days. Immediately after that, all of the apples contained in each sample were individually examined for any external signs of superficial BP symptom. The incidence of BP of each sample was calculated as the percentage of fruit with BP symptoms.

2.5. Data analysis

2.5.1. Chemometric analysis

Principal component analysis (PCA) of all of the measurements taken during the three seasons was performed (without including the BP incidence) to provide exploratory data analysis and a visualisation of all the data set information. The purpose of the PCA was to detect various clusters in terms of BP occurrence and to determine which variables were related to each other, as well as classification of the samples. Then, partial least squares regression (PLS) was calculated at each sampling time (40, 60, 80 DAFB and harvest) for the three seasons (2010, 2011, and 2012) and for the different clusters detected by the PCA model. The accuracies of the models (R^2) were compared to designate the best time to perform early mineral analysis of fruitlets for BP prediction.

In all cases, data were centred and weighted with the inverse of the standard deviation of each variable to allow all the variables the same chance to affect the estimates of the components. The data were represented in terms of the first two principal components (PCs), which represent the most important portion of the variability of the data (Johnson and Wichern, 2007). The software used was The Unscrambler® (Version 10.4; Camo Process AS, Oslo, Norway).

2.5.2. Linear regression

The linear correlation was calculated using the Ca concentration in the fruit as the independent variable, and the percentage of BP after storage as the dependent variable. The correlation was calculated at each early stage time (at 40, 60, 80 DAFB) and at harvest. The significance level and R^2 value were calculated for each linear regression model. The data were analysed using the JMP® statistical software package (Version 8.0.1; SAS Institute Inc., Cary, North Carolina).

3. RESULTS AND DISCUSSION

3.1. Sources of bitter pit variability

Two PCs described 54% of the total variance (a third PC explained approximately 20% more) in the PCA model that was carried out to detect different clusters according to BP occurrence. Two groups of samples were clearly differentiated on the PC1 axis in the scores plot. One group corresponded to samples from 2012 and had a low BP occurrence (50% of the orchard showed BP symptoms but only a 3% presented a BP incidence >10%), and a second group corresponded to samples from 2010 and 2011, and had a high occurrence of BP (90% of the orchards were affected and a 70–80% presented a BP incidence >10%) (Fig. 1).

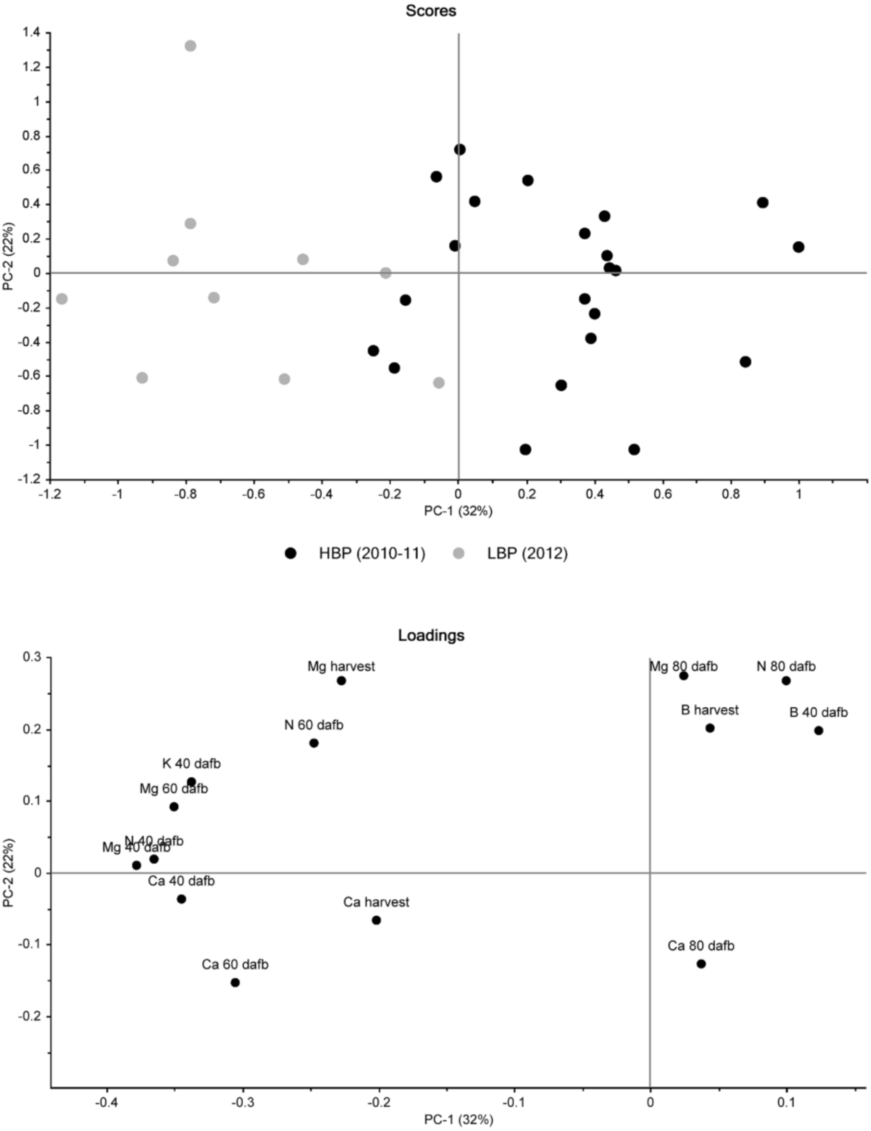

Fig. 1. Score plot (above) and loading plot (below) of PC1 vs. PC2 from a full data PCA model. In the score plot, the samples are grouped according to the occurrence of bitter pit: LBP group corresponds to the samples (orchards) from 2012, with a low occurrence of BP (only 5% of the orchard showed BP symptoms), and HBP corresponds to samples from 2010 and 2011, with a high occurrence of BP (90% of affected orchards). In the loading plot, 20 variables were included: B, Ca, Mg, N and K at 40, 60, 80 days after full bloom (DAFB) and at harvest, respectively.

In the loadings plot, the measurements at 40 DAFB and 60 DAFB showed a strong effect on PC1, especially the Ca (negative), Mg (negative), and N (positive) concentrations; by contrast, Ca at 80 DAFB was closer to the origin, which suggested a lesser effect in the model (Fig. 1). Finally, the results of the scores plot (in which two clusters can be differenced by PC1 according to the frequency of BP and the results of the loadings plot) suggested that the best sampling date to predict BP was between 40–60 DAFB.

According to the loadings plot, the Ca concentration seemed to be negatively correlated with B, independently of the sampling date (Fig. 1). These results are in line with those of Benavides et al. (2002), who observed a negative correlation between Ca and B at harvest, with a multivariate analysis that evaluated the relationships between textural parameters, quality attributes, and mineral elements in 'Golden Smoothee' apples. A relationship between Ca at harvest, and at 40 or 60 DAFB, was also showed by the loading plot. These results coincide with other studies regarding BP prediction and Ca content at harvest through an early stage analysis (Brooks, 2001; Conway et al., 1994; Ferguson and Triggs, 1990).

3.2. PLS models

3.2.1. PLS at harvest

The accuracy of the PLS model at harvest was $R^2 = 0.424$ for two PCs (Table 1). Increasing the number of PCs did not significantly improve the accuracy (data not shown). The Ca and Mg concentrations contributed negatively to the BP incidence, especially for Ca, which had the largest (negative) coefficient. On the other hand, the B, N, and K concentrations contributed positively to BP incidence (Fig. 2).

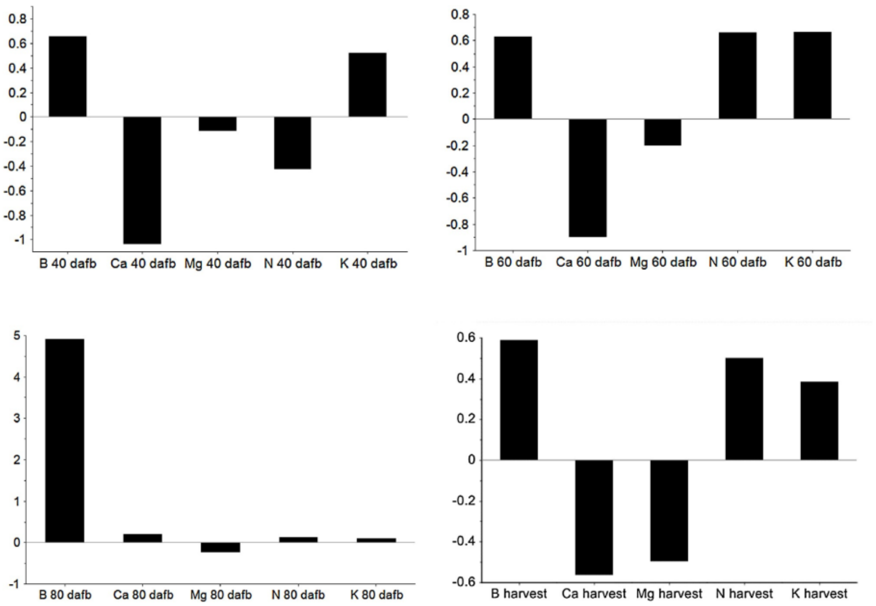

Fig. 2. Weighted regression coefficients of the PLS models to predict bitter pit incidence at 40 days after full bloom (DAFB) (above-left), 60 DAFB (above-right), 80 DAFB (below-left) and at harvest (below-right), based on the B, Ca, Mg, N and K concentrations in fruit. Data set from three seasons (2010, 2011 and 2012).

According to the literature, BP is more common when a B deficiency is present, since B improves Ca movement to the fruit and the cell wall structure, as well as maintaining membrane stabilization (Granelli et al., 1989; Rosenberger et al., 2004; Wojcik et al., 1999; Wojcik and Cieslinski, 2000). However, our results suggested the opposite, showing an increase in BP when a B surplus existed. This was confirmed by the PCA model mentioned above, in which Ca and B concentrations were negatively correlated. The role of B in plant physiology is unique among other nutrients because of the small difference that exists between deficiency and excess (Nable et al., 1997; Paparnakis et al., 2013). In this study, the B levels in the fruit could be considered to be excessive according to the recommendations of Peryea and Drake (1991), who considered excessive >40 ppm of B in fruit.

The reported symptoms of B toxicity are internal necrosis in fruit and stems (Nable et al., 1997), but BP symptoms are not generally present. Perhaps, the association of B with BP prediction may be indirectly related to Ca uptake or transport into the fruit. Recent studies have shown a relationship between xylem functionality, the Ca content, and the BP incidence in apples (Miqueloto et al., 2014). This evidence suggests that high levels of B could reduce the xylem functionality in the fruit stem—B toxicity is related to internal necrosis in the fruit or stems—and, consequently, reduce Ca uptake into the fruit, triggering the development of BP. However, this hypothesis has not been tested and more studies are needed to understand the relationship of B and Ca nutrition, as well as for BP incidence, especially in arid or semi-arid climates.

The other tested nutrients (K, Mg and N) showed expected behaviour (Fig. 2). In general, apples with higher K, Mg and/or N levels at harvest are more likely to exhibit BP. The effect of K and Mg in the development of BP has been explained by their antagonist effect with Ca. In the case of N it has been suggested that high levels may trigger faster cell expansion and rapid fruit growth, leading to a reduction in the fruit Ca concentration and, consequently, fruit susceptibility to BP (de Freitas et al., 2015; Ferguson and Watkins, 1989; Saure, 2005).

3.2.2. PLS at 40 DAFB

The variance explained by the PLS model at 40 DAFB using two PCs had a $R^2 = 0.389$, slightly less than at harvest (Table 1). Additional PCs did not significantly improve the accuracy of the model. The Ca concentration at 40 DAFB contributed negatively to the BP incidence and had the largest (negative) coefficient. The B and K concentrations were also significant and contributed positively to BP. The N concentration had a slight negative effect, whereas the effect of Mg was not significant (Fig. 2). As previously mentioned, the positive effect of N on the BP incidence may be related to rapid fruit growth; therefore, subsequent observations could show a positive effect when the growth rate of the fruit is larger.

3.2.3. PLS at 60 DAFB

The variance explained by the PLS model at 60 DAFB for two PCs had a $R^2 = 0.464$, which indicated that this was a better sampling time than 40 DAFB, and even better than at harvest (Table 1). The accuracy of the model did not improve when the number of PCs was increased. The Ca concentration, again, had the largest (negative) weighed regression coefficient, whereas the B and K concentrations contributed positively to the BP. The weight of the Ca concentration was almost twice that of the K concentration, suggesting a larger effect of Ca in the model than for K and the other nutrients. The N and Mg concentrations produced positive and negative effects, respectively, in the model, although they were not significant in comparison to the other nutrients (Fig. 2).

3.2.4. PLS at 80 DAFB

The predictive power of a PLS model with measurements at 80 DAFB was lower than using harvest measurements, or even 40 DAFB and 60 DAFB (Table 1). The optimum number of PCs was three, one more than at 40 DAFB or 60 DAFB, and had a $R^2 = 0.321$, whereas with two PCs had a $R^2 = 0.117$. The B concentration contributed strongly (positive) in the model, while the influence of the rest of the variables was minor (Fig. 2). The possible causes of this lack of influence could be the instability of the Ca content in the fruit during this period, since is a period with high leaf transpiration and high shoot and fruit growth rates, especially in semi-arid regions (Lakso et al., 1999). Deviations within few days during this period could cause a great variability in the Ca concentration and, consequently, meaningless results. These could be corrected just before harvest, when the growth rate is reduced and the Ca concentration in fruit is stabilized.

These results suggested that an early mineral analysis of fruitlets at 60 DAFB was the best approach to predict BP incidence at early stages of 'Golden Smoothe' apples. This coincides with Lotze et al. (2008), who reported a better correlation with BP incidence for the Ca concentration in fruit at 41–81 DAFB than at 83–118 DAFB.

The regression coefficients for the B, Ca, Mg, N and K concentrations, as well as the regression coefficient for the intercept (B_0) and coefficient of determination (R^2) for each model, are shown in Table 1. We must note that the accuracy of the models can vary within cultivar and region.

Table 1. Regression coefficients (B_i) of the PLS models based on the B concentration (expressed as mg 100 g^{-1} of dry weight) and the Ca, Mg, N and K concentrations (expressed as mg 100 g^{-1} of fresh weight) in fruit, as well as the regression coefficient for the intercept (B_0), to predict bitter pit incidence at 40 days after full bloom (DAFB), 60 DAFB, 80 DAFB and at harvest. Data set from three seasons (2010, 2011 and 2012).

Sampling date	Num. PCs	B_0	B	Ca	Mg	N	K	R^2
40 DAFB	2	14.655	1.740	-0.627	-0.115	-0.038	0.075	0.389
60 DAFB	2	-14.859	2.597	-3.877	-0.885	0.266	0.226	0.464
80 DAFB	3	-24.350	4.923	0.2113	-0.235	0.138	0.099	0.321
Harvest	2	-33.827	5.003	-2.652	-0.432	0.474	0.223	0.424

3.3. PLS model from HBP seasons

The sample grouping in the PCA model—one group corresponding to a season with a low occurrence of BP (LBP) and a second group corresponding to seasons with high occurrences of BP (HBP)—suggests that seasonal factors (e.g., weather conditions) could contribute to the differences either in the mineral fruit composition or in the BP incidence. These outcomes are also supported by our previous studies that showed that either BP or mineral content were strongly related to the season of growth (Torres et al., 2015, 2017). Considering this, we separately studied the HBP samples to improve the accuracy of the BP prediction. We discarded a PLS model with data from the LBP season by having a low number of observations and, consequently, yielding erratic results.

The R^2 of the models at 40 DAFB (0.390), 60 DAFB (0.580) and at harvest (0.574) slightly improved with respect to the data set from the three seasons (Table 2). By contrast, the accuracy of the model at 80 DAFB ($R^2 = 0.108$) was lower than that of the model for the three seasons. Consequently, according to our results, a model for the prediction of BP based on mineral analyses of fruitlets at 80 DAFB could not be recommended for our growing conditions, as Lotze et al. (2008) suggested.

The goodness-of-fit values of the HBP models were similar to the model that used the three-seasons data set. However, the weight of B (positive) and K (negative) in the HBP model at 40 DAFB decreased and increased, respectively, compared to the three-seasons model (Table 2).

Table 2. Regression coefficients (B_i) of the PLS models based on the B concentration (expressed as mg 100 g^{-1} of dry weight) and the Ca, Mg, N and K concentrations (expressed as mg 100 g^{-1} of fresh weight) in fruit, as well as the regression coefficient for the intercept (B_0), to predict bitter pit incidence at 40 days after full bloom (DAFB), 60 DAFB, 80 DAFB and at harvest. Data set from 2010 and 2011 (high occurrence of bitter pit: 90% of orchard affected).

Sampling date	Num. PCs	B_0	B	Ca	Mg	N	K	R^2
40 DAFB	2	14.124	0.562	-1.623	0.311	-0.091	0.204	0.390
60 DAFB	2	26.548	0.879	-5.333	-1.565	0.248	0.158	0.580
80 DAFB	2	16.477	2.727	-1.111	0.344	-0.192	0.092	0.135
Harvest	2	60.589	-0.532	-6.050	-2.068	0.220	-0.040	0.574

On the other hand, the regressions of the weighted coefficients of the HBP model at 60 DAFB were similar to those of the three seasons model—the weight of Ca was twice that of the N or K concentration—which suggested greater reliability than at 40 DAFB. The weight of the B, Mg, N and K at harvest was not significant in comparison to the Ca weight (Fig. 3). This was a significant change with respect to the three-seasons model in which all of the tested nutrients showed a relevant weight in the model.

Ferguson et al. (1999) and Ferguson and Triggs (1990) reported that Ca in fruit at harvest was the primary determinant of BP risk, whereas Mg and K may be useful only in specific cases where the Ca vs. BP relationship did not fit the expected pattern.

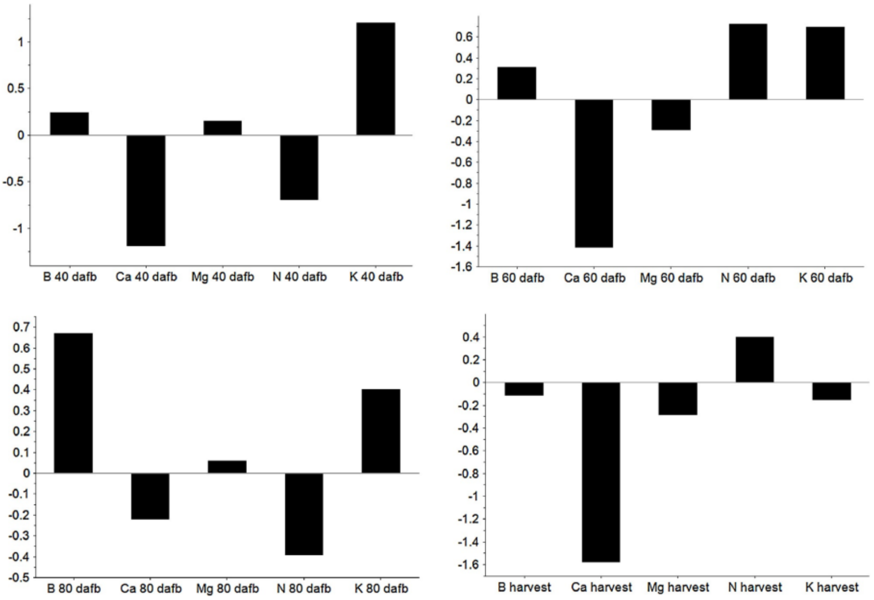

Fig. 3. Weighted regression coefficients from the PLS models to predict bitter pit incidence at 40 days after full bloom (DAFB) (above-left), 60 DAFB (above-right), 80 DAFB (below-left) and at harvest (below-right) based on B, Ca, Mg, N and K concentrations in fruit. Data set from 2010 and 2011 seasons (high occurrence of bitter pit: 90% of orchard affected).

3.4. Bitter pit prediction through Ca content

According to the various PLS models, Ca seemed to be the nutrient that had the largest contribution to BP incidence, which suggested that Ca concentration alone could be sufficient to predict BP in most cases, as reported by Ferguson et al. (1999) and Ferguson and Triggs (1990) for analysis performed at harvest. We calculated the linear regressions between BP and the Ca content in fruit at early stages and at harvest to confirm this observation. Some authors have also suggested that the N/Ca, K/Ca and/or Mg/Ca ratios might predict BP (Casero et al., 2010; de Freitas et al., 2015; Drahorad and Aichner, 2003; Miqueloto et al., 2014; Van der Boon, 1980). However, our results using these ratios were negative and inconsistent (data not shown), in agreement with Ben (2006), Ferguson et al. (1999) and Ferguson and Triggs (1990), who did not observe an improvement in the BP correlation by using Ca ratios with other nutrients.

The linear correlations were significant for 40 DAFB and 60 DAFB (Table 3). However, the accuracy decreased in comparison to their respective PLS models, especially at 40 DAFB. The accuracy at 40 DAFB in 2010 and 2011 was $R^2 = 0.409$ and 0.358, respectively, but decreased to $R^2 = 0.151$ when both HBP seasons were analysed together. The decrease in accuracy was lower at 60 DAFB, especially for the HBP seasons. The accuracy at 60 DAFB was $R^2 = 0.618$ and 0.742, in 2010 and 2011, respectively, and decreased to $R^2 = 0.503$ when both seasons were analysed together (Table 3). This suggested that although the Ca concentration in fruit is strongly correlated with BP, other triggers, different than mineral composition, may exist and could vary depending on the season.

Table 3. Linear correlation of the Ca concentration in fruit at 40, 60, 80 days after full bloom (DAFB) and at harvest with the bitter pit incidence. ** $P < 0.01$; * $P < 0.05$; NS $P > 0.05$.

Sampling date	2010		2011		2012		2010-11 [1]		2010-12	
	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
40 DAFB	0.409	*	0.358	*	0.026	NS	0.151	*	0.110	*
60 DAFB	0.618	**	0.742	**	0.105	NS	0.504	**	0.274	**
80 DAFB	0.282	NS	0.011	NS	0.134	NS	0.055	NS	0.022	NS
Harvest	0.557	**	0.180	NS	0.010	NS	0.218	*	0.231	**

The linear correlation at 80 DAFB was non-significant, as expected according to our previous multivariate analyses (Table 3). Our results for 80 DAFB differed from the recommendation of Brooks (2001) or Drahorad and Aichner (2001) who used 50 g and 70 g of fruitlet, respectively, to analyse the Ca concentration and to predict the BP level. These fruitlet weights are achieved at approximately 80–90 DAFB under our growing conditions.

According to our results, 60 DAFB is the date recommended to perform a mineral analysis to predict BP based either on a PLS model or on the Ca concentration alone, as suggested by Lotze et al. (2008). Sampling at 60 DAFB would allow growers to know the potential risk of BP development approximately 100 days before the expected harvest date. If a high risk of BP is detected at that moment, there would be enough time to take measures to reduce its incidence. According to our previous studies, increasing the number of sprays that occurred closer to harvest can improve BP control (Torres et al., 2017).

The accuracy of Ca alone was lower than that of the PLS model, especially when many orchards showed a low BP incidence.

However, a threshold for Ca content to predict BP, at least qualitatively, will be more useful for farmers and advisors than a multivariate model. In general, the fruit mineral analysis approach is able to provide a threshold value above that the risk of BP increases. We observed that at 60 DAFB, Ca concentrations lower than 11.0 mg Ca 100 g^{-1} FW suggested a BP incidence higher than 10%, independently of the season, with a 73% chance of occurrence (Fig. 4). This accuracy was better than that of the traditional standard of 5–6 mg Ca 100 g^{-1} FW in fruit at harvest (Terblanche et al., 1980; von Bennewitz et al., 2015), which had an accuracy of 57%, and similar to the 'passive method' (71%) recently proposed by Torres et al., 2015. The 'passive method' is an easy and inexpensive way for predicting BP between 30 and 10 days before harvest, therefore, could be used after the early mineral analysis in order to improve prediction accuracy. A more effective prediction would help growers and packing houses to decide whether to increase the number of Ca sprays or to use $CaCl_2$ dips, which would mean an optimization of the BP control.

The proposed threshold seemed robust when tested with a diferent data set from seven orchards in different seasons and from the same region, with an accuracy of 71%. Nevertheless, with data from other regions with weather conditions not as extreme as in the present study, the level of Ca at 60 DAFB did not achieve the proposed threshold and BP incidence was lower than 10%. In this case, it was not possible to predict the BP incidence using a multivariate model (data not shown). According to this, many studies have found no relationship in seasons with a low frequency of BP (Autio et al., 1986; Hewett and Watkins, 1991; Le Grange et al., 1998; Van der Boon, 1980).

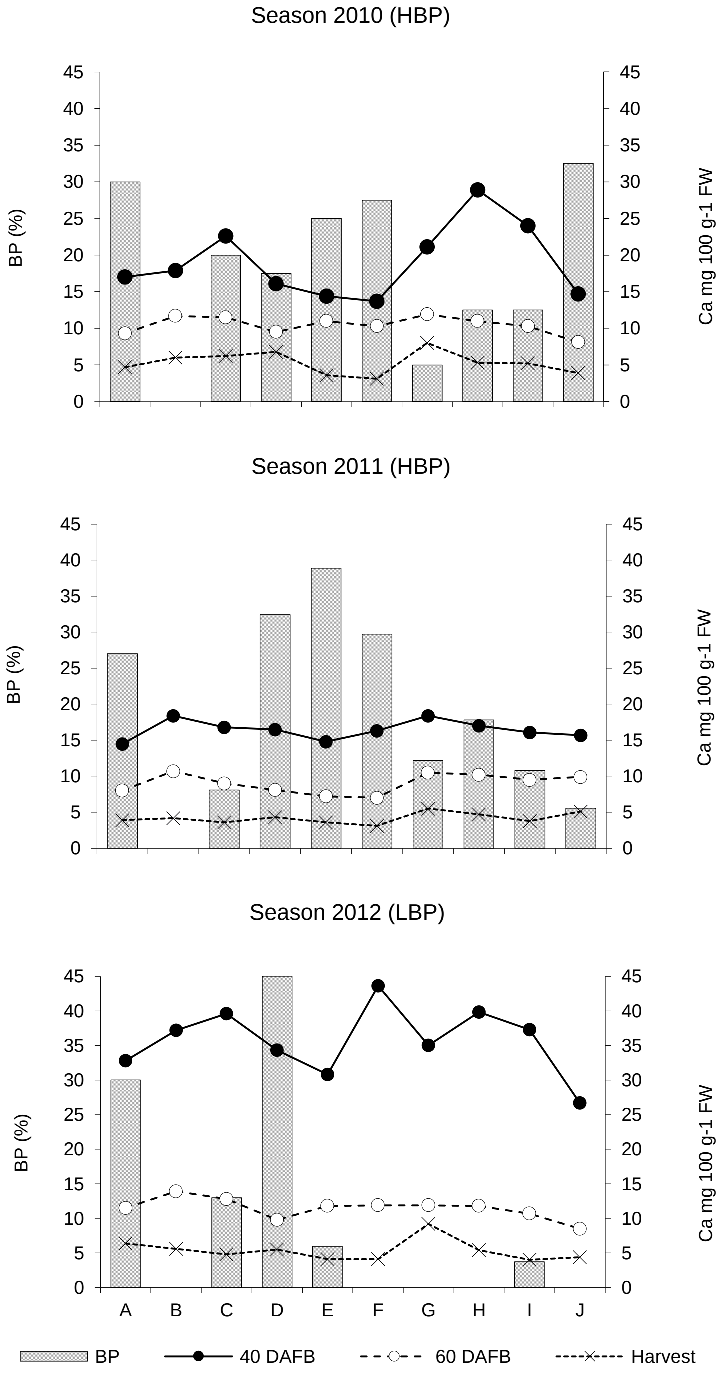

Fig. 4. Bitter pit (BP) incidence for each orchard (A-J), and the Ca concentrations in the fruit (Ca mg 100 g^{-1} of fresh weight) at 40 and 60 days after full bloom (DAFB) and at harvest for each season (210, 2011 and 2012).

In any case, Ca concentrations at 60 DAFB higher than 11.0 mg Ca would indicate a low risk of BP, whereas the prediction of a lower threshold depends on the region and/or year. There was a similar occurrence when we used fruit Ca at harvest to predict BP: values higher than 6 mg 100 g^{-1} FW indicated a low BP incidence, whereas at lower values, the BP incidence depended on the orchard and/or the year. Our previous studies indicated that a low Ca concentration in fruit (<4 mg 100 g^{-1} FW) is not enough to develop BP (Torres et al., 2017). Some authors question whether the Ca nutrition is the main cause of BP and propose that abiotic stress situations could have a more important role than the Ca concentration on the mechanisms that trigger BP development (Autio et al., 1986; Krawitzky et al., 2016; Marcelle et al., 1989; Saure, 2014). Stress increases the production of reactive oxygen species, which cause lipid peroxidation with an increase in membrane leakiness, leading to rapid vacuolation of parenchyma cells and the loss of ions, such as apoplastic Ca. Therefore, a final deficiency of Ca could only be considered to be a result, but not a cause (Saure, 2014). However, the proper balance of Ca with the rest of the nutrients would help to reduce the susceptibility to BP by improving cell wall stability and membrane integrity (Saure, 1996; Saure, 2014; Witney and Kushad, 1990), explaining the strong relationship between Ca in fruit and BP. Future research must address the relationship between different sources of abiotic stress and BP to gain a better understanding of BP triggers and improve the accuracy of the predictions.

4. CONCLUSIONS

The relationship between BP and the mineral concentration of nutrients that have a direct effect on BP (Ca, Mg, K, N) was examined using multivariate analysis (PCA and PLS models), and linear correlation at various early stages (40, 60, 80 DAFB) and at harvest. The correlations carried out using data at 40 and 60 DAFB were significant, whereas the correlations at 80 DAFB were non-significant, suggesting that 60 DAFB was the best time to perform early mineral analysis in fruitlets to predict BP due to a higher accuracy. Of all of the nutrients analysed, the Ca concentration in fruit contributed the most to BP incidence, either at early stages or at harvest. Other tested nutrients showed a smaller effect on the prediction models, and/or an irregular pattern, suggesting that the Ca content alone can be an indicator of the risk of BP incidence. Finally, our results showed a good chance of a low BP incidence (<10%) when the Ca level at 60 DAFB was equal or higher than 11.0 mg 100 g^{-1} FW, whereas for a lower Ca concentration, the diagnosis depended on the region or season. This threshold value early in the season was a better indicator of the BP risk than the threshold value at harvest, which is traditionally promoted (5–6 mg Ca 100 g^{-1} FW). The relationship between the mineral concentration in apples and BP continues to be a complex and poorly understood process, and its influence in triggering BP could be minor. Considering factors other than the nutritional balance as the main cause could provide a better understanding and more effective prediction and control of BP.

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CAPÍTULO 2

Induction of symptoms pre-harvest using the passive method: an easy way to predict bitter pit

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ABSTRACT

An effective method for predicting bitter pit incidence implemented before harvest could be a useful instrument for both the fruit industry and growers. Between 2009 and 2011, various methods for the prediction of bitter pit were evaluated at 60, 40, and 20 days before harvest (DBH) and at a commercial harvest in 'Golden Smoothee' apples. Four methodologies, two new ones (the development of natural bitter pit before harvest through the 'passive method' and bagging detached fruit before commercial ripening), and two of known efficacy under other conditions (infiltration with magnesium salts and maturity enhancement with ethephon dips) were assessed. To estimate the predictive accuracy of each method, bitter pit-like symptoms were related to the postharvest presence of bitter pit. The 'passive method' assesses bitter pit-like symptoms that appear naturally in fruit once they are picked from the tree, and left at room temperature during its evaluation. The 'passive method', as well as the infiltration with magnesium salts and ethephon dips, recognized bitter pit-like symptoms approximately 5–7 days after sampling (at 40 DBH) and showed significant correlation with the incidence of bitter pit after three months of cold storage. The 'passive' and 'ethephon' methods were also validated over two additional seasons in 30 and 16 different orchards, respectively. The results of these validations supported the efficacy of using the 'passive method' as the main method for predicting bitter pit, without having to use either reactive products or specialized equipment.

1. INTRODUCTION

Bitter pit is the main physiological disorder affecting apples (*Malus domestica* L. Borkh). Currently, the most effective way to reduce bitter pit is by spraying the fruit with calcium (Ca) during the growing season (Ferguson et al., 1999; Lotze and Theron, 2007; Peryea et al., 2007; Casero et al., 2010). Although this treatment can significantly reduce the incidence of bitter pit, it cannot eradicate the disorder. The responses of fruit crops to foliar Ca sprays may vary; therefore, fruit growers often obtain inconsistent results after applying treatments (Serrano et al., 2004; Manganaris et al., 2006; Val et al., 2008; Fernandez et al., 2009). The negative impact of bitter pit could be reduced if its incidence could be predicted or previously assessed. An effective prediction of bitter pit would allow for measures to be taken to reduce its incidence by either increasing the application of Ca sprays or by reducing the application of fertilizers involving nitrogen and potassium (Retamales and Lepe, 2000). If predictions could be made close to harvest time, it would be helpful to packing houses that have to make management decisions: whether to use Ca salt dips, design appropriate storage strategies, etc. (Manganaris et al., 2005; Torres and Alegre, 2012).

The main predictive method that has been used for many years to assess the risk of bitter pit, is based on analysis of Ca content in fruit, and also on that of nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) contents, and their relationship with Ca content in fruit (Autio et al., 1986; Wolk et al., 1998). However, the concentration of Ca is usually very low and variable, both within the same fruit and between fruit on the same tree; as a result, large sample sizes are necessary to assess the risk of bitter pit (Ferguson and Triggs, 1990). Even so, this does not always explain the incidence, or absence, of symptoms in a particular orchard. There are, however, other methods which are based on inducing symptoms before they naturally occur.

An inversely proportional relationship between Ca and Mg established the basis for developing a prediction method based on infiltrating fruit with $MgCl_2$ (Burmeister and Dilley, 1993; Retamales et al., 2000). Burmeister and Dilley (1993) speculated that extracellular Mg supplied by infiltration could disrupt cellular homeostasis via the key enzymes that regulate intracellular Ca. This would result in the appearance of visible spots reminiscent of bitter pit after 10–15 days (bitter pit-like symptoms). They suggested that the pitting symptoms induced by Mg infiltration are physiologically synonymous with the bitter pit disorder. Since 1997, this methodology has been applied commercially in some packing houses in Chile for the 'Gala', 'Braeburn', and 'Fuji' varieties (Retamales and Valdes, 2001). Nevertheless, very few studies have been conducted on 'Golden' apples, which is the most important cultivar in Europe (Casals and Iglesias, 2013). It is therefore possible that the effectiveness and/or accuracy of the methodology could differ between regions and/or cultivars (Retamales et al., 2000).

Another method for predicting bitter pit involves the acceleration of maturity through the application of ethylene. Ethylene plays an essential role in regulating the ripening process of climacteric fruit (Recasens et al., 2004) and is also known to enhance the expression of bitter pit-like symptoms in apples (Eksteen et al., 1977). Two ethylene-related methods were studied by Eksteen et al. in South

Africa during the 1970s (Eksteen et al., 1977; Lotze and Theron, 2006; Lotze et al., 2010): the Ginsburg method, in which apples were treated with 1% of acetylene gas, and the Bangerth method, in which they were immersed in a water solution containing 0.2% ethephon. The results obtained showed that the Ginsburg method tended to be less accurate in the majority of cases. Lotze et al. (2010) compared the effectiveness and accuracy of the Mg infiltration and the Bangerth method to predict the incidence of bitter pit in 'Golden Delicious' and 'Braeburn' apples in South Africa, with the Bangerth method proving more effective.

Another possible method involves bagging fruit before the climacteric peak. It is possible to advance the climacteric onset (onset of ethylene production) by exposing pre-climacteric fruit to the ethylene that is self-produced when they are placed inside closed bags (Mattheis, 1996; Recasens et al., 2004). However, this effect has not yet been assessed. In contrast, postharvest reductions in bitter pit have been reported in association with modified atmospheres and low O_2 levels (Khan et al., 2006; Torres and Alegre, 2012). Pesis et al. (2010) and Val et al. (2009) showed that pre-treatments prior to cold storage involving low O_2 and short-term storage at 20 °C were able to reduce the incidence of apple scald and bitter pit during cold storage.

Another method to predict bitter pit involves assessing bitter pit that appears naturally in fruit picked from the tree before harvest, and keeping the fruit at room temperature. Prior to the study, we observed that it was possible to observe symptoms of bitter pit in some apple orchards, in fruit that had dropped to the ground approximately 3 weeks before harvest. From this, we developed the hypothesis that apples picked from the trees before harvest could offer a good method for predicting bitter pit. We have called this way of predicting bitter pit the 'passive method' because there is no need to use any product or additional treatment to trigger the mechanism of bitter pit development. In the 1970s, England and Larsen (1973) conducted a study involving the varieties 'Goldspur' and 'Wellspur', in which the incidence of bitter pit was evaluated in apples removed from the tree before harvest, and before there were any visible symptoms on the apples on the tree. This study suggested that the incidence of storage bitter pit could be observed 1–3 weeks prior to harvest, but no analyses were conducted to assess the relationship between the incidence of pre- and post-harvest bitter pit. Despite being an easy and inexpensive way for predicting bitter pit, the method has not been tested in commercial orchards and, to the best of our knowledge, similar studies have not been conducted to date.

The aim of the present study was to find a good methodology for predicting the incidence of bitter pit, after harvest and under the typical conditions found in Northeast Spain, choosing either one of the existing methodologies (infiltration with magnesium salts and maturity enhancement with ethephon dips) or a new one (development of natural bitter pit through the 'passive method' and the effect of bagging fruit picked before commercial ripening). This is the first time that the 'passive method' and the effect of bagging fruit are tested and that all four approaches are compared to each other.

2. MATERIAL AND METHODS

2.1. Plant material

The first experiment was conducted over three consecutive seasons (2009–2011) in 10 commercial orchards growing 'Golden Smoothee' apples (*Malus domestica* L. Borkh) in the Lleida area (NE Spain), with antecedents of different levels of susceptibility to bitter pit. All of the orchards studied were mature and their trees were normally spaced (approximately 4 m 1.2 m), grafted onto M9 rootstock and fully irrigated. They all received standard cultural practices in terms of pruning, fertilization, irrigation, and crop management.

2.2. Fruit sampling

All of the orchards were sampled four times in two of the three seasons: 2009 and 2010, and three times in 2011. In the first two seasons (2009 and 2010), four 40-fruit samples were collected from each orchard at 60, 40, and 20 days before harvest (DBH) and at the commercial harvest. In the third season (2011), the samples were taken at 40 and 20 DBH and at the commercial harvest when the starch index was within 7–8 (starch chart EC-Eurofru). Each sample was composed of 40 apples without symptoms of bitter pit. Apples were taken from 40 selected trees with standard crop loads and vigor. One average-sized, undamaged apple was taken from each tree at a height of 130–170 cm above the ground.

2.3. Sample preparation and treatments

Each 40-fruit sample taken from each orchard received a different treatment. These were performed as follows:

- Infiltration of apples with $MgCl_2$ (MG): the first 40-fruit sample was treated under vacuum conditions (250 mm Hg) during 2 min with a solution of 0.10 M $MgCl_2$ $6H_2O$, 0.4 M sorbitol as osmoticum, and 0.01% Tween-20 as a surfactant, in accordance with the recommendations of Chilean apple exporters (Retamales and Valdes, 1996). The apples were subsequently placed on plastic fruit trays and left at room temperature (approximately 25 ºC) to allow for the development of bitter pit-like symptoms. This method was evaluated for the three seasons (2009–11).
- Ethephon dips (ET): the second 40-fruit sample was dipped in an ethephon solution (2000 ppm) for 5 min. The apples were then left at room temperature as described above. This method was evaluated for the three seasons (2009–11).
- Passive method (PS): the third 40-fruit sample was left untreated at room temperature, under the same conditions as described above. This method was also evaluated for the three seasons (2009–11).
- Bagging of fruit (BG): the fourth 40-fruit sample was packaged on two plastic 20-fruit trays. Each tray was then left inside a transparent plastic bag without holes, which was subsequently sealed, and left at room temperature. To maintain the atmosphere inside the bag, the subsequent assessments were performed visually

without opening the bag. This method was evaluated in two of the seasons (2009 and 2010).

To detect the point at which the incidence or severity of bitter pit-like symptoms stopped increasing, all of the apples contained in each sample were individually examined at intervals of 5–7 days over a 40-day period for any external signs of superficial bitter pit-like symptoms. The incidence of bitter pit was calculated as the percentage of fruit with bitter pit-like symptoms. The severity of bitter pit was assessed by classifying each apple according to a grading of its bitter pit-like symptoms (Fig. 1): level 0, healthy fruit; level 1, or slight, when the fruit had between 1 and 6 pits on its surface; level 2, or moderate, when less than one-third of the surface of the fruit was affected (approximately between 7 and 15 pits per fruit); and level 3, or severe, when more than one-third of the surface of the fruit was affected (>15 pits per fruit).

2.4. Storage treatment

To evaluate the postharvest incidence of bitter pit, another 80-fruit sample was collected from each orchard at the maturity status recommended for long-term storage. The percentage of bitter pit associated with each sample was recorded after three months of storage in air at 0 °C and at 80% relative humidity plus a further seven days at 20 °C and 45% relative humidity.

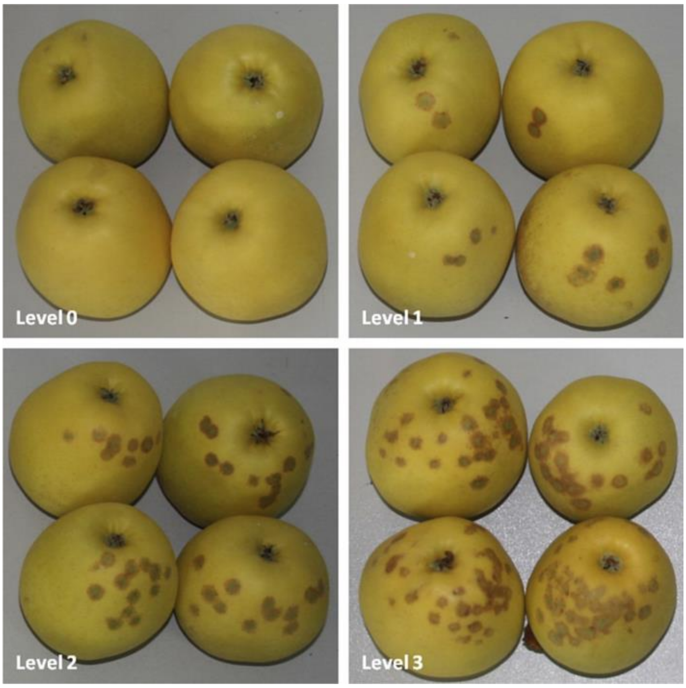

Fig. 1. Scale to assess the severity of bitter pit symptoms. Level 0 (no-bitter pit): without symptoms; level 1 (slight): 1–6 pits on surface; level 2 (moderate): one-third of the surface of the fruit affected (approximately between 7 and 15 pits per fruit); level 3 (severe) one-third of the surface of the fruit affected (>15 pits per fruit).

2.5. Validation at the commercial scale

The PS and ET methods were validated in two seasons (2011 and 2012), and in relation to 30 and 16 commercial orchards, respectively, which were different from those used above. They also received standard cultural practices in terms of pruning, fertilization, and irrigation. Two 40-fruit samples were collected from each orchard at 40 and 20 DBH in 2011, and at 40 DBH in 2012. One 40-fruit sample was treated according to the PS method, while the other was treated according to the ET method. Another 80-fruit sample was collected at harvest to evaluate the percentage of post-harvest bitter pit per orchard, with the same methodology as described above.

2.6. Data analysis

Data were analyzed by linear correlation using the percentages of bitter pit-like symptoms (BPLS) associated with each method (MG, ET, PS, and BG) at pre-harvest, as the independent variable. The dependent variable was the percentage of bitter pit (BPAS) after 4 months of storage in air at 0 °C. Correlations were calculated for each method-sampling time combination and reported for each year. We then calculated the coefficient of determination (R^2) and the level of significance of each linear regression model. The data were analyzed using the JMP[1] 8.0.1 program (SAS Institute Inc., 2009).

3. RESULTS AND DISCUSSION

3.1. Development of bitter pit-like symptoms

Bitter pit-like symptoms were visible on the apples treated by all methods within 5 days of the initial application of the method. Beyond 10 days after treatment, there was no increase in either the size or incidence of lesions in most of the methods. The only exception was the BG method; in this case, there was a tendency for symptoms to develop more slowly than in the other methods (Fig. 2). Other researchers (Burmeister and Dilley, 1991; Lotze et al., 2010) have previously observed that beyond 10 days after Mg infiltration or ethylene treatment, bitter pit-like symptoms tended not to increase. Nevertheless, the recommendation made by packing houses in Chile involved performing evaluations 16 days after Mg infiltrations (Valeria Lepe and Jose Antonio Yuri, personal communication). Our results suggest the possibility of predicting bitter pit using either the MG, ET or PS methods within 7–10 days of treatment. However, we observed some differences between methods regarding the severity and/or development of symptoms, which are discussed below.

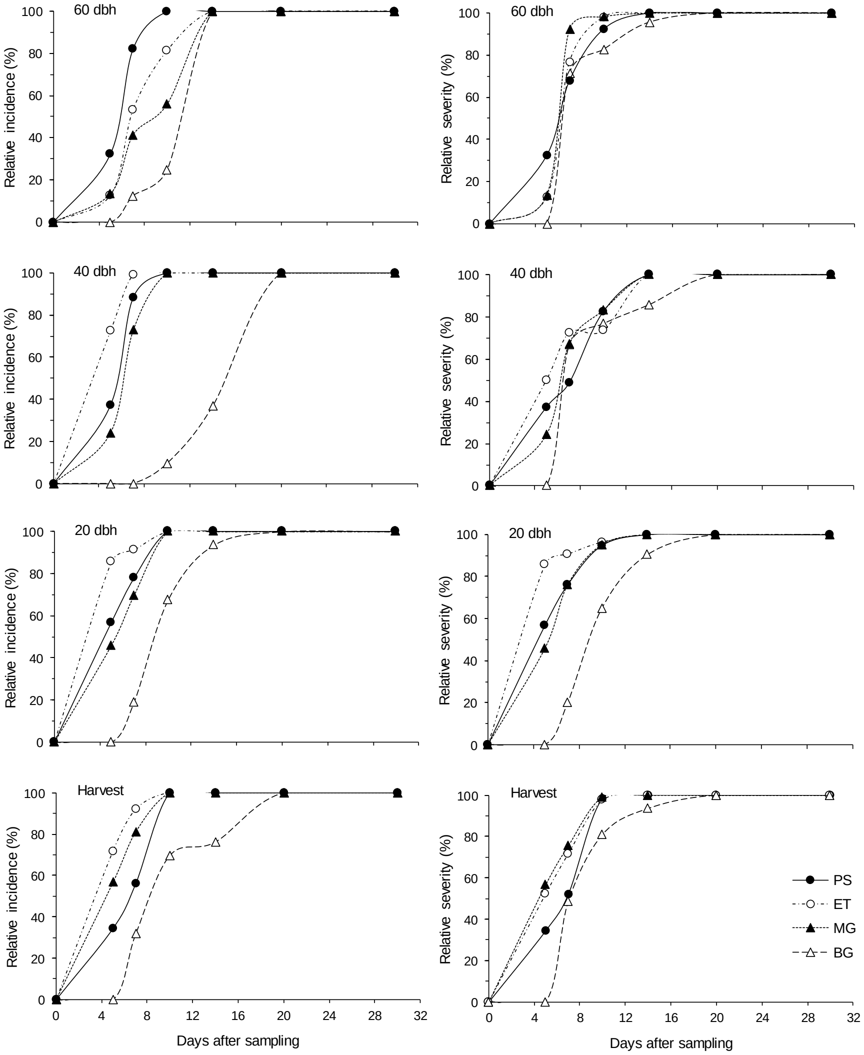

Fig. 2. Progress curves of the relative incidence (relative to the incidence at 30 days after sampling; left) and relative severity (relative to the severity at 30 days after sampling; right) of bitter pit-like symptoms beyond the treatment to predict bitter pit: passive method (PS), ethephon dips (ET) infiltration with $MgCl_2$ (MG), and bagging of fruit (BG). A graph for each sample date: 60, 40, and 20 days before harvest (DBH) and at commercial harvest. Each data point represents the mean for all of the orchards over two seasons (2009 and 2010).

Bagging apples at the pre-climacteric stage did not increase either the incidence or severity of bitter pit; in fact, it did quite the opposite. The BPLS associated with the BG method were less pronounced for all sample groups and seasons. Other methods consisting of modifying the atmosphere and achieving low O_2 levels to reduce postharvest bitter pit have also been reported (Hewett, 1984; Hewett and Thompson, 1989; Khan et al., 2006; Val et al., 2009; Pesis et al., 2010). Pesis et al. (2010) and Val et al. (2009) reported that low O_2 and 20 °C short-term pre-treatments prior to cold storage, could help to reduce bitter pit during cold storage. This bitter pit reduction could be due to a decrease of the respiratory rate. Recasens et al. (2004) demonstrated that respiratory rate and ethylene production correlate negatively with Ca content in apples, and Sharma et al. (2012) reported that rates of ethylene production and respiration were lower in disorder-free fruit than in disorder-affected fruit. On the other hand, Saure (2014) considered the susceptibility to stress, rather than Ca deficiency, as the main cause of the development of bitter pit. According to Saure (2014), the influence of stress on the occurrence of 'Ca-related disorders', such as bitter pit, could be based on an increased production of reactive oxygen species (ROS) in the fruit. ROS are known to trigger cell death, which is characterized by a progressive loss of membrane integrity and release of cellular constituents (Van Breusegem and Dat, 2006; Saure, 2014). The bagging could reduce the oxidative stress into the fruit and, therefore, decrease the production of ROS and the incidence of BPLS.

Unlike the bagging, the BPLS associated with the MG method were more pronounced than with the other methods, especially closer to harvest. There were more pits per fruit; therefore, a greater fruit surface was affected. Researchers do not agree as to whether Mg-induced pits are directly comparable with those associated with the natural bitter pit disorder (Burmeister and Dilley, 1991). It has been suggested that Mg-induced pits result from a breakdown caused by either Mg toxicity at the point of entry into the fruit (Ferguson and Watkins, 1989), or by polyphenol oxidase (PPO) activity (Hopfinger et al., 1984). The Mg could generate an increase of ROS as result of PPO activity, and cause cell death (Saure, 2014). Results from Burmeister and Dilley (1991) supported the hypothesis that Mg-induced pits are quite similar to those associated with the actual bitter pit disorder when the $MgCl_2$ concentration was 0.18 M. Our results at concentrations of 0.10 M indicate that Mg-induced pits are also quite similar to those associated with BPAS and that the major differences with the other methods are in the severity of the symptoms rather than in the incidence.

No relevant differences were observed between the other two methods, ET and PS, in terms of the development of symptoms. We should, however, underline that ethephon-dipped fruit, after 5–7 days of treatment, had a yellower background color than other treatments. This helped us to observe bitter pit-like lesions more clearly, thanks to the contrast between the green color of the pits and the yellow of the background. From our existing data, it is not possible to conclude whether applying ethephon dips stimulated the appearance of BPLS because we observed that BPLS were also enhanced if the apples were left at room temperature without any additional treatment (PS method).

According to our results, final bitter pit potential could be determined within 7–10 days of the application of the method, whether using the MG, ET or PS method. This implies that bitter pit could potentially be predicted between 6 and 9 days earlier than the Chilean method based on Mg infiltration, or 4–7 days earlier than the

South African method based on ethephon dips. This would offer important benefits for both farmers and packing houses as they would have more time for decision making.

3.2. Accuracy of the predictions in relation to sampling time

There were no significant correlations between the prediction methods at 60 DBH and BPAS, either in 2009 or 2010 because of the lack of BPLS at this early sampling stage (Table 1). According to our observations, the lack of accuracy of the methods at 60 DBH could be because the sampled fruit were still at an unripe stage. As fruit maturity is an important factor in the subsequent development of bitter pit (Ferguson and Watkins, 1989; Prinja, 1990), this could have masked the full appearance of BPLS. At this sampling time, it was not possible to improve either the accuracy of prediction or the incidence of BPLS by accelerating fruit maturation with ethephon. It is evident that sampling at 60 DBH is not useful for predicting bitter pit, and for this reason we decided not to use it in the next season.

At 40 DBH, the MG, ET, and PS methods produced significant linear correlations with BPAS, with $R^2 = 0.4$ ($P < 0.05$) over the three seasons (Table 1). When all of the years were analyzed together, the MG, ET, and PS methods applied at 40 DBH also showed significant linear correlations with BPAS. However, the maximum visual expression of bitter pit-like symptoms and the best correlations did not occur until 20 DBH, ($R^2 = 0.7$, $P < 0.01$) (Table 1). Moreover, when we analyzed the 2009 and 2010 seasons separately, the best correlation was also observed at 20 DBH. Other researchers have obtained good linear correlations between BPLS at pre-harvest up to 20 DBH and the BPAS (Eksteen et al., 1977; Lotze et al., 2010). Nevertheless, from our data, it can be observed that both the ET and PS methods at 20 DBH produced non-significant linear correlations with BPAS in one of the seasons (2011), whereas at 40 DBH, the linear correlations were always statistically significant in the three seasons and regardless of the method used.

At harvest, the linear correlations between the MG, ET, and PS methods with BPAS were significant for all three seasons ($P < 0.05$), but with a lower R^2 than at 20 DBH in 2009 and, particularly, in 2010 (Table 1). The apparent lack of accuracy for the 2010 harvest could be due to the decrease in the incidence of bitter pit. The mean percentage of BPAS in 2010 season was approximately 7%, whereas in 2009 and 2011 the corresponding values were 21% and 17%, respectively. Other trials with incidences of bitter pit below 10% also produced low correlation coefficients (Lotze et al., 2010). The lower accuracy at harvest could be improved by considering the apples with evident signs of bitter pit on the tree. Another cause of the apparent lack of accuracy at harvest could be the fruit maturity stage. Most reports have shown that, in general, bitter pit is more severe in early-picked than in later-picked apple fruit (Perring, 1986; Volz et al., 1993; Prange et al., 2011) Therefore, BPLS could also be more severe in early-picked than in later-picked apple fruit.

From our data, we observed that the orchards in which BPLS appeared earliest were those with a high incidence of BPAS, regardless of the method used. Furthermore, there were no cases of high levels of BPLS before harvest associated with low levels of BPAS in any sampling time or season. Therefore, early sampling (and always after

60 DBH) could perhaps detect orchards with a high risk of bitter pit, at least qualitatively.

Table 1. Correlations between bitter pit after storage and bitter pit-like symptoms using the passive method (PS), ethephon dips (ET), infiltrations with $MgCl_2$ (MG), and bagging of fruits (BG) during three seasons (2009–2011), and sampling at 60 days before harvest (DBH), 40 DBH, 20 DBH and harvest time.

Method	2009		2010		2011		All seasons	
	R^2	*P*	R^2	*P*	R^2	*P*	R^2	*P*
60 DBH								
MG	0.023	NS	0.075	NS	-	-	0.069	NS
ET	0.061	NS	0.100	NS	-	-	0.000	NS
PS	0.062	NS	0.000	NS	-	-	0.004	NS
BG	0.004	NS	0.000	NS	-	-	0.048	NS
40 DBH								
MG	0.398	0.043	0.567	0.005	0.435	0.043	0.315	0.002
ET	0.599	0.024	0.542	0.010	0.417	0.044	0.297	0.002
PS	0.564	0.032	0.614	0.003	0.623	0.004	0.599	<.001
BG	0.064	NS	0.246	NS	-	-	0.017	NS
20 DBH								
MG	0.814	0.001	0.599	0.003	0.638	0.006	0.754	<.001
ET	0.887	<.001	0.656	0.001	0.312	NS	0.656	<.001
PS	0.792	0.001	0.601	0.003	0.375	NS	0.670	<.001
BG	0.303	NS	0.119	NS	-	-	0.395	0.002
Harvest								
MG	0.733	0.003	0.350	0.054	0.645	0.022	0.581	<.001
ET	0.465	0.043	0.320	0.054	0.424	0.041	0.376	<.001
PS	0.603	0.014	0.439	0.019	0.458	0.003	0.527	<.001
BG	0.164	NS	0.144	NS	-	-	0.296	0.013

Consequently, sampling at 40 DBH seems to be the best option both in terms of earliness and accuracy. Sampling at 40 DBH would allow growers to know the potential risk of bitter pit development approximately 30 days before the expected harvest date. If a high risk of bitter pit could be detected at that moment, there would be enough time to take measures to reduce the incidence of bitter pit (Casero et al., 2010).

3.3. Accuracy of predictions in relation to the method used

There were no significant correlations between the BG method and BPAS in any season when these were separately analyzed (2009 and 2010). When the two years were correlated together, the BG method showed significant linear correlations with BPAS ($P < 0.01$), but with a R^2 lower than 0.4, whereas the other methods reached $R^2 = 0.7$. The lack of accuracy in relation to the BG method was due to the lack of BPLS, as discussed in the preceding Section 3.1. It is evident that the BG method is not useful for predicting bitter pit, and for this reason we decided not to use it in the next season.

In determining the most accuracy of the remaining three methods (MG, ET, and PS) analyzed for predicting bitter pit, our results suggest that they would all be sufficiently accurate for commercial purposes. However, the variability between methods was high in terms of accuracy (Table 1). In the 2009 season, the ET method applied at 40 DBH showed the highest coefficient of determination with respect to BPAS ($R^2 = 0.60$, $P < 0.05$), whereas in the 2010 and 2011 seasons, this was associated with the PS method ($R^2 = 0.61$ and 0.62, respectively, $P < 0.01$). At 20 DBH, the highest correlations in the 2009 and 2010 seasons were obtained with the ET method ($R^2 = 0.89$ and 0.66, respectively, $P < 0.01$), and in 2011 with the MG method ($R^2 = 0.64$, $P < 0.01$). At harvest, the MG method produced the highest coefficient of determination for the 2009 season ($R^2 = 0.73$, $P < 0.01$) and the 2011 season ($R^2 = 0.73$, $P < 0.01$), and the PS method for the 2010 season ($R^2 = 0.44$, $P < 0.05$). Even so, our results with the MG and ET methods were quite similar to those obtained in Chile during the 1990s (Mg infiltration $R^2 = 0.67–0.87$; ethephon $R^2 = 0.60–0.84$) (Retamales and Valdes, 1996), and better than those reported by Lotze et al. (2010) for ethephon dips in the 'Golden Delicious' cultivar. Regarding the latter, the different sample size between both trials (40-fruit samples vs. 20-fruit samples) could have influenced the results. Obtaining a wide range of incidence of bitter pit among the different samples might also have had a positive influence on the accuracy of the models. The ranges that we obtained were quite wide: from 0% to 59% (2009), from 0% to 23% (2010), and from 0% to 38% (2011).

A prediction method would only be applied commercially by packing houses and/or growers if it is easy to implement and offers a low-cost solution. For these reasons, the PS method seems to offer a better alternative for bitter pit prediction. Unlike the other alternatives, this method does not require the use of either reactive products or specialized equipment. However, the accuracy of the method may vary from cultivar to cultivar, from region to region (Retamales et al., 2000; Lotze et al., 2010), and also between seasons, as we have seen in this study, but its simplicity suggests that it could be the first real commercial approach for predicting bitter pit before harvest. The method should therefore be evaluated in different regions and with different cultivars to determine its accuracy of prediction under varied conditions.

3.4. Validation of the passive and ethephon methods in commercial orchards

The results of the validations of the PS and ET methods in different commercial orchards in the 2011 and 2012 seasons showed a clear and significant relationship with BPAS, regardless of the sample time (40 and 20 DBH) and season (2011 and 2012). In all of the cases studied, the PS and ET methods produced significant R^2 with BPAS ($R^2 > 0.6$, $P < 0.01$), without any important differences between the two methods (Fig. 3).

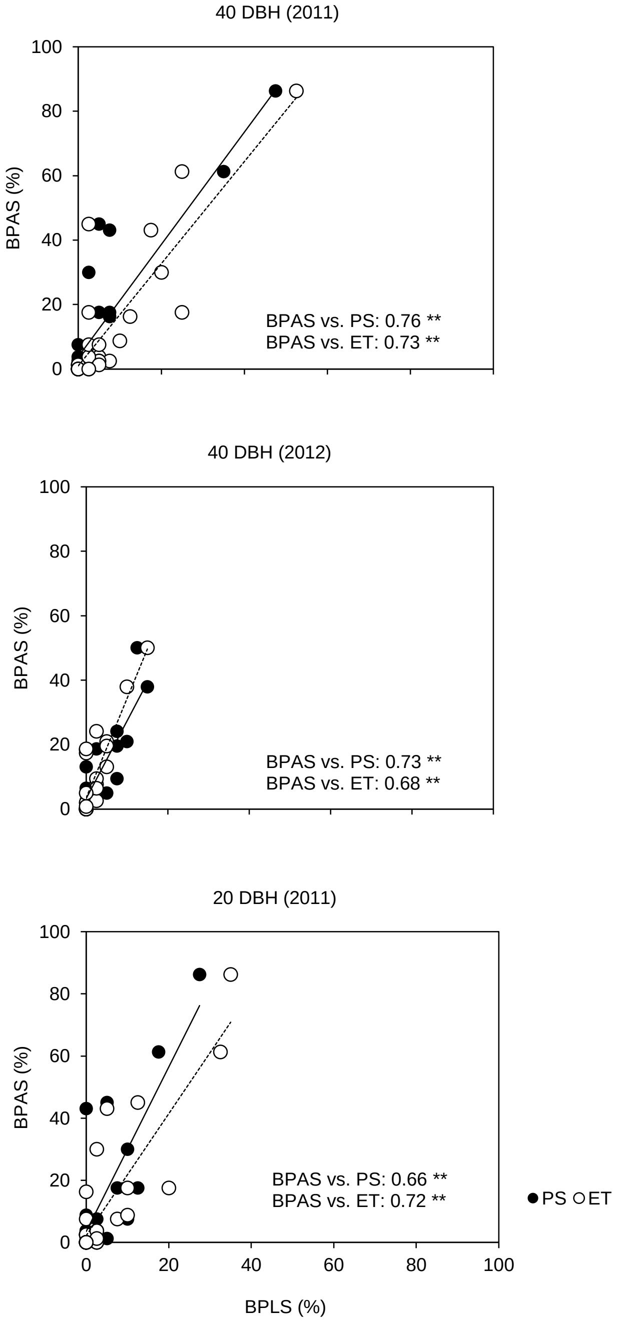

Fig. 3. Linear relationships and coefficients of determination (R^2) between the incidences of bitter pit after storage (BPAS) and of bitter pit-like symptoms (BPLS) using the passive (PS) and ethephon (ET) methods in two seasons (2011 and 2012). In the 2011 season, the pre-harvest samples were collected 40 and 20 days before harvest (DBH) and in the 2012 season only at 40 DBH.

The accuracy of the validations was even better than in previous tests; this could be attributable to the use of more orchards and, as a consequence, a wider range of incidence of bitter pit (from 0% to 80%). As explained in the discussion in the preceding subsections, sampling at 40 DBH and using the PS method would therefore offer the best alternative. In terms of practical use and taking an incidence scale of three degrees (<5% or low; 5–10% or medium; >10% or high), the percentage of orchards well classified was 71% in 2011 and 62% in 2012. The rest of orchards were all underestimated. The results indicate that we should assume the risk of having some orchards with a high incidence of BPAS if no BPLS are recorded by the PS method, but that the identification of significant BPLS levels (>5%) are a guarantee of high incidence of BPAS. From all of the above it can be stated that neither the PS method nor the other methods would permit an accurate quantitative prediction of bitter pit, but they would allow a qualitative prediction of BPAS.

4. CONCLUSION

We conclude that the 'passive method' (PS) offers a reliable and relatively inexpensive method for assessing the risk of bitter pit in 'Golden Smoothee' apples after storage between 10 and 30 days before harvest. Furthermore, unlike the other methods studied, the PS method requires neither reactive products nor specialized equipment. This makes it a more interesting option than the other methods for commercial and/or industrial purposes. Nevertheless, the method would need to be assessed for different regions and for different cultivars before it can be adopted on a wider scale.

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CAPÍTULO 3

Process to predict bitter pit in ‘Golden Smoothee’ apples based on different diagnostic methods

Torres E., Recasens I., Alegre S.

ABSTRACT

Bitter pit is the most important physiological disorder in apples, but there is no fully effective control strategy available. An effective prediction method could be a good instrument either for growers or industry in order to identify orchards with a high risk and prevent economical losses associated labour and packaging costs. On the one hand, if the prediction could be made at an early stage of fruit development, this would allow optimizing the resources during the fruit development (sprays of calcium, fertilization of nitrate, potassium, etc). On the other hand, if that prediction could be done close to harvest, this would help to packing houses to take important decisions in the management, such as whether to realise dips with calcium salts or not, or to design the storage strategy. In the last years, we have investigated different methods to predict the bitter pit (mineral analysis of fruitlets and fruit, ethephon dips, magnesium infiltration and passive method) in different period of fruit growth of 'Golden Smoothee' apples. Finally, early season analysis of fruitlets and passive method are proposed to predict bitter pit, at early stage of fruit development and close to harvest respectively. Regarding the early analysis, a Ca threshold at 60 days after full bloom equal to or greater than 11.0 mg 100 g^{-1} fresh weight would indicate a low risk of bitter pit; however, lower fruit Ca concentration resulted in a high probability of false positives. In these cases, we recommend using the 'passive method' from 40 days before harvest to track the development of bitter pit in orchards with a low level of Ca.

1. INTRODUCTION

Bitter pit is the main physiological disorder in apples. It is characterised by sunken lesions, which may develop on fruit as they mature on the tree or during storage, and the tissue below the skin in the pitted area becomes discoloured and dehydrated. Certain ambient and cultural conditions contribute to increase bitter pit, such as light cropping, calcium (Ca) deficiency, excessive tree vigour, excessive nitrogen nutrition and/or moisture stress. Therefore, adjusting the tree nutrition, regulating the tree vigour and incrementing Ca levels in fruit are the main practices to reduce levels of bitter pit. Nevertheless, control of bitter pit is highly variable and not always complete. Because of the absence of an effective control method for bitter pit, some studies have focused on its prediction before harvest period.

Effective bitter pit prediction would allow optimizing control measures (e.g., increasing Ca sprays, or decreasing N and K fertilization), or helping the packinghouse in choosing the right management approach. Mineral analyses based on Ca level within the fruit just before harvest is a standard approach to predict bitter pit (Terblanche et al., 1980; Ferguson et al., 1999), but it can only provide valuable information just before harvest. Some authors have suggested early season analysis of fruitlets in order to gain time and then implement corrective action (Brooks, 2001; Drahorad and Aichner, 2001; Torres et al., 2017a). However, fruit Ca content does not always explain the incidence, or absence, of symptoms in a particular orchard. Some authors have suggested that approaches for forcing symptoms, before they naturally appear—such as the maturity enhancement with ethephon dips, the infiltration with magnesium salts and passive method—, are more accurate at predicting bitter pit than standard test of pre-harvest fruit Ca levels.

Ethylene is known to enhance ripening of fruit and this acceleration of maturity can also enhance the expression of bitter pit-like symptoms in apples (Eksteenet al., 1977). The inversely proportional relationship between Ca and Mg established the basis for developing a prediction method using the fruit infiltration with $MgCl_2$ (Retamales, et al., 2000). The development of bitter pit in 'Golden' apples detached from the tree before harvest was associated with post-harvest bitter pit and proposed as predictive method of bitter pit; this method was named 'passive method' (Torres et al., 2015).

The aim of this study was to compare the accuracy of these methods—fruit Ca tests, at 60 days after full bloom (DAFB) and at harvest, and ethephon dips (ET), magnesium infiltration (MG) and passive method (PS), at 40 and 20 days before harvest (DBH)—in order to define an optimal process to predict bitter pit in 'Golden Smoothee' apples.

2. MATERIALS AND METHODS

2.1. Plant material

The experiment was carried out during four seasons in 10 'Golden Smoothee' commercial orchards in the Lleida area (NE Spain) with different bitter pit susceptibility antecedents.

2.2. Analysis of Ca content in fruit

A sample of 40 fruits per orchard was taken at 60 DAFB and at harvest to analyze the Ca concentration in fruit. Two longitudinal opposed slices per fruit were analyzed, excluding the core and seeds. The sample from each orchard was weighed, dried, and then re-weighed to know the percentage of dry mass. This dried tissue was then ground and a sub-sample was wet-digested with concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a microwave oven (Millestone MCR). The calcium (Ca) concentration was determined using inductively coupled Plasma-optical emission spectroscopy (ICP-OES). A high risk of bitter pit (incidence $\geq$ 10%) was considered when the Ca concentration was < 11 mg 100 g^{-1} fresh weight (FW) at 60 DAFB (Torres et al., 2017a) or < 6 mg 100 g^{-1} FW at harvest (Terblanche et al., 1980).

2.3. Symptom induction (ethephon dips, magnesium infiltration and passive method)

Three lots of 40 fruits per orchard at 40 and 20 DBH were taken to test the ET, MG and PS. The samples corresponding to the ET lots were dipped in an ethephon solution (2000 ppm) for five minutes; subsequently, fruit were put in plastic fruit trays and left at room temperature (approximately 25 °C) to develop bitter pit-like symptoms. The MI lots were treated under vacuum (250 mm Hg) for two minutes with a solution that contained 0.10 M $MgCl_2$, 0.4 M sorbitol as osmoticum and 0.01 % Tween-20 as surfactant and afterwards fruit were left at room temperature. The PS lots were left untreated at room temperature, under the same conditions as the ET and MG lots. The apples were examined individually for external signs of superficial bitter pit-like symptoms at 7-10 days after sampling. The percentage of fruit with bitter pit-like symptoms was calculated for each treatment and orchard. Samples with incidences higher or equal than 10% were considered of high risk of bitter pit.

2.4. Storage treatment

To evaluate the real incidence of bitter pit, a sample (approx. 100 fruit) was harvested from each orchard at commercial harvest. The percentage of bitter pit at post-harvest associated with each sample was recorded after four months of storage in air at 0 °C and at 80% relative humidity plus a further seven days at 20 °C and 45% relative humidity.

Orchards were classified as negative (low level of bitter pit) when bitter pit incidence at post-harvest was < 10%, and as positive (high level of bitter pit) when bitter pit incidences was ≥ 10%.

2.5. Statistical analysis

A one-way blocked ANOVA was performed, with the year serving as block, to determine whether the methods yielded different accuracies of classification and to determine if accuracies were different for negative, positive and overall classifications. Duncan's multiple range tests were used for the mean separation of significant effects from ANOVA models. Significance was declared at $P < 0.05$.

3. RESULTS

Throughout the period of this study, a wide range of bitter pit incidence among orchards was obtained (Fig. 1). The percentage of orchards with high bitter pit incidence (classified as positives) was of 56, 42, 64 and 50% for the 4 years of trial. This fact conferred validity to the study to evaluate the tested methods to predict bitter pit.

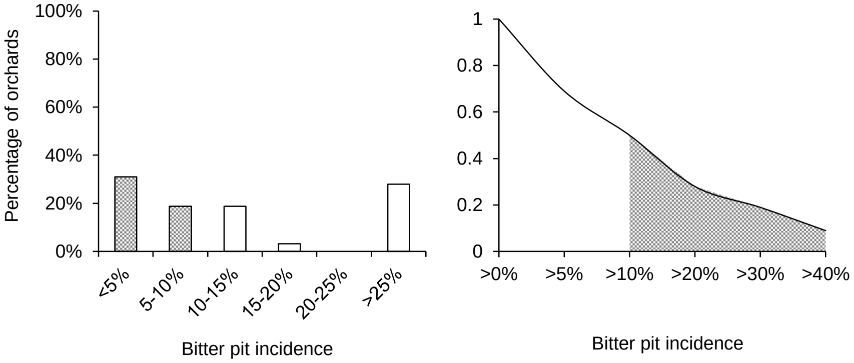

Fig. 1. Ranges of bitter pit incidence (percentage of orchards in four years of study).

3.1. Classification of orchards with high level of bitter pit (positive classification)

The methods based on forcing symptoms (PS, MG, and ET) proved more accurate at detecting positive orchards than fruit Ca testing at 60 DAFB or at harvest, (Fig. 2). The average accuracy for the positive classification was significantly higher using the PS, MG or ET methods than Ca tests (82% vs. 33%). Therefore, fruit Ca testing yielded a percentage of false positives (orchards with low level of bitter pit classified as positive) significantly higher than the methods based on inducting symptoms (67% vs. 18%).

No significant differences were observed between the different methods based on inducting symptoms at predicting positive orchards, independently of application time (40 or 20 DBH). Regarding fruit Ca tests, the samplings at 60 DAFB proved significantly more accurate than at harvest at predicting positive orchards (47% vs. 19%).

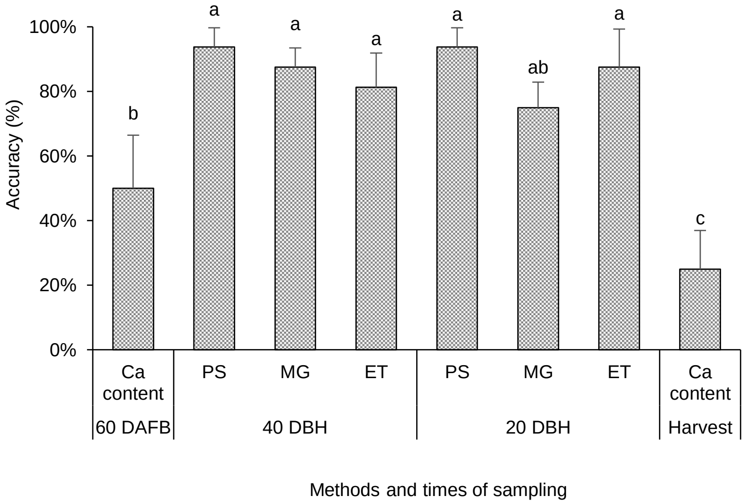

Fig. 2. The average classification accuracies of orchards with a high level of bitter pit (true positive classification) using the Ca content in fruit, at 60 days after full bloom (DAFB) and at harvest, and the passive method (PS), infiltrations with $MgCl_2$ (MG), and ethephon dips (ET), at 40 days before harvest (DBH) and 20 DBH. Mean values followed by same small letters do not differ significantly according to Duncan test ($P = 0.05$). Error bars represent standard deviation.

3.2. Classification of orchards with low level of bitter pit (negative classification)

The classification of orchards with low level of bitter pit was more accurate through fruit Ca testing (either at 60 DAFB or at harvest). Fruit Ca tests yielded an average accuracy of true negative classification of 94%, whereas for the PS, MG and ET tests was of

53% (Fig. 3). There were significant differences between fruit Ca testing at 60 DAFB and at harvest. Nevertheless, the samplings at 20 DBH showed a tendency to be more accurate for the different methods based on inducting symptoms, independently of method.

Finally, the methods based on forcing symptoms yielded percentages of false negatives (orchards with high level of bitter pit classified as negatives) significantly higher than fruit Ca tests at 60 DAFB or at harvest (47% vs. 6%). This lack of accuracy to classify negative orchards from forcing symptoms could be due to the natural variation in bitter pit incidence between samples of a same orchard. A simple and low-cost test as PS could help to performance several sampling from 40 DBH to harvest in order to provide more accurate predictions.

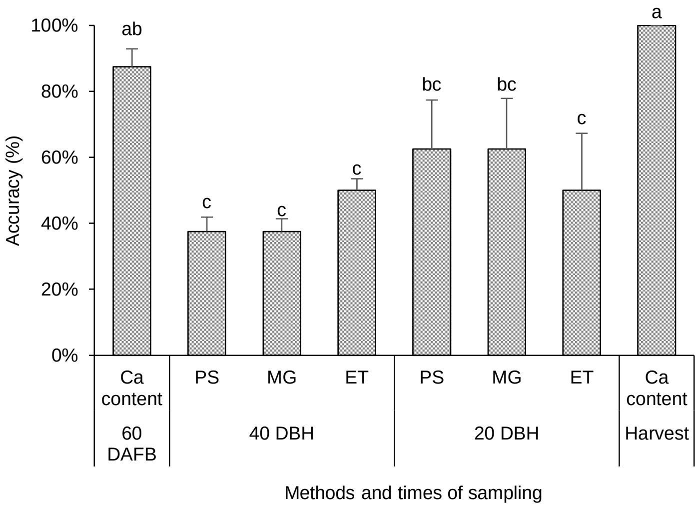

Fig. 3. The average classification accuracies of orchard with a low level of bitter pit (true negative classification) using the Ca content in fruit, at 60 days after full bloom (DAFB) and at harvest, and the passive method (PS), infiltrations with $MgCl_2$ (MG), and ethephon dips (ET), at 40 days before harvest (DBH) and 20 DBH. Mean values followed by same small letters do not differ significantly according to Duncan test (*P* = 0.05). Error bars represent standard deviation.

3.3. Overall classification

The overall classification accuracies were all greater than 63% (Fig. 4). In general, for the methods based on forcing symptoms, the accuracies were greater at 20 DBH than at 40 DBH (average of 65% vs. 72%), but only PS showed significant differences between both sampling times. These results keep in line with previous findings reported for Mg infiltration and ethephon dips (Retamales and Valdes, 2000; Lotze et al., 2010; Torres et al., 2015). No significant differences were observed between fruit Ca test at 60 DAFB (67%) and forcing symptoms at 40 or 20 DBH, independently of method used. Nevertheless, the average overall classification accuracy was significantly higher using PS test at 20 DBH than PS, MG and ET tests at 40 DBH and fruit Ca testing at harvest (78% vs. 66-63%).

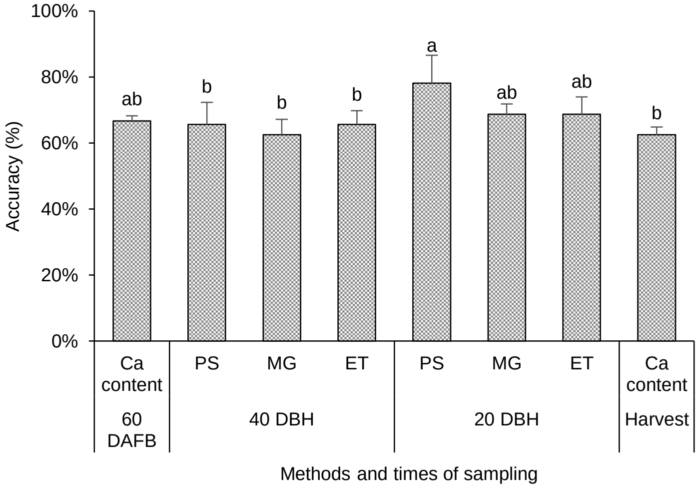

Fig. 4. The average overall classification accuracies using the Ca content in fruit, at 60 days after full bloom (DAFB) and at harvest, and the passive method (PS), infiltrations with $MgCl_2$ (MG), and ethephon dips (ET), at 40 days before harvest (DBH) and 20 DBH. Mean values followed by same small letters do not differ significantly according to Duncan test (P = 0.05). Error bars represent standard deviation.

4. DISCUSSION

Although, in general, there were no significant differences for the overall classification between methods, classification accuracies were different for positive and negative class. The accuracy to classify negative orchards was of 88% using fruit Ca test at 60 DAFB, but we have to consider that the number of false positives was significantly higher than using the methods based on forcing symptoms (67% vs. 18%). Even so, several authors suggest early season analysis of fruitlets in order to gain time and then implement corrective action (Brooks, 2001; Drahorad and Aichner, 2001; Torres et al., 2017a; Torres et al., 2017b).

Among the methods based on forcing symptoms, the PS test proved accuracies equal or higher than the other ones at predicting the level of bitter pit throughout the period of this study. In addition, PS shows a number of advantages with respect to other methods:

- Cost: PS testing is cheaper than the MG, ET and fruit Ca test since no equipment or reactive products are necessaries.
- The test is simple to implement: growers or technician advisors attached to packinghouses would easily be able to carry out the procedure themselves.
- With respect to fruit Ca test, the results are very visual which and helped in conveying the risk message to growers.

For all these reasons, we recommend using the PS in ‘Golden Smoothee’ apples from 40 DBH to track the development of bitter pit in orchards with a low level of fruit Ca at 60 DAFB, in order to provide more accurate predictions (Fig. 5). We should point out that the accuracy of these methods at predicting bitter pit can vary between cultivars or regions.

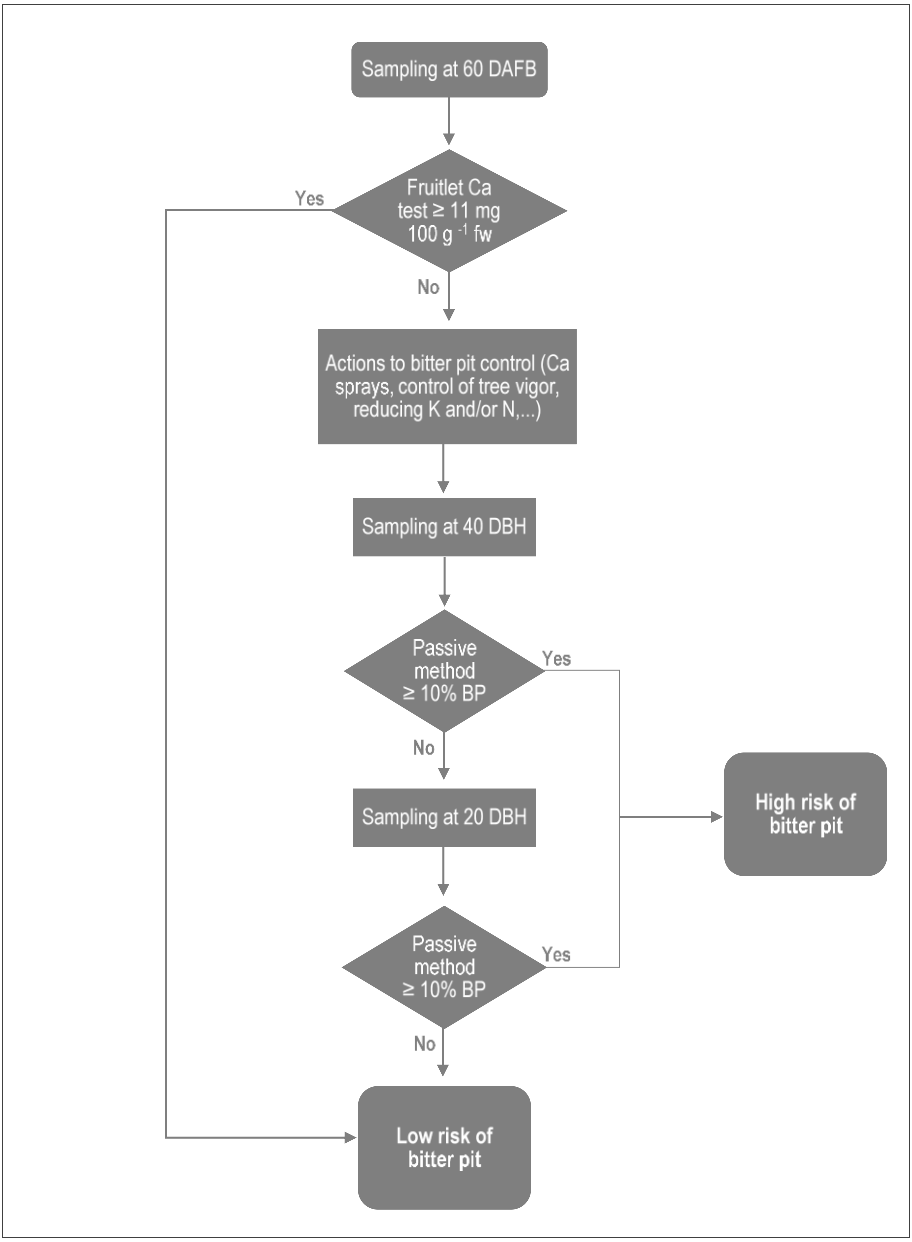

Fig. 5. Flow diagram of predict bitter pit process for 'Golden' apple orchards

4. CONCLUSION

Fruitlet Ca testing at early stage of fruit development (60 days after full bloom) and 'passive method' closer to harvest (around 50 days later) are proposed to predict bitter pit in orchards of 'Golden Smothee' apples. Regarding the early analysis, a Ca threshold at 60 days after full bloom equal to or greater than 11.0 mg 100 g^{-1} fresh weight would indicate a low risk of bitter pit. However, in orchards with a low level of Ca, we recommend using the 'passive method' from 40 days before harvest to track the development of bitter pit.

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CAPÍTULO 4

Potential of VIS/NIR spectroscopy to detect and predict bitter pit in 'Golden Smoothee' apples

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Enviado a: Postharvest Biol. Technol.

ABSTRACT

An evaluation was made of the use of VIS/NIR spectroscopy for the detection of bitter pit in ‘Golden Smoothee’ apples. Two experiments were carried out on apples from a bitter-pit-prone orchard. In experiment #1, VIS/NIR measurements were carried out at postharvest on apples previously classified according to 3 classes (class 1: non-bitter pit; class 2: slight symptoms; class 3: severe symptoms) to evaluate the suitability of the spectral feature extraction in discriminating the identified classes. Two measures were performed per fruit to determinate the classification capacity depending on the measured area: close to pitted tissue, or on healthy tissue, or both. In experiment #2, VIS/NIR measurements were carried out on healthy apples collected 20 days before harvest or at commercial harvest to determinate bitter pit prediction capacity prior to the appearance of symptoms. After 7 days at room temperature or after 4 months in cold storage, respectively, bitter pit was evaluated using a binary-class classification (symptomatic and asymptomatic apples). In all cases, three different classification algorithms were used (LDA, QDA and SVM). Bitter pitted ‘Golden Smoothee’ apples could be distinguished through VIS/NIR spectroscopy only when the symptoms were visible and for two different classes of apples (class 1 and 3). The linear classifier LDA performed better than the multivariate non-linear QDA and SVM classifiers in discriminating between healthy and bitter pitted apples (75% vs. 57–65%). No significant differences in accuracy were observed when taking 1 or 2 measurements on the fruit, independently of the area measured.

1. INTRODUCTION

Bitter pit is considered one of the key physiological disorders in apple crop, causing serious losses in certain apple varieties. The symptoms are characterized by depressions on the skin, generally located at the calyx end of the fruit. The tissue under these depressed areas is darkened, dry and corky with a bitter taste. Traditionally, research studies have related apple bitter bit with calcium deficiency and the balance between calcium and other nutrients such as nitrogen, potassium or magnesium (Amarante et al., 2013; de Freitas et al., 2015; Ferguson and Watkins, 1989). However, some works have shown a lack of relationship between calcium content level and bitter pit, indicating that the disorder could be affected by other causes such as climate and/or growing conditions (Krawitzky et al., 2016; Lotze et al., 2008; Saure, 1996, 2005; Saure, 2014; Torres et al., 2017a).

Although the process that causes bitter pit usually starts during the period of fruit growth, the symptoms may not be evident in the orchard and generally appear during fruit storage or transport. This can result in extensive losses associated with labor and packing costs (Kafle et al., 2016; Val et al., 2010). Identifying fruit prone to bitter pit before export or shipment would help to reduce economic losses caused by market rejection (Nicolaï et al., 2006; Torres et al., 2015). Mineral analyses based on calcium levels within the fruit or approaches which force the appearance of symptoms before they naturally occur have been studied by different researchers to predict bitter pit (Ferguson et al., 1999; Kalcsits, 2016; Torres et al., 2017b; Torres et al., 2015). The drawbacks of these techniques include the fact they are destructive, which means the same fruit cannot be monitored over time, and a delay of between 5 and 14 days before obtaining the results.

Spectral reflectance in the visible and near-infrared regions (VIS/NIR spectroscopy technology) has several advantages (rapid measurement, repeatability, chemical-free and ability to measure multiple attributes simultaneously) over the methods described above which could help to overcome these difficulties. The VIS/NIR radiations penetrate the object, and changes in the spectral characteristics due to scattering and absorption depend on its chemical composition. These spectral changes provide information about microstructural properties of the object, including stiffness and internal damage.

Many studies have explored the possibility of using VIS/NIR spectroscopy for studying quality and disorders or for detecting defects in apples (Ariana et al., 2006; Mehl et al., 2002; Upchurch et al., 1990; Xing et al., 2006; Xing and De Baerdemaeker, 2007; Xing et al., 2003; Zhang et al., 2017). Kleynen et al. (2005) observed that the 750 and 800 nm bands offered good contrast for detecting internal tissue damage like hail damage, bruises, and so forth. This system was subsequently used by Unay and Gosselin (2007) and Unay et al. (2011) to detect surface defects and to classify apples in different categories.

As for bitter pit detection and/or prediction, very few studies have been published. Lotze (2005) made a classification between healthy and bitter pitted 'Braeburn' apples using fluorescence imaging with an accuracy of 75–100%. In another study, Nicolaï et al. (2006) used a line scan near-infrared camera with a spectrograph to capture spectral images and successfully identify visible and non-visible bitter pit lesions. Similarly, Ariana et al. (2006) presented an imaging model of reflectance and fluorescence to differentiate between pitted and non-pitted apples with accuracies of up to 87%. Recently, Kafle et al. (2016) and Jarolmasjed et al. (2017) distinguished bitter pitted apples by means of NIR spectrometry with an average accuracy in the range of 70–100%.

Most of the above-mentioned studies used laboratory equipment in the NIR region (780–2500 nm) and are of little practical use in orchards or packing houses. In recent years, as the result of rapid technological developments, handheld VIS/NIR spectrometers have been designed to specifically control and monitor the quality and maturity of different fruits, either in the orchard or in postharvest storage. These spectrometers are portable and compact, but usually offer measurements only in limited wavelength ranges (<1100 nm). ElMasry et al. (2008) developed a hyperspectral imaging system based on a spectral region between 400 and 1000 nm for early detection of bruises on apples. Packing lines for fruit and vegetable sorting are now being built with this technology incorporated to identify different quality parameters such as sugar content or dry matter.

The present study aims to evaluate the VIS/NIR spectroscopy technology for detecting and predicting bitter pit in apples. The specific goals of the study are: (1) to determine the capacity to classify different levels of bitter pit; (2) to determinate the capacity to classify healthy and bitter pitted apples, measuring close to pitted tissue, or on healthy tissue, or both; and (3) to determine the capacity to predict bitter pit prior to the appearance of symptoms. To our knowledge, no results related to these specific objectives have been published to date.

2. MATERIALS AND METHODS

2.1. Plant material and selection of samples

‘Golden Smoothee’ apples were harvested on September 10th, 2015, and September 13th, 2016, from a bitter-pit-prone orchard located at Gimenells (Lleida, NE Spain). The orchard was planted in 1994 and trees were grafted onto M9 rootstock. Plant and row spacing were 4 × 1.4 m spacing (1786 trees/ha). Fertigation was applied through drip irrigation. The soil was characterized as a calcareous loam with excellent drainage characteristics. Trees were managed according to the guidelines for apple integrated production, including the application of mineral fertilizers that were estimated to cover the nutrient requirements.

2.1.1. Sampling of apples with visible bitter pit symptoms (experiment #1)

In 2015, average-sized apples at commercial harvest were collected and placed in packaged fruit boxes and stored in cold storage at 0 °C for 4 months. Bitter pit was then evaluated using a category scale with 3 classes of bitter pit depending on the amount of pitted area: class 1 with no bitter pit symptoms; class 2 with slight symptoms (fruit having 1–6 pits on the surface); class 3 with moderate/severe symptoms (more than 7 pits per fruit). Two 276-apple samples were selected. Each sample comprised 100 apples of class 1, 100 apples of class 3, and 76 apples of class 2. Two VIS/NIR measurements were carried out on each fruit (see section *2.2. Data collection*).

2.1.2. Sampling of apples when bitter pit symptoms were not visible (experiment #2)

In 2016, healthy apples were collected at 20 days before harvest (at preharvest) and at commercial harvest (at harvest). One average-sized and undamaged apple was taken from different trees with standard crop loads and vigor at a height of 130-170 cm above the ground. The apples collected were subsequently placed on plastic fruit trays. Then, VIS/NIR measurements were carried out on each fruit (see section *2.2. Data collection*).

After the VIS/NIR measurements, the apples collected at preharvest were left at room temperature (approximately 25 °C) to allow the development of bitter pit symptoms according to the passive method (Torres et al., 2015). After 7 days, bitter pit was evaluated using a binary-class classification: class 1 and class 3 (apples classified as class 2 were discarded for this experiment). Two 40-apple samples were selected for bitter pit prediction at preharvest; each sample comprised 20 apples of class 1 and 20 apples of class 3.

The apples collected at commercial harvest were kept, after the VIS/NIR measurements, in cold storage at 0 °C for 4 months; after this, bitter pit was evaluated using the binary-class classification described above. Two 40-apple samples were selected for bitter pit prediction at harvest; each sample comprised 20 apples of class 1 and 20 apples of class 3. Two VIS/NIR measurements were carried out on each fruit (see section *2.2. Data collection*).

2.2. Data collection

The spectral absorbance data were collected under laboratory conditions using a UT-5001 portable handheld VIS/NIR spectrometer (UT instruments, Lugo, Ravenna, Italy) with measurement range of 650-950 nm. The spectral resolution of the spectrometer was 2 nm. Fruits were equilibrated at room temperature approximately half a day before spectral acquisitions. A blank scan was performed using Teflon® before starting the spectral measurements of each sample. The VIS/NIR measurements were carried out from two opposite sides along the equator of the fruit.

In experiment #1, the first measurement was carried out on the most affected side when symptoms were visible, but on healthy area close to symptoms (symptomatic side); the second measurement was carried out on healthy area from the opposite side (non-symptomatic side).

For each experiment, two datasets were obtained from the measurement of each sample. The two datasets were combined to develop a new set of data. Finally, the spectral absorbance data of each experiment were analyzed as three datasets.

2.3 Classification

2.3.1. Experiment #1: identification of bitter pitted apples

Each dataset was analyzed to evaluate the suitability of the spectral feature extraction in discriminating the identified class of bitter pit using a multiclass classification (classes 1, 2 and 3) or a binary-class classification (classes 1 and 3). Differences between classification accuracies using measurements from different fruit sides (symptomatic, non-symptomatic and both fruit sides) were also analyzed. Each dataset was separated into a balanced training and testing dataset. The ratio of each dataset was 7:3 for model development and validation, respectively. The spectral feature extractions were evaluated using three classification algorithms (LDA, QDA and SVM). The software used was The Unscrambler® (version 10.4; Camo Process AS, Oslo, Norway). The datasets were randomized three times each for evaluation of classifier performance.

2.3.2. Experiment #2: prediction of bitter-pit-prone apples

Each dataset was analyzed to evaluate the suitability of the spectral feature extraction in predicting the appearance of bitter pit—at preharvest and harvest—before the appearance of symptoms on the fruit surface. For this experiment, a binary classification was used (apples of class 1 and class 3). The VIS/NIR measurements were carried out from two opposite sides along the equator of the fruit. The spectral feature extractions were evaluated using three classification algorithms (LDA, QDA and SVM) for each case. Each dataset was separated into a balanced training and testing dataset as described above (training-testing ratio of 7:3).

2.4 Statistical analysis

Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., 2009). For experiment #1, a three-way ANOVA was performed with the GLM procedure to test the main effects on classification accuracies of 'algorithm' (LDA, QDA and SVM classifier models), 'bitter pit class' (1, 2, 3, overall or 1, 3, overall) and 'fruit side' (symptomatic, non-symptomatic and both fruit sides), and their interactions. For experiment #2, a two-way ANOVA was performed, for the pre- and harvest data, to test the main effects of ‘algorithm’ and ‘bitter pit class’ (1, 3, overall), and their interaction, on prediction accuracies. Duncan’s multiple range tests were used for the mean separation of significant effects from ANOVA models. Significance was declared at $P < 0.05$.

3. RESULTS

3.1. Experiment #1

3.1.1. Multiclass classification accuracies

No significant differences were found between multiclass classification accuracies from different measured fruit sides (symptomatic, non-asymptomatic and both fruit sides). Nevertheless, the three-way ANOVA indicated a significant ‘algorithm × class’ interaction effect on classification accuracies. Because of this significant interaction, algorithms were compared in each individual class (class 1, class 2 and class 3) and ‘overall’ (Table 1).

The average individual (class 1, 2 and 3) and overall multiclass classification accuracies, using the different classifier models (LDA, QDA and SVM), are shown in Figure 1. The average overall classification accuracy was significantly higher using the LDA classifier than the QDA and SVM classifiers (50% vs. 42%). No significant differences between algorithms were found for class 1 (mean accuracy of 51%). The three classifier models (LDA, QDA and SVM) showed the lowest accuracies in class 2; LDA yielded an average class 2 classification accuracy significantly higher than QDA and SVM, and

QDA obtained an accuracy significantly higher than SVM (37%, 22% and 1%, respectively). For class 3, the average classification accuracy was significantly higher for SVM (65%) than LDA and QDA; no significant differences were found between LDA and QDA (52%).

Table 1. *P*-values from three-way ANOVA of the effect of factors (algorithm, fruit side measured and class of classification, and their interactions) on multiclass (class 1, class 2 and class 3) and binary class (class 1 and class 3) classification accuracies, using VIS/NIR data when bitter pit symptoms were visible.

Factor	Multiclass classification accuracy	Binary class classification accuracy
Algorithm	<.0001	<.0001
Fruit side measured	NS	NS
Algorithm × fruit side	NS	NS
Classification	<.0001	NS
Algorithm × class classification	<.0001	<.0001
Fruit side × class classification	NS	NS
Algorithm × fruit side × classification	NS	NS

NS indicates the *P*-value > 0.05.

3.2. Binary classification

Binary classification showed significantly higher accuracies than multiclass classification, independently of the classifier model. No significant differences were found for the effect of different fruit sides (symptomatic, non-asymptomatic and both fruit sides) on binary classification accuracies (Table 1). As in the multiclass classification, the three-way ANOVA indicated a significant ‘algorithm × class’ interaction effect on classification accuracies. Because of this significant interaction, algorithms were compared in each individual class (healthy-class and bitter pit-class) and overall.

The average individual (class 1 and 3) and overall binary-class classification accuracies, using the different classifier models (LDA, QDA and SVM), are shown in Figure 2. As in the multiclass classification, the average overall classification accuracy was significantly higher using the LDA classifier than the QDA and SVM classifiers; QDA produced an overall classification accuracy significantly higher than SVM (75%, 65% and 57%, respectively).

The average class 1 classification accuracy was also significantly higher using LDA than QDA and SVM, and QDA showed a significantly higher accuracy than SVM (81%, 59% and 49%, respectively). The classification accuracy of class 3 using QDA was significantly higher than when using LDA or SVM (71% vs. 67%); no significant differences between LDA and SVM were observed. The accuracy average of class 1 was significantly higher than of class 3 using LDA (81% vs. 68%), whereas using QDA and SVM the accuracies were higher for class 3 than for class 1 (59% vs. 71% and 49% vs. 65%).

These results indicate that the LDA classifier yielded a higher number of false positives (healthy apples identified as bitter pitted apples), whereas the QDA and SVM classifiers yielded a higher number of false negatives (bitter pitted apples classified as healthy).

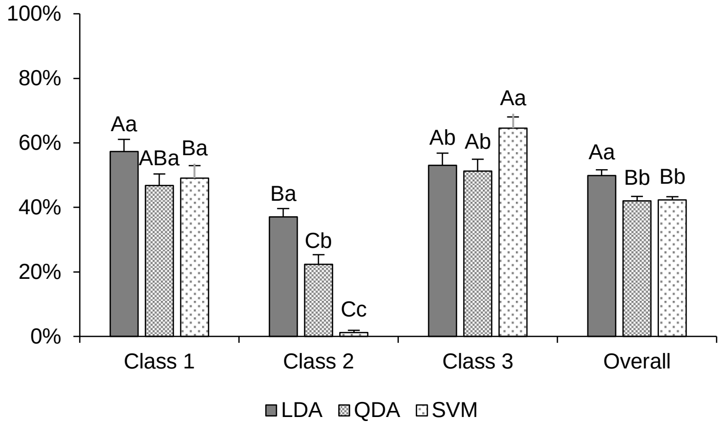

Fig. 1. Comparison of average LDA-, QDA- and SVM-based multiclass classification accuracies, for each bitter pit class classification (class 1, class 2, class 3 and overall), using VIS/NIR data when bitter pit symptoms were visible. For each class, different lowercase letters above the bars indicate significant differences between algorithms for a Duncan test (*P* = 0.05). For each algorithm, different capital letters indicate significant differences between classes for a Duncan test (P ≤ 0.05). Error bars indicate the standard error of the mean. Class 1: no bitter pit symptoms; class 2: slight/moderate symptoms (1–6 pits on the surface); class 3: severe symptoms (more than 7 pits per fruit).

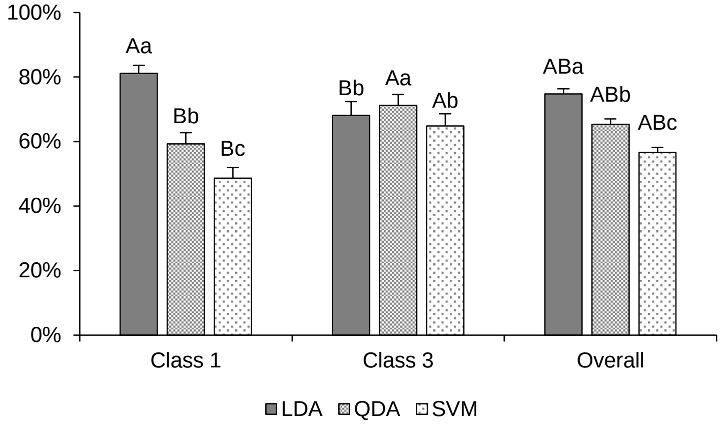

Fig. 2. Comparison of average LDA-, QDA- and SVM-based binary classification accuracies, for each bitter pit class classification (class 1, class 3 and overall), using VIS/NIR data when bitter pit symptoms were visible. For each class, different lowercase letters above the bars indicate significant differences between algorithms for a Duncan test (*P* = 0.05). For each algorithm, different capital letters indicate significant differences between classes for a Duncan test (P ≤ 0.05). Error bars indicate the standard error of the mean. Class 1: no bitter pit symptoms; class 3: severe symptoms (more than 7 pits per fruit).

3.3. Experiment #2

Within each prediction time (preharvest and harvest), no significant differences in classification accuracies were found between the different classifier models or the different classification classes (Table 2). The average individual (class 1 and 3) and overall classification accuracies, using the different classifier models (LDA, QDA and SVM), are shown in Figures 3 and 4, for both prediction times (at preharvest and at harvest, respectively). In both prediction times, the classification accuracies prior to the appearance of bitter pit were lower than when bitter pit was visible, independently of the classifier model. The average prediction accuracy was 45% at preharvest and 54% at harvest. These results indicate that VIS/NIR spectra were not capable of accurately detecting bitter pit prone fruit.

Table 2. *P*-values from two-way ANOVA of the effect of factors 'algorithm' and 'classification class' (binary class classification), and their interaction, on accuracies of bitter pit prediction using VIS/NIR data when bitter pit symptoms were not visible. '$\text{NIR}_{\text{preharvest}}$ vs. Bitter $\text{pit}_{\text{preharvest}}$': NIR measured at 20 days before harvest on healthy apples and bitter pit symptoms assessed after 7-10 days at room temperature (22-25 ºC). '$\text{NIR}_{\text{harvest}}$ vs. Bitter $\text{pit}_{\text{postharvest}}$': NIR measured at harvest on healthy apples and bitter pit symptoms assessed after 4 months in cold storage (0 ºC).

Factor	$\text{NIR}_{\text{preharvest}}$ vs. Bitter $\text{pit}_{\text{preharvest}}$	$\text{NIR}_{\text{harvest}}$ vs. Bitter $\text{pit}_{\text{postharvest}}$
Algorithm	NS	NS
Classification	NS	NS
Algorithm × Classification	NS	NS

NS indicates the *P*-value > 0.05.

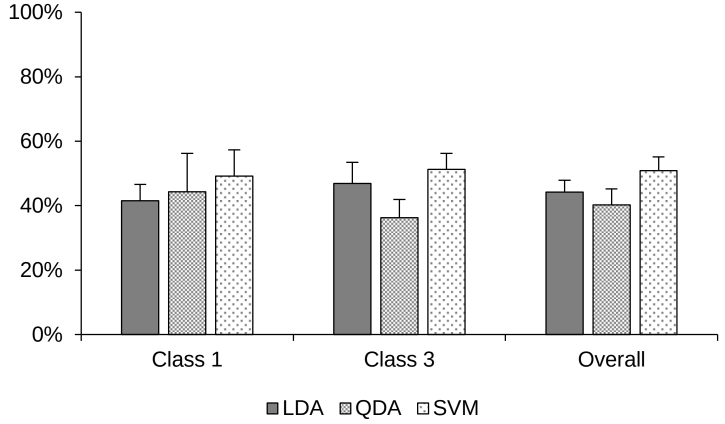

Fig. 3. Comparison of average LDA-, QDA- and SVM-based binary classification accuracies, for each bitter pit class classification (class 1, class 3 and overall), using VIS/NIR data on healthy apples at 20 days before harvest (bitter pit symptoms assessed on the same fruits after 7-10 days at room temperature). Error bars indicate the standard error of the mean. Class 1: no bitter pit symptoms; class 3: severe symptoms (more than 7 pits per fruit).

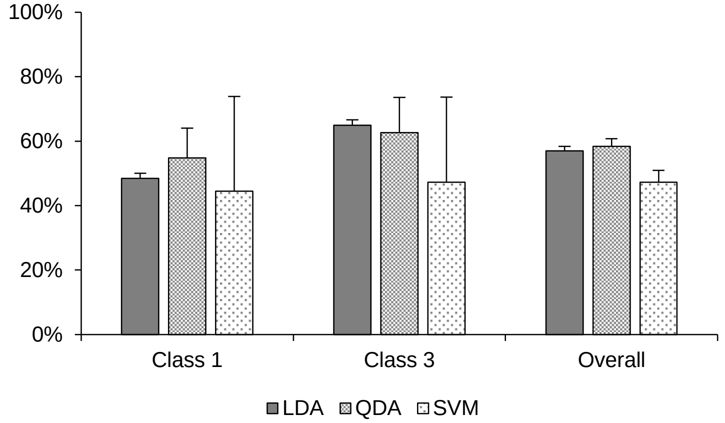

Fig. 4. Comparison of average LDA-, QDA- and SVM-based bitter pit prediction accuracies, for each bitter pit class classification (class 1, class 3 and overall), using VIS/NIR data on healthy apples at comercial harvest (bitter pit symptoms assessed on the same fruits after 4 months in cold storage at 0°C). Error bars indicate the standard error of the mean. Class 1: no bitter pit symptoms; class 3: severe symptoms (more than 7 pits per fruit).

4. DISCUSSION

A visual inspection of the absorbance curves indicates that the greatest differences between pitted and non-pitted apples were in the VIS region of 650-700 nm and in the NIR region of 900-950 nm (Fig. 5). Absorption valleys around 500 and 680 nm represent pigment such as carotenoids and chlorophyll which represent the color characteristics in the fruit (Abbott et al., 2010). ElMasry et al. (2008) found that the wavelengths in the NIR at 750, 820, and 960 nm could potentially be implemented in multispectral imaging systems for detection of bruises on apples; they defined the absorption valleys in the NIR at 840 and 960 nm as sugar and water absorption bands. In bruised areas, water replaces the intercellular air spaces in the plant tissue and, consequently, could cause a decrease in NIR-reflectance of these areas (Lotze, 2005).

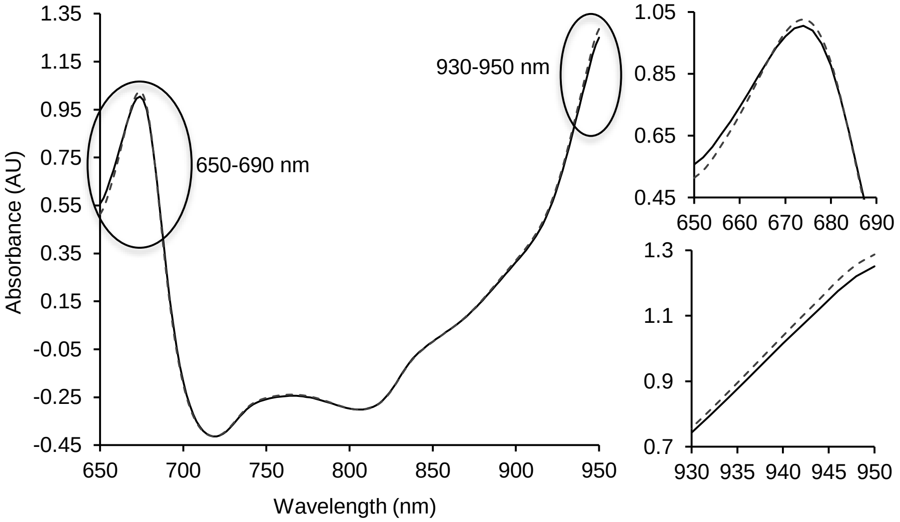

Fig. 5. Average VIS/NIR spectra of healthy (—) and bitter pitted (---) apples.

Multiclass classification overall accuracies were 42-50%. Class 2 (slight symptoms, with fruit having 1–6 pits on the surface) showed accuracies significantly lower than for the other two classes and overall accuracies (1-37%). This lack of accuracy could be attributed to the low degree to which the apples were affected. In order to reduce this error, we rejected class 2 apples for the binary-class classification. The binary-class classification resulted in accuracies significantly higher than for multiclass classification (overall accuracies of 57-75%). However, further studies need to be performed to analyze the effect of including slightly affected apples.

The overall accuracies of the binary-class classification were 75-81% using the LDA classifier model. These results are similar to those obtained by other researchers for discriminating between healthy and bitter pitted apples using a binary-class classification (Jarolmasjed et al., 2017; Kafle et al., 2016; Nicolaï et al., 2006). The LDA classifier accuracies were significantly higher than those of the QDA and SVM classifiers, and QDA was found to yield higher accuracies than SVM. Kafle et al. (2016) also reported that QDA had a higher classification accuracy than SVM of healthy and bitter pitted apples, with accuracy values similar to those obtained in the present study.

The accuracy level in each class depended on the classifier model used. QDA and SVM showed higher accuracies for class 3 (bitter pitted apples), whereas classification accuracies of LDA were significantly higher for class 1 (asymptomatic apples). This indicates that LDA classifiers yielded a higher number of false positives (healthy apples identified as bitter pitted apples), whereas QDA and SVM classifiers yielded a higher number of false negatives (bitter pitted apples classified as healthy). These results contrast with those obtained in a similar experiment with Honeycrisp apples performed by Kafle et al. (2016), who obtained a significant increase for healthy-apple class accuracies compared to bitter-pitted-apple class using QDA and SVM algorithms. Jarolmasjed et al. (2017) obtained a higher number of false negatives than false positives using partial least square regression. Further studies with larger datasets need to be performed to validate these aspects because algorithm performance arguably depends on several factors which might be responsible for the different results (Kafle et al., 2016; Sankaran and Ehsani, 2011).

It was observed that classification accuracies did not change significantly when measuring different fruit sides (symptomatic, asymptomatic or both). VIS/NIR spectroscopy point meter readings measure a limited area (10 mm diameter circle) per measurement. This aspect may limit the application of NIR technology to detect affected apples due to the inability to take measurements on affected areas (Lotze, 2005). However, the signature of organic and complex compounds of major chemical contents associated with bitter pit (calcium, magnesium, and potassium) might exist in spectral data obtainable by VIS/NIR spectroscopy (Jarolmasjed et al., 2017). Hence, it would be possible to detect—even prior to the appearance of visible symptoms—bitter pitted apples based on the chemical contents of the fruits, independently of whether the measurement is made on the bitter pit lesion. Our results indicate that the VIS/NIR-spectroscopy method offers potential for non-destructive discrimination of bitter pitted from healthy apples, independently of measured fruit area. This would facilitate the development of a model for implementation in an automatic apple sorting system. Over the last few years, VIS/NIR technology has been implemented in automatic fruit and vegetable sorting machines to determine different quality parameters and, according to the results of this research, could also be used to discriminate between bitter pitted and healthy apples.

VIS/NIR spectroscopy readings were however unable to identify immature (preharvest) or mature apples (harvest) more prone to bitter pit development prior to the appearance of visible symptoms. Lotze (2005) proposed that the inability of VIS/NIR spectroscopy point meter readings to identify bitter pit prone fruit could be due to not being able to take measurements on the affected areas. However, we did not observe this limitation in experiment #1 when the bitter pit symptoms were visible. The identification of apples with visible bitter pit through VIS/NIR spectroscopy may be due to chlorophyll degradation or changes in intercellular water in fruit tissue (Lotze, 2005) and, from our point of view, these changes would not have developed before the appearance of symptoms. We suggest that bitter pit prone apples could be detected through the relationship of mineral content associated with bitter pit (calcium, magnesium, and potassium) but, for these cases, a higher wavelength range will be necessary. Jarolmasjed et al. (2017) obtained a strong relationship between the spectral features and the magnesium-to-calcium ratio in three different apple varieties using a wavelength range of 935-2500 nm. Along this line, a future work using a higher wavelength range (>900 nm) will investigate a possible relationship between NIR spectral features and calcium, potassium and magnesium content at an early stage of fruit growth to predict bitter pit potential.

5. CONCLUSIONS

The findings support the possibility of using VIS/NIR spectral features to detect bitter pit when symptoms are well developed. Overall, the results indicate that the LDA classifier performed better than the multivariate non-linear classifiers QDA and SVM in discriminating between healthy and bitter pitted apples (75%, 65% and 57%, respectively). No significant differences in accuracies were observed when taking 1 or 2 measurements on the fruits. Hence, these outcomes confirm the applicability of using NIR reflectance spectra for the detection of bitter pit in apples with automatic apple sorting systems. Although VIS/NIR spectroscopy showed great potential in pitted apples detection, it could not identify apples more prone to bitter pit development prior to the appearance of visible symptoms. Further studies using NIR spectra with a higher wavelength range are necessary to explore the applicability of this technology in predicting bitter pit.

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CAPÍTULO 5

Combination of strategies to supply calcium and reduce bitter pit in ‘Golden Delicious’ apples

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ABSTRACT

Calcium (Ca) sprays and Ca applications to soil throughout the growing season or Ca solution dips at post-harvest are widespread practices to supply Ca and decrease bitter pit in apples. However, published results conflict, and there is no information about the effectiveness of combining all these treatments. In the present study, the following treatments were assessed during four growing seasons: early-season (April) Ca soil applications applied 4 times, mid-season (May) $CaCl_2$ sprays applied 7 or 13 times, late-season (June) $CaCl_2$ sprays applied 7 times, and the combination of late-season sprays and soil applications. In addition, post-harvest dips were evaluated in the latter two growing seasons. Notably high bitter pit incidences were monitored for the first and fourth year of study (>20%), while the second and third year were almost without incidence. Post-harvest dips mitigated bitter pit incidence to a greater extent than pre-harvest treatments, and the sprays mitigated bitter pit to a greater extent than Ca soil applications. The combination of sprays and soil applications did not improve the results relative to Ca sprays alone. No detectable advantage for starting spray programmes earlier than June was observed. Our results showed a trend towards reduced bitter pit with an increasing number of $CaCl_2$ sprays, but this was not clearly an effect of maximizing fruit Ca. Finally, applying 13 $CaCl_2$ sprays in combination with a Ca solution dip at post-harvest appeared to be the most effective practice for minimizing the risk of bitter pit development.

1. INTRODUCTION

Calcium (Ca) deficiency has been implicated in several disorders fruits, such as bitter pit in apples (*Malus domestica* L. Borkh) (Casero et al., 2010; Ferguson et al., 1999; Lotze and Theron, 2007; Peryea et al., 2007). Although bitter pit develops during post-harvest storage, the underlying process stems from the period of fruit growth and development (Val et al., 2010). Ca supplied during the growing season through spray applications is currently the most common method of reducing bitter pit and/or increasing the Ca content of fruit. However, its effectiveness is irregular and can depend on the number of applications, salt type (nitrate, chloride or others) and period in which it is supplied (Val et al., 2008; Wojcik and Borowik, 2013; Wooldridge et al., 1998). The general consensus is that no Ca-based product has consistently shown greater efficacy in reducing bitter pit than $CaCl_2$ (Schönherr, 2001). Nevertheless, there is no general agreement on when is the best time to apply Ca sprays or on the efficacy of Ca supplied to the roots. Some studies have found that the best time for Ca sprays is immediately after full bloom, when fruits are small. Early applications may be advantageous because a less developed cuticle favours the penetration of Ca into the fruit (Neilsen et al., 2005; Wilsdorf et al., 2012). However, in a great number of studies, Ca sprays have been most effective in the second half of the fruit growth period (Benavides et al., 2001; Casero et al., 2010; Lotze et al., 2008; Peryea et al., 2007; Wilsdorf et al., 2012). According to Casero et al. (2002), starting Ca sprays at 10 days after full bloom (DAFB) did not increase Ca accumulation in apples, while starting at 70 DAFB increased the Ca absorption rate and accumulation in fruit. This effect is thought to occur because Ca is provided mainly by root absorption during the first period of fruit growth, whereas during the second period of growth, when fruit Ca absorption is reduced, Ca sprays may be more effective (Casero et al., 2002).

In this context, Ca root applications during the first period of fruit growth and Ca sprays during the second half of fruit growth appear to be the most effective management strategy for maximizing fruit Ca and minimizing the risk of bitter pit development. Nevertheless, there are no published reports about the effectiveness of Ca root applications in controlling bitter pit and very few reports about the effectiveness of combining Ca root applications with Ca sprays to increase the Ca concentration in fruit. Wilsdorf et al. (2012) evaluated the contributions of Ca root applications and sprays to increases in the Ca content of fruit. Ca sprays were more effective than soil Ca applications in increasing Ca in fruit, but the treatments could not be evaluated with respect to bitter pit because there was no incidence of the disorder. Although soil Ca application is a common practice and many studies have evaluated its effects on Ca content in fruits, there are no researches that specifically relate this practice to bitter pit reduction.

Another technique to control bitter pit in apples is to dip or drench fruit in Ca solutions post-harvest. This technique can also increase the Ca content of fruit and has shown effectiveness both in decreasing bitter pit and improving fruit quality (Manganaris et al., 2007). Guerra and Casquero (2010) observed a decrease in bitter pit of 16.7% with a post-harvest Ca treatment in the 'Reinette de Canada' cultivar.

Most of the published works about Ca fertilization have separately evaluated these approaches (soil application, sprays and dips) and have obtained insufficient results. The combination of these three practices might improve the efficacy of bitter pit control, but, as far as we know, there are no up-to-date published studies in which pre-harvest Ca application to soil, sprays and post-harvest dips have been combined, a strategy that may be the most effective practice for controlling bitter pit. Taking all these techniques into account, we investigated the effects of combining Ca applications pre-harvest (using soil and/or sprays) and Ca dips post-harvest on 'Golden Delicious', a cultivar that is susceptible to bitter pit (Moscetti et al., 2013). This is the first study combining these approaches to supply Ca to apples during several consecutive growing seasons.

2. MATERIALS AND METHODS

2.1. Plant material and crop technology

The trial was carried out in an apple tree (*Malus domestica* L. Borkh.) orchard of the IRTA Experimental Station of Lleida (Mollerussa, NE Spain) over four consecutive growing seasons (from 2007 to 2010). Mature, uniform, 'Golden Delicious' trees grafted onto 'M9' rootstock and planted in 1994 at 4 × 1.4 m spacing (1786 trees/ha) were selected from a bitter pit-prone orchard. Fertigation was applied through drip irrigation. The interrows were grassed down, but herbicide strips were kept along the rows. The soil was characterized as a calcareous loam with excellent drainage characteristics. During the four-year study, trees were managed according to the guidelines for apple integrated production, including the application of mineral fertilizers that were estimated to cover the nutrient requirements.

2.2. Treatments

2.2.1. Pre-harvest treatments

Ca root applications were applied during the whole vegetative stage, whereas Ca sprays were applied during the mid and/or late fruit growing stages according to each treatment. A total of 6 pre-harvest treatments were assessed: i) control with no calcium application; ii) early-season root applications (R_{Early}) consisted of four calcium applications to the soil through drip irrigation and were performed at full bloom, fruit set, the cell multiplication phase and the beginning of maturation (CaO total: 6.0 kg ha^{-1} per season); iii) Ca sprays from mid-season ($F7_{Mid}$) consisted of 7 Ca sprays every 12–14 days starting 30 days after full bloom (DAFB) (CaO total: 5.9 kg ha^{-1} per season); iv) Ca sprays during late season ($F7_{Late}$) consisted of 7 Ca sprays every 5–10 days starting 60 DAFB (CaO total: 5.9 kg ha^{-1} per season); v) Ca root applications and sprays (R_{Early} $F7_{Late}$) consisted of a combination of both R_{Early} and $F7_{Late}$ treatments (CaO total: 11.9 kg ha^{-1} per season); vi) additional mid stage Ca sprays ($F13_{Mid}$) consisted of 13 Ca sprays every 5–10 days starting 30 DAFB (CaO total: 11.0 kg ha^{-1} per season).

For the Ca root applications, a commercial $CaCl_2$ solution of 15% water-soluble CaO (Timac Agro, Spain) was used at a rate of 10 L ha^{-1} per application. For the Ca sprays, a commercial $CaCl_2$ solution of

16.9% water-soluble CaO (Yara Iberian SA, Spain) was used at 5 L ha^{-1} per application. Sprays were applied very early in the morning, when air temperatures were below 25 °C, to minimize phytotoxicity, which could cause visible necrosis or marks on fruits or leaves. A high-pressure handgun sprayer (25 atm) was used at a rate of 1000 L ha^{-1}.

2.2.2. Post-harvest treatment

In each of the four growing seasons, in each elemental plot, 100 fruits of uniform size (70–75 mm in diameter) and without any disorder were picked at commercial harvest and placed in cold storage at 0 °C for 90 days. In addition, within 24 h of harvest in 2009 and 2010, another 100 fruits per elemental plot were dipped into a solution with $CaCl_2$ (15%) for 30 s and received a dose of 3.5 L of $CaCl_2$ per 100 L of water. Immediately after, the dipped fruits were placed in cold storage at 0 ° C. After 90 days in cold storage, all samples were transferred to room temperature (20–23 °C) for 7 days, during which time the samples were evaluated.

2.3. Bitter pit assessment

Bitter pit was evaluated using a category scale with 4 classes of bitter pit depending on the amount of pitted area (Torres et al., 2015): class 0 demonstrated no bitter pit symptoms; class 1 demonstrated slight symptoms, with fruit having 1–6 pits on the surface; class 2 demonstrated moderate symptoms, with less than one-third of the surface of the fruit affected (approximately 7–15 pits per fruit); and class 3 demonstrated severe symptoms, with more than one-third of the surface of the fruit affected (>15 pits per fruit). Bitter pit incidence was calculated as the percentage of apples with at least one pit. In addition, a relative index of severity (S) was calculated for each sample using the formula $S = \sum_{n=1}^{N} \frac{I_n}{3 \times N}$, where *S* is the relative index of bitter pit severity (from 0 to 1), I_n is the severity class of each apple, *N* is the total number of apples assessed, and 3 is the maximum level of severity.

2.4. Fruit calcium content

In 2008, 2009 and 2010, 20 apples per elemental plot were collected at harvest for mineral analyses. The samples were taken from spurs on 2-year-old shoots. The apples were then carefully washed, and two longitudinal slices were cut from opposite sides of each fruit, excluding the cores and seeds. The complete sample from each elemental plot was weighed, dried, and then re-weighed to determine the percentage of dry mass. The dried tissue of each sample was wet digested with concentrated nitric acid (HNO_3) and hydrogen peroxide ($H_2 O_2$) in a microwave oven (Millestone MCR). The Ca content was then determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

2.5. Fruit yield parameters

Every season, all apples from each tree were separately harvested and weighed at commercial harvest by automatic fruit sorting equipment. The harvest and sampling were carried out when the starch index of the fruit was 6–7 (starch chart EC-Eurofru 1–10). Finally, for each treatment and season, fruit yield (kg tree^{-1}), fruit load (number of fruits tree^{-1}) and fruit weight (g fruit^{-1}) were calculated.

2.6. Fruit quality parameters

In 2008, 2009 and 2010, 20 apples from each elemental plot, of uniform size and without bitter pit symptoms, were taken for fruit quality assessments at harvest. The parameters measured were fruit firmness, starch index, soluble solid content and titratable acidity. Firmness was measured at two opposite sides on the fruit equator using a digital firmness tester (Penefel®; Ctifl, France). The starch index was visually measured using the EC-Eurofru scale (1–10). Soluble solid content (°brix) and titratable acidity (malic acid g L^{-1}) were determined using the freshly prepared juice of the whole subsample. Soluble solid content was measured using a digital temperature compensated refractometer (model PR-101, Atago Co. Tokyo Japan), and titratable acidity (expressed as malic acid) was determined by titrating 10 mL of juice with 1.0 M NaOH to pH 8.2. In the 2009 and 2010 seasons, fruit firmness at post-harvest was also measured.

2.7. Experimental design and statistical analysis

The experimental design of the trial was a randomized complete block with 4 blocks and 6 elemental plots per block. Each elemental plot consisted of 3 rows of 6 trees, and the measurements were carried out on the two central trees in the central row of each elemental plot.

Analyses were performed in SAS 9.2 (SAS Institute Inc., 2009). A two-way ANOVA was performed with the GLM procedure to test main effects of season and pre-harvest treatments, and their interaction, on bitter pit and the parameters analysed at harvest.

Duncan's multiple range tests were used for the mean separation of significant effects and, if pre-harvest treatment effect from ANOVA models were significant ($P < 0.1$), single degree of freedom and polynomial contrast were performed to compare specifics groups of pre-harvest treatments. Besides, a three-way ANOVA was performed with the GLM procedure to test main effects of season, pre-harvest and post-harvest treatments, and their interaction, on bitter pit and the fruit firmness analysed at post-harvest.

3. RESULTS AND DISCUSSION

3.1. Effect of pre-harvest treatments

3.1.1. Effect on bitter pit

The two-way ANOVA indicated that bitter pit was strongly related to the season of growth (Table 1). The incidence of bitter pit was higher than 20% in two seasons (2007 and 2010) and almost inappreciable in other two seasons (2008 and 2009) (Fig. 1). The pre-harvest treatments also showed a significant effect on bitter pit incidence and severity. The interaction season × pre-harvest treatment was non-significant.

Table 1. *P*-values from two-way ANOVA of factors season and pre-harvest Ca applications, and its interaction, on bitter pit incidence and severity and on the parameters analysed at harvest (Ca concentration in fruit, number of fruit per tree, kg tree^{-1}, fruit weight, starch index, acidity sugar content and fruit firmness).

Factor	Season	Pre-harvest treatment	Season × Pre-harvest treat.
Bitter pit incidence	<.0001	0.0651	NS
Bitter pit severity	<.0001	0.0156	NS
Ca concentration	<.0001	0.0624	NS
No. fruits per tree	<.0001	NS	NS
Fruit yield	<.0001	NS	NS
Fruit weight	<.0001	NS	NS
Starch index	<.0001	NS	NS
Acidity	<.0001	NS	NS
Sugar content	<.0001	NS	NS
Firmness harvest	<.0001	NS	NS
Firmness post-harvest	<.0001	NS	NS

NS indicates *P*-value was greater than 0.1.

A contrast confirmed a significant difference between the control and the pre-harvest treatments. In general, the sprays decreased bitter pit to a greater extent than root applications (Fig. 1). The root applications alone (R_{Early}) did not show a significant decrease of bitter pit with respect to the control treatment. A contrast confirmed significant differences between the R_{Early} treatment vs. the sprays treatments ($F7_{Mid}$, $F7_{Late}$ and $F13_{Mid}$). The combination of root applications and sprays ($R_{Early}F7_{Late}$) did not bring any significant improvement with respect to the rest of treatments (Table 2). Considering all these findings, it appears not to be detectable advantage on bitter pit control as a result of applying Ca through fertigation.

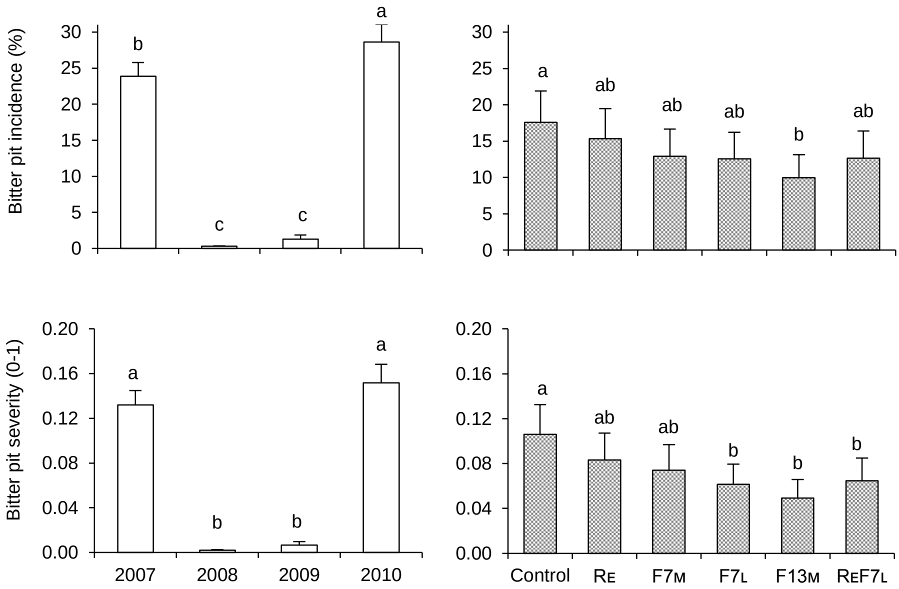

Fig. 1. Mean value of bitter pit incidence (left) and relative index of severity (right) for each season (above) and pre-harvest treatment (below). Columns not sharing the same letter indicate significant differences according to the Duncan test (*P* = 0.05). Error bars indicate the standard error of the mean. R_E: early-season root applications; $F7_M$: 7 Ca sprays from mid-season (every 12–14 days starting 30 DAFB); $F7_L$: 7 Ca sprays during late season (every 5–10 days starting 60 DAFB); $F13_M$: 13 Ca sprays from mid-season (every 5–10 days starting 30 DAFB); R_E $F7_L$: combination of both R_E and $F7_L$ treatments.

Table 2. *P*-values of the contrasts to compare specific groups from pre-harvest treatments on parameters bitter pit incidence, bitter pit severity and Ca concentration in fruit (parameters with significant effect according the two-away ANOVA).

Contrasts	Bitter pit incidence	Bitter pit severity	Ca content in fruit
Ctrl vs. Ca-treat. (R_E, $F7_M$, $F7_L$, $F13_M$ and R_EF7_L)	0.0138	0.0022	0.0097
Root (R_E) vs. sprays ($F7_M$, $F7_L$ and $F13_M$)	0.0909	0.1068	0.0977
Mid ($F7_M$) vs. late-season ($F7_L$)	NS	NS	NS
13 ($F13_M$) vs. 7 Ca sprays ($F7_M$ and $F7_L$)	NS	NS	NS
Combination (R_EF7_L) vs. separately (R_E and $F7_L$)	NS	NS	NS

NS indicates the *P*-value was greater than 0.1.

No significant differences were observed between applying 7 and 13 sprays, although the $F13_{Mid}$ treatment showed a trend towards a lower incidence of bitter pit in comparison to the other treatments (Fig. 1). These results are in agreement with those obtained by Casero et al. (2010), who did not observe a significant decrease in bitter pit by increasing the number of applications of $CaCl_2$ from 6 to 12 sprays using similar doses to those used in the present trial. Lotze et al. (2008) did not obtained either a significant improvement by increasing from 6 to 8 the number of sprays. Significant differences were neither observed between starting the sprays at mid-season and at late-season (Table 2). Casero et al. (2002), Peryea et al. (2007), Lotze et al. (2008), and Lotze and Theron (2007) did not observed either any improvement by beginning sprays before June. Instead, we observed a trend towards a lower incidence of bitter pit when the applications were made later in the season. In line with this, only the $F7_{Late}$, $R_{Early}F7_{Late}$ and $F13_{Mid}$ treatments significantly decreased bitter pit severity in comparison to the control treatment (Fig. 1). Thus, our results suggest that increasing the number of sprays from June until harvest could more effectively minimize the risk of bitter pit than starting spray programmes earlier. However, not only the number or moment of applications can be important but also the concentration of $CaCl_2$ in solution. Yuri et al. (2002) achieved significant efficacy in controlling bitter pit by carrying out only 6 $CaCl_2$ sprays (an 81% reduction with respect to the control treatment) but with a higher total amount of CaO per hectare than the amount used in the present study (18 vs. 11 kg CaO ha^{-1}).

3.1.2. Fruit calcium concentration

The Ca content of fruit was significantly different among growing seasons (Table 1). The highest Ca content was recorded in 2008 (5.1 mg 100 g^{-1} FW) when it was not observed any incidence of bitter pit (Fig. 2). On the other hand, no differences in the Ca content were observed between 2009 and 2010 (3.9 mg 100 g^{-1} FW) although the bitter pit incidence was significantly different between these seasons (1% vs. 19%).

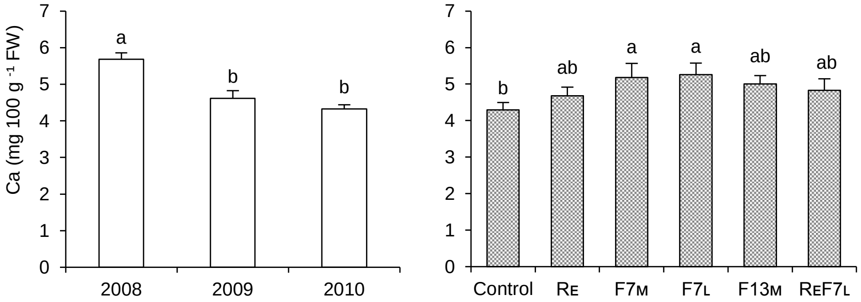

Fig. 2. Mean value of calcium (Ca) content in fruit for each season (left) and pre-harvest treatment (right). Columns not sharing the same letter indicate significant differences according to the Duncan test ($P = 0.05$). Error bars indicate the standard error of the mean. R_E: early-season root applications; $F7_M$: 7 Ca sprays from mid-season (every 12–14 days starting 30 DAFB); $F7_L$: 7 Ca sprays during late season (every 5–10 days starting 60 DAFB); $F13_M$: 13 Ca sprays from mid-season (every 5–10 days starting 30 DAFB); R_E $F7_L$: combination of both R_E and $F7_L$ treatments.

There was no clear relationship between Ca content and bitter pit incidence. Generally, a threshold of 5–6 mg Ca 100 g $^{-1}$ FW in apples at harvest is required to minimize bitter pit, but there are many papers reporting the failure of predictions based on this level of Ca content (Lotze et al., 2008) which is in line with our results. To explain this lack of relationship, some researchers have suggested that bitter pit is dependent on abnormal Ca partitioning and distribution in the cell (Pesis et al., 2010). According to Pavicic et al. (2004), the water-soluble Ca fraction is a more significant factor than the water-insoluble Ca fraction or the sum of these fractions. Freitas et al. (2010) reported that the development of bitter pit is a complex process that involves not only the total input of Ca into the fruit but also Ca homeostasis at the cellular level, Ca accumulation into storage organelles and Ca binding to the cell wall. Some researchers have achieved better results when analysing skin instead of tissue from fleshy fruit (Amarante et al., 2013; Peryea et al., 2007; Val et al., 2008). Saure (2014) reported that stress was the main cause of 'Ca disorders' in different fruits and vegetables. Stress increases the production of reactive oxygen species (ROS), which causes lipid peroxidation and an increase in the leakiness of membranes, leading to the rapid vacuolation of parenchyma cells and the loss of ions, such as apoplastic Ca. Therefore, in the context of bitter pit development, the final deficiency of Ca might be considered a result rather than a cause. Ca applications could increase antioxidant capacity, including the total content of phenols and ascorbic acid. This effect would explain the efficacy of treatments in the reduction of bitter pit symptoms. Based on our results, a low Ca concentration in fruit (< 4 mg 100 g^{-1} FW) is not enough to develop bitter pit. Considering the differences observed between seasons in the present study, it is important for future research to expand the knowledge on the relationship between weather conditions and bitter pit to gain a better understanding of bitter pit triggers.

All Ca treatments led to a significant average increase of 14% in the Ca content of fruit compared to the control treatment. No interaction effect was found between season and pre-harvest treatments (Table 1). Increasing fruit Ca content was more successfully achieved with Ca sprays than with root applications. Only the increase in the $F7_{Late}$ and $F7_{Early}$ treatments was significant in comparison to the control treatment (Fig. 2). Advancing the start date of sprays, increasing the number of sprays or combining root applications and sprays did not improve the fruit Ca content (Table 2). Wilsdorf et al. (2012) also observed a greater effect of sprays in increasing Ca in comparison with root applications. Some research has found that the Ca reserves from the permanent structure of the trees make an important contribution to new growth during the period of rapid shoot extension (Himelrick and McDuffie, 1983; Wilsdorf et al., 2012). Along these lines, Wilsdorf et al. (2012) observed that root applications in the previous season, after harvest, were more efficient than root applications during the growing season. We should note that our study was carried out on calcareous soil, and thus, there were high levels of Ca in the soil. However, in most of the growing season, the Ca concentration in fruit is lower than the minimum Ca requirement to avoid the risk of bitter pit (5–6 mg 100 g^{-1} FW) (Johnson et al., 1987; von Bennewitz et al., 2015). Leaves do not experience such a low Ca concentration, possibly because water and mineral moves from roots to leaves via xylem through transpiration and the leaves have a higher transpiration rate than the fruits. Therefore, under the conditions of our study (calcareous soil and/or high temperatures), fruit sprays appeared to be the most effective practice for maximizing fruit Ca.

3.1.3. Fruit yield parameters

The pre-harvest Ca treatment did not affect fruit yield parameters. Contrariwise, there was a significant effect of growing season (Table 1). The 2007 and 2010 seasons, which had the lowest fruit yield, also had the highest incidences of bitter pit. Therefore, the fruit yield may affect bitter pit incidence, but we did not observe a statistically significant relationship between fruit yield and bitter pit incidence (data not shown). In general, heavy crop load relates toes a lower incidence of bitter pit (Ferguson and Triggs, 1990). A possible explanation for the higher bitter pit incidence observed in trees with light crops is that such trees have a higher proportion of large fruits (Schumacher et al., 1980). Large fruits generally have lower Ca concentrations and a higher risk of bitter pit than small fruits (Peryea et al., 2007). Nevertheless, our results showed higher Ca concentration in the season with the largest fruits and a higher incidence of bitter pit in the seasons when fruit were smaller. Generally, trees with lower crop load provide larger fruits (Corollaro et al., 2015; Delong et al., 2006), but this did not occur in 2007 and 2010 (Fig. 3). In both growing seasons, there were cloudier days and days with cooler temperatures during the bloom period. These conditions may have resulted in poor pollination and, consequently, a decrease in crop load and poor quality of set fruit, as poor pollination leads to the development of fewer seeds (de Freitas et al., 2015). Bramlage et al. (1990) and Buccheri and Di Vaio (Buccheri and Di Vaio, 2004) showed a negative relationship between the number of seeds and the incidence of bitter pit; unfortunately, we did not count the number of seeds for this purpose.

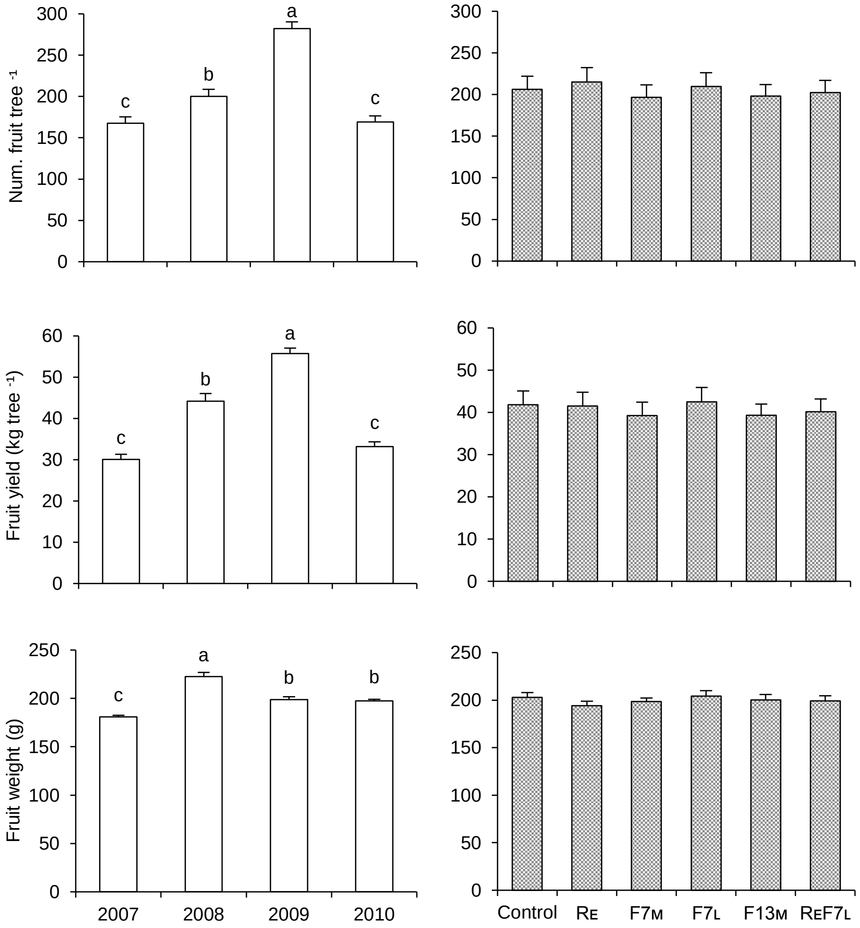

Fig. 3. Mean value of number of fruit per tree (above), fruit yield (center) and fruit weight (below), for each season (left) and pre-harvest treatment (right). Columns not sharing the same letter indicate significant differences according to the Duncan test ($P = 0.05$). Error bars indicate the standard error of the mean. R_E: early-season root applications; $F7_M$: 7 Ca sprays from mid-season (every 12-14 days starting 30 DAFB); $F7_L$: 7 Ca sprays during late season (every 5-10 days starting 60 DAFB); $F13_M$: 13 Ca sprays from mid-season (every 5-10 days starting 30 DAFB); R_EF7_L: combination of both R_E and $F7_L$ treatments.

3.1.4. Fruit quality parameters

All tested fruit quality parameters were significantly different among growing seasons. The starch index was significantly higher in 2008 and 2010 than in 2009 (Fig. 4). Most reports have shown that, in general, bitter pit is more severe in early-picked than in later-picked apple fruit (Delong et al., 2006; Ferguson et al., 1999). However, we found no consistent effect of fruit maturity on the incidence of bitter pit. The sugar content was significantly lower in 2009 than in 2008 or 2010 as expected, according to the results of starch index. Generally, sugar content in fruits tends to increase with maturation due to the hydrolysis of starch. The highest content of malic acid content was observed in 2008, whereas the lowest content was observed in 2010. In 2010, fruit firmness was significantly higher than in 2008 and 2009 and no consistent relation could be established between fruit firmness and starch index (Fig. 5).

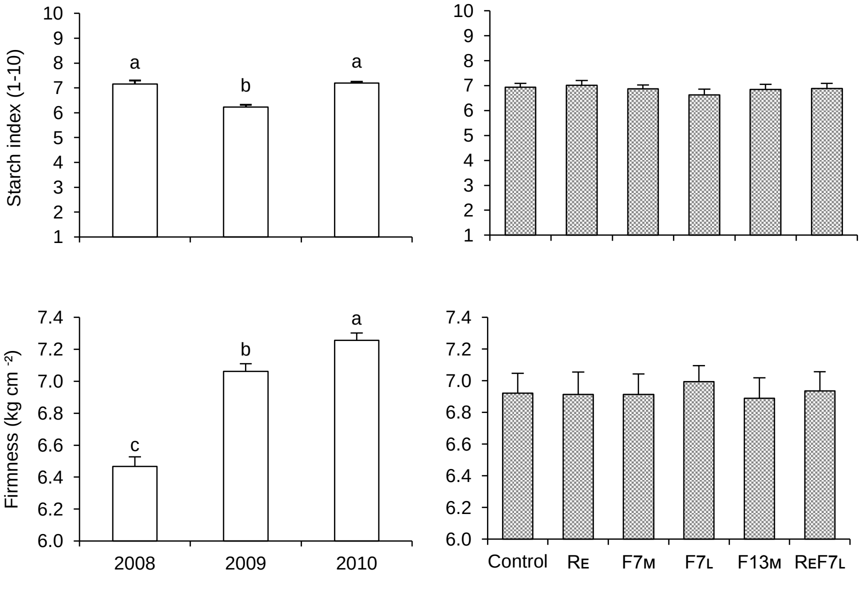

Fig. 4. Mean value of number of starch index (above) and fruit firmness (below), for each season (left) and pre-harvest treatment (right). Columns not sharing the same letter indicate significant differences according to the Duncan test (*P* = 0.05). Error bars indicate the standard error of the mean. R_E: early-season root applications; $F7_M$: 7 Ca sprays from mid-season (every 12–14 days starting 30 DAFB); $F7_L$: 7 Ca sprays during late season (every 5–10 days starting 60 DAFB); $F13_M$: 13 Ca sprays from mid-season (every 5–10 days starting 30 DAFB); $R_E\,F7_L$: combination of both R_E and $F7_L$ treatments.

The pre-harvest Ca treatment did not affect any fruit quality parameter. According to other authors, the application of Ca might delay the changes associated with ripening and senescence in fruits (Beavers et al., 1994; Fallahi et al., 1997; Mason and Drought, 1975; Vicente et al., 2007). Therefore, applications of Ca can decrease the sugar content and increase starch index in apples by slowing senescence. In the same way, Ca treatments can increase acidity in fruit and/or affect perception of fruit flavour compounds (El Hadi et al., 2013; Recasens et al., 2004) and, consequently, affect in sensorial acceptability.

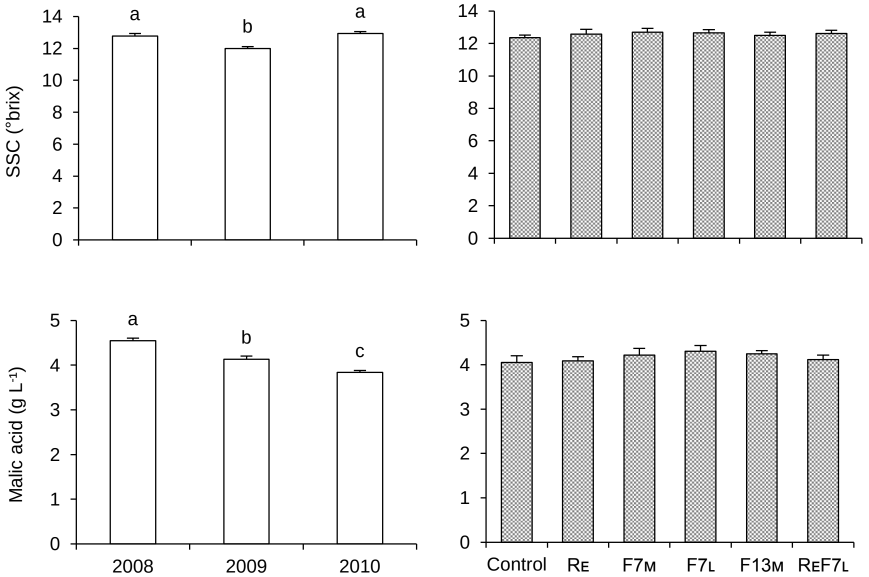

Fig. 5. Mean value of number of soluble solid (above) and malic acid (below) content in fruit, for each season (left) and pre-harvest treatment (right). Columns not sharing the same letter indicate significant differences according to the Duncan test (*P* = 0.05). Error bars indicate the standard error of the mean. R_E: early-season root applications; $F7_M$: 7 Ca sprays from mid-season (every 12–14 days starting 30 DAFB); $F7_L$: 7 Ca sprays during late season (every 5–10 days starting 60 DAFB); $F13_M$: 13 Ca sprays from mid-season (every 5–10 days starting 30 DAFB); R_E $F7_L$: combination of both R_E and $F7_L$ treatments.

The relationship between fruit Ca concentration and fruit firmness is well documented (Glenn and Poovaiah, 1990; Vicente et al., 2007). However, in 2008, fruit firmness was significantly lower than in 2010 and 2009, and fruit had a significantly higher Ca content. There are many papers reporting the failure of Ca applications to increase or keep fruit firmness (Dris and Niskanen, 1999; Glenn and Poovaiah, 1990; Recasens et al., 2004). Casero et al. (2010), when applying similar amounts of Ca to the amounts applied in the present study, achieved an increase in fruit firmness at harvest only in one out of three growing seasons. The Ca application rate may play an important role. In accordance with this hypothesis, Weis et al. (1980) and Wojcik (2001) obtained significant effects on fruit firmness by applying 68% and 50% more Ca, respectively, than in the present study, but damage to apple skin was observed.

Based on our results, there are likely factors other than minerals that affect the physiological and biochemical changes that take place during fruit development. However, little is known about the contributions of pre-harvest factors to fruit quality.

3.2. Effect of post-harvest treatment

3.2.1. Effect on bitter pit

Dipping in $CaCl_2$ solution significantly decreased the incidence and severity of bitter pit (Table 3). Globally, dips at post-harvest showed a higher efficacy in decreasing bitter pit incidence and relative index of severity than the pre-harvest treatments. No interaction between Ca applications at pre-harvest × dips at postharvest was observed. Therefore, the pre-harvest treatments $F7_{Late}$ and $F13_{Mid}$

might contribute to improve the effectiveness of the dips like when there was no treatment at post-harvest.

Table 3. *P*-values from three-way ANOVA of factors season, pre-harvest and post-harvest treatment, and its interaction, on bitter pit incidence and severity and fruit firmness at post-harvest.

Factor	Bitter pit incidence	Bitter pit severity	Firmness post-harvest
Season	<.0001	<.0001	<.0001
Pre-harvest	0.0737	0.0337	NS
Season × Pre-harvest	NS	NS	NS
Post-harvest	<.0001	<.0001	NS
Season × Post-harvest	<.0001	<.0001	NS
Pre- × Post-harvest	NS	NS	NS
Season × Pre- × Post-harvest	NS	NS	NS

NS indicates the *P*-value was greater than 0.1.

There was a significant interaction between season × dips at post-harvest since the effect of dips was higher in the season with higher incidence of bitter pit (Fig. 6). In 2010, the growing season with higher bitter pit incidence, the dips significantly decreased the incidence and severity of bitter pit whereas no significant effect was observed in 2009, the growing season with low bitter pit. An effective prediction even close to harvest time could help to packing houses to decide whether to use $CaCl_2$ dips, which would mean an important saving (Torres et al., 2015).

Other researchers have also reported significant effects of Ca dips in decreasing bitter pit. Mignani et al. (1983), and more recently Guerra and Casquero (2010) and Guerra et al. (2011), obtained a reduction of 17%, but they did not compare their results with Ca applied in pre-harvest treatments. As the first example of a combination of pre-harvest treatments and post-harvest dips, our results showed that spraying later in the season, especially with a high number of sprays, in combination with dips at post-harvest, was the most effective approach to mitigate bitter pit, with a reduction of 30% with respect to the untreated control.

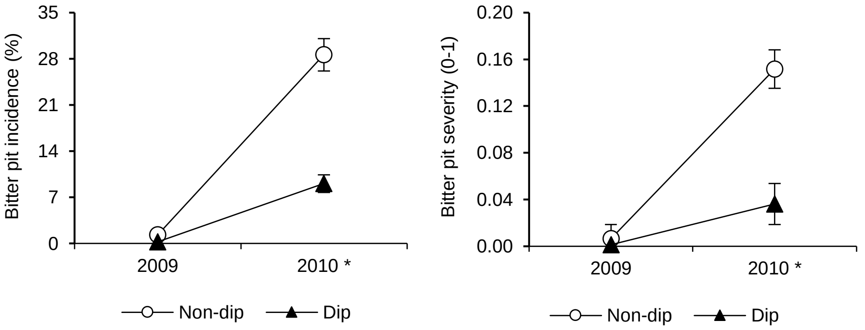

Fig. 6. Interaction effect of season × post-harvest treatments on bitter pit incidence (left) and relative severity index (right). The asterisk denotes a significant difference between the two treatments (ANOVA, $P < 0.001$). The error bars represent the standard error of the mean. Error bars indicate the standard error of the mean.

3.2.2. Effect on fruit firmness

As expected, the firmness of apples decreased during storage. Significant differences were observed between seasons (Table 3). Fruit firmness at harvest and post-harvest was significantly lower in 2009 than in 2010 (Fig. 7). There are likely pre-harvest factors other than fruit Ca content that affect the physiological and biochemical changes that take place during storage. Some research has found that the regulation of water transpiration from ripe fruits may represent an important strategy to prolong fruit firmness (Vicente et al., 2007).

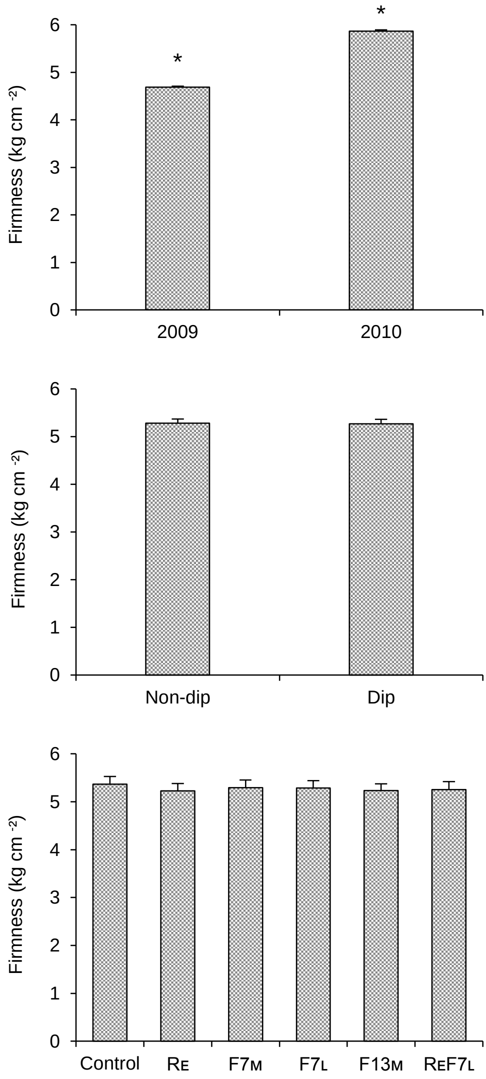

Fig. 7. Mean value of fruit firmness at post-harvest for each season (above), pre-harvest treatments (centre) and pre-harvest treatments (below). The asterisk denotes a significant difference between the two seasons (ANOVA, $P < 0.001$). Error bars indicate the standard error of the mean. R$_E$: early-season root applications; F7$_M$: 7 Ca sprays from mid-season (every 12–14days starting 30 DAFB); F7$_L$: 7 Ca sprays during late season (every 5–10days starting 60 DAFB); F13$_M$: 13 Ca sprays from mid-season (every 5–10 days starting 30 DAFB); R$_E$ F7$_L$: combination of both R$_E$ and F7$_L$ treatments.

Neither pre-harvest nor post-harvest treatments had an effect on retention of fruit firmness during storage (Table 3). Casero et al.

(2010) did not either retain the firmness of apples during storage when applying at pre-harvest similar amounts of Ca to the amounts applied in the present study. The $CaCl_2$ concentration application rate and dipping duration may play an important role in the post-harvest dips. Jan et al. (2016) found that the higher these parameters, the firmer the apples were. Besides, they observed a significant effect of dips on other quality parameters, such as ascorbic acid or weight loss. According to the authors, the retention of firmness can be attributed to the formation of Ca pectate, which leads to increased rigidity of the cell wall and improved turgor pressure. In the present study, the Ca concentration and the dipping duration were equal and lower, respectively, than the lowest rates using by Jan et al. (2016). Our results showed that dips into a 3.5% $CaCl_2$ solution for a period of 30 s is enough to significantly reduce the bitter pit incidence in seasons with high incidence, but with no effect on fruit firmness. It is therefore important for future research to investigate how dipping duration can affect fruit quality parameters during post-harvest storage of fruits.

4. CONCLUSIONS

Although the literature concerning calcium application is extensive, there are very few works dealing with the combination of different techniques for applying Ca to reduce bitter pit and increase fruit Ca content, and no previous studies have combined the three main techniques of root applications, sprays and dips at post-harvest. In this work, these three practices were tested during four consecutive years.

Year had more influence on tested parameters than Ca application. This finding highlights the need to increase our knowledge of the effects of environmental factors on the triggering of bitter pit, the maturation and development of fruits. However, Ca applications had a significant effect on bitter pit and on Ca concentration in fruit. When the different techniques were compared globally, Ca dips at post-harvest decreased bitter pit to a greater extent than pre-harvest treatments (sprays or root applications). No interaction was observed between the pre- and post-harvest treatments. Regarding the pre-harvest treatments, sprays decreased bitter pit and increased Ca content in fruit to a greater extent than root applications. Combining root applications and sprays did not provide an improvement with respect to sprays alone. Increasing the number of sprays or applying closer to harvest resulted in an improvement in bitter pit control, but they did not lead to an increase in fruit Ca content in fruit with respect to earlier sprays. Neither pre-harvest treatments nor postharvest treatments had an effect on fruit quality parameters. From our findings, we concluded that increasing the number of sprays that occurred closer to harvest can improve bitter pit control and that their combination with dips at post-harvest results in the most efficient way to reduce the bitter pit incidence.

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DISCUSIÓN GENERAL

5. DISCUSIÓN GENERAL

Mediante la presente tesis se pretendía desarrollar un sistema de predicción de la incidencia del *bitter pit* para plantaciones de manzanas del cultivar 'Golden Smoothee', a partir de métodos de predicción basados en el diagnóstico del contenido mineralógico del fruto (Capítulo 1 y 5), la inducción de síntomas (Capítulo 2 y 5) y la utilización de herramientas no destructivas como la espectroscopía VIS/NIR (Capítulo 3). Los estudios realizados han permitido evaluar y validar en condiciones locales la eficacia de distintos métodos basados en dichas técnicas, así como la evaluación y desarrollo de nuevas metodologías. Paralelamente, se evaluó y cuantificó la eficacia de distintas estrategias para la mitigación del *bitter pit* basadas en diferentes tipos de aportaciones de Ca al fruto (Capítulo 4). A continuación, se discuten los resultados obtenidos en cada uno de los capítulos, así como los aspectos más relevantes a tener en cuenta en la utilización de las distintas metodologías evaluadas a partir de la experiencia y resultados adquiridos.

5.1. Análisis mineralógico de fruto

El análisis de fruto temprano tiene como principal ventaja la precocidad en la predicción. A día de hoy, los estudios publicados en referencia a la utilización del análisis mineralógico del fruto en estadios de desarrollo tempranos se basaban en predecir los contenidos de Ca en recolección a partir del contenido de Ca en estadios tempranos, normalmente en torno a los 80 DDPF, con la finalidad de utilizar los umbrales conocidos en recolección (5-6 mg Ca 100 g^{-1} PF) para el diagnóstico del *bitter pit* (Brooks, 2001; Drahorad y Aichner, 2003; Ferguson y Triggs, 1990).

En la presente tesis se evaluaron distintos momentos para realizar el análisis de fruto temprano (40, 60 y 80 DDPF), así como determinar el mejor indicador para obtener la mayor precisión posible en la predicción del *bitter pit*. Para ello, durante 4 años se correlacionó la incidencia del *bitter pit* de 10 plantaciones distintas con el contenido en fruto de los principales nutrientes con mayor relevancia en la aparición del *bitter pit* (Ca, N, P, K y B). Se comparó la precisión de predicción de modelos multivariantes, obtenidos mediante regresión parcial por mínimos cuadrados (PLS), con la precisión obtenida mediante la correlación lineal de la incidencia del *bitter pit* con cada uno de los nutrientes analizados por separado y de sus respectivos cocientes con el Ca.

Los contenidos en fruto de Ca, N, P y K mostraron un comportamiento en concordancia con los valores de referencia, respecto la incidencia de *bitter pit* (de Freitas et al., 2015; Ferguson y Watkins, 1989). Sin embargo, el contenido de B en fruto mostró un comportamiento distinto a lo que cabría esperar según la mayoría de trabajos publicados al respecto. En el presente estudio, el contenido de B mostró una correlación positiva respecto a la incidencia de *bitter pit* y negativa respecto al contenido de Ca en fruto. Como norma general, la deficiencia de B se correlaciona con una mayor incidencia de fisiopatías tipo *bitter pit*. Dicho efecto se relaciona con la capacidad del B de mejorar el movimiento de Ca en el interior de la planta y por sus funciones en la estructura de la membrana celular (Granelli et al., 1989; Rosenberger et al., 2004; Wojcik et al., 1999; Wojcik y Cieslinski, 2000). Sin embargo, Benavides et al. (2002), en un estudio realizado en manzana 'Golden Smothee' en las mismas condiciones locales del presente estudio, observaron una correlación negativa entre el contenido de Ca y de B en fruto. Cabe remarcar que el B es un elemento único entre el resto de nutrientes ya que la diferencia que presenta entre los niveles considerados deficientes con los que podrían considerarse excesivos, y en consecuencia provocar fitotoxicidad, sobretodo en hojas, es muy estrecha (Paparnakis et al., 2013). Según Nable et al. (1997), en algunas especies de árboles como *Prunus*, *Malus* o *Pyrus*, donde la movilidad del B también puede ser por el floema, los frutos y brotes jóvenes son los primeros a mostrar síntomas de toxicidad por exceso de B. Sin embargo, dichos síntomas suelen ser manchas necróticas internas sin ningún parecido al *bitter pit*. La relación del incremento de B y la aparición de *bitter pit* podría deberse a un deterioro de los vasos xilemáticos que nutren al fruto debido a un exceso de B. Miqueloto et al. (2014) demostraron recientemente una relación entre la funcionalidad del xilema, el contenido de Ca y la incidencia de *bitter pit*.

El Ca fue el elemento que mejor se correlacionó con la incidencia de *bitter pit*. Modelos multivariantes basados en el contenido del Ca, K, Mg, N y B no mejoraron significativamente la precisión de predicción en comparación a la utilización del Ca por sí solo. En este sentido, existen diversidad de resultados. Mientras que Ferguson y Triggs (1990) y Ben (2006) contrastaron que la utilización del Ca por si sólo era suficiente para el diagnóstico del *bitter pit*, Van der Boon (1980), Drahorad y Aichner (2003), Miqueloto et al. (2014) o de Freitas et al. (2015), por poner unos ejemplos, proponen la utilización del cociente entre el Ca y alguno de sus antagonistas, como el Mg, K o N.

El mejor momento para realizar los análisis de fruto temprano se posicionó entorno a los 60 DDPF, a diferencia de otros autores (Brooks, 2001; Drahorad y Aichner, 2003). Estos resultados están en línea con los que obtuvo Lotze et al. (2008), quienes observaron que el contenido de Ca en recolección se correlacionaba mejor cuando era analizado entre 41 y 82 DDPF que entre 83 y 118 DDPF. Las posibles causas de esta falta de correlación alrededor de los 80 DDPF pueden ser debidas a la inestabilidad del contenido de Ca en el fruto. Cabe remarcar que éste es un periodo con una alta transpiración por parte de las hojas y una alta tasa de crecimiento tanto por parte de los brotes como de los frutos. Esto implica que, durante dicho periodo, pueda haber una gran variabilidad en la concentración de Ca en frutos de una misma plantación. Esta variabilidad se podría corregir justo antes de la recolección, cuando la tasa de transpiración y de crecimiento se reducen y, en consecuencia, la concentración de Ca se estabiliza (Casero et al., 2017).

La existencia de un umbral de referencia facilita el diagnóstico e interpretación de los análisis mineralógicos y, en consecuencia, su implementación y utilización. Los resultados obtenidos indican 11,0 mg 100 g^{-1} de PF como umbral de referencia del contenido de Ca en fruto a 60 DDPF para determinar el riesgo de *bitter pit*. Valores por encima de dicho umbral indicarían un bajo riesgo de *bitter pit* y viceversa. Los umbrales de referencia utilizados hoy día para el diagnóstico del potencial de *bitter pit* en una plantación están referenciados al momento de recolección, por lo que hasta la fecha no existía una referencia contrastada para la predicción del *bitter pit* para estadios tempranos. Cabe remarcar que el umbral propuesto a 60 DDPF presentó una precisión estadísticamente igual o mejor que la referencia utilizada tradicionalmente en recolección (5-6 mg Ca 100 g^{-1} PF).

Finalmente, a partir de los distintos estudios realizados en la presente tesis se puede concluir que, en nuestras condiciones, la predicción de *bitter pit* mediante el análisis de Ca en fruto en estadios tempranos tiene una probabilidad de acierto entorno al 71%. Cabe remarcar que el momento de la predicción se sitúa alrededor de los 60 DDPF, lo que permite, en el caso de alto riesgo, poder empezar con una estrategia para mitigar la incidencia de *bitter pit*. No obstante, se debe matizar que la precisión del análisis mineralógico dependió de la región y, en menor medida, del año de evaluación. El efecto de la región se observó en el momento de validar el modelo, utilizando datos del área de Girona y de Lleida, resultando en un número significativo de falsos positivos (plantaciones con una incidencia real de *bitter pit* baja, clasificadas de alto riesgo) en las predicciones realizadas en Girona. El efecto del año vino dado en función de si las condiciones eran propicias para el desarrollo de *bitter pit* o no. En este sentido, los años con un mayor número de plantaciones con altas incidencias de *bitter pit* las predicciones fueron mejores, mientras que los años con menor presencia de plantaciones afectadas se incrementó la probabilidad de falsos positivos. En definitiva, la utilización del análisis mineralógico comporta un alto riesgo de pronosticar falsos positivos, lo que podría llevar a desechar producción de calidad. Es por ello que su diagnóstico se debería combinar con los resultados obtenidos a partir de los métodos de predicción basados en la inducción de síntomas, aplicados en estadios de desarrollo del fruto más avanzados y con una menor probabilidad de falsos positivos. De los métodos analizados basados en la inducción de síntomas, recomendamos el método pasivo al ser igual de preciso que el resto, pero de menor coste y mayor simplicidad, lo que le convierte en el complemento idóneo para el seguimiento de la incidencia de *bitter pit* en las plantaciones con contenidos de Ca por debajo del umbral óptimo de referencia, a partir de la propuesta desarrollada al final del Capítulo 5.

Trabajos futuros

Debería utilizarse el análisis de fruto en estadios de desarrollo temprano para conocer la evolución y estado nutricional del fruto antes de que éste alcance su total desarrollo y, por lo tanto, poder tomar medidas correctoras en el caso que fuera necesario, optimizando en todo momento la fertilización. Sin embargo, la utilización de análisis de fruto temprano es minoritario por dos motivos principales. En primer lugar, los análisis pueden resultar costosos ya que deben ser realizados por laboratorios especializados con el personal y equipos adecuados. En segundo lugar, aunque se conoce el estado nutricional óptimo de un fruto en recolección, existen muy pocos trabajos sobre el estado nutricional de los frutos durante su desarrollo, lo que dificulta el diagnóstico y la correcta interpretación de los resultados.

El avance de la tecnología hacia nuevos instrumentos adaptados para la medición en campo de nutrientes, como el Ca, en los tejidos vegetales, facilitará la utilización del análisis mineralógico para el diagnóstico de deficiencias nutricionales, así como para la predicción de fisiopatías como el *bitter pit*. Investigaciones en la utilización de la espectroscopía NIR o rayos X en la agricultura van en esta línea (Jarolmasjed et al., 2017; Kalcsits, 2016). Dicha tecnología o similares permitirán conocer en todo el ciclo de desarrollo del fruto su contenido nutricional de manera no destructiva, sin embargo, para una correcta interpretación de los resultados, será necesario conocer los umbrales de referencia en cada momento. Es por ello que futuros trabajos deberían ir dirigidos al conocimiento del estado nutricional de los frutos durante su desarrollo con el objetivo de poder utilizar el análisis mineralógico de fruto temprano para un correcto seguimiento y optimización de la fertilización. Cabe recordar que el contenido de nutrientes com el N, K y Mg es esencial para evitar problemas de *bitter pit*.

Los resultados del presente trabajo han manifestado un efecto significativo del año en la incidencia del *bitter pit*, habiendo años, en los que la incidencia es elevada y años en que prácticamente no aparecen síntomas para una misma plantación y condiciones de cultivo. Todo ello parece indicar que existen situaciones intrínsecas del propio año favorecedoras de la aparición del *bitter pit*. Dichas situaciones podrían estar relacionadas con las condiciones meteorológicas del año. Algunos autores han determinado que temperaturas estivales elevadas son favorables para el desarrollo de la fisiopatía (Ferguson et al., 1999). Futuras investigaciones deberían enfocarse a determinar qué condiciones meteorológicas, y en qué momento, son determinantes en la aparición del *bitter pit* con el objetivo de, por un lado, poder caracterizar el año en función del potencial de *bitter pit* y, por otro lado, entender los fenómenos asociados a su aparición con la finalidad de poder determinar estrategias de mitigación.

5.2. Inducción de síntomas

Los métodos basados en la inducción de síntomas, antes de que aparezcan de manera natural en campo o durante la conservación, permiten poder realizar un seguimiento del riesgo de *bitter pit* en estadios más avanzados que el propuesto para el análisis mineralógico de fruto temprano. En general, los métodos evaluados (infiltración de Mg, baño con etefón, embolsado de frutos y método pasivo) fueron capaces de provocar la aparición de síntomas solamente a partir de los 40 DAR, observando la máxima sensibilidad a los 20 DAR. Estos resultados coinciden con los obtenidos por Kim and Lee (2000) en manzanas 'Tsugaru', quienes mediante la infiltración de Mg a 60 y 90 DAR no observaron el desarrollo de síntomas. Dicha fecha de muestreo se posiciona, aproximadamente, 50 días después del análisis mineralógico temprano a 60 DDPF.

Los dos métodos más conocidos para inducir síntomas de *bitter pit* son la infiltración de Mg y el baño con etefón. La utilización de la infiltración de Mg para la predicción del *bitter pit* a nivel industrial fue desarrollado en Chile en los años 90s (Retamales y Valdes, 1996; Retamales y Valdes, 2001). La inducción de síntomas mediante baños de etefón fue desarrollada en Sudáfrica a finales de la década de los 70s (Eksteen et al., 1977); aunque es un método bastante popular y recomendado en bastantes manuales de fruticultura, no se tiene constancia de su implementación a nivel comercial. A diferencia de los métodos anteriores, el método pasivo era prácticamente desconocido, habiéndose desarrollado a partir del trabajo realizado durante la presente tesis doctoral. Se basa en contabilizar los frutos que desencadenan síntomas de *bitter pit* de manera natural después de haber sido recolectados sanos del árbol antes de su madurez comercial y dejándolos a temperatura ambiente. Dicho método presenta ventajas significativas respecto a los dos anteriores. La principal ventaja es que no requiere de soluciones o reactivos químicos para provocar la aparición de los síntomas, lo que le convierte, entre todos los métodos evaluados, en el más sostenible desde un punto de vista medioambiental al no generar ningún tipo de residuo más allá del de la propia muestra; además, tampoco precisa de personal y equipos especializados para provocar o evaluar la aparición de los síntomas, lo que facilita significativamente su implementación, tanto a nivel de campo como industrial. La única publicación encontrada en referencia a dicho fenómeno corresponde a England y Larsen (1973), quienes observaron que manzanas de las variedades 'Goldspur' y 'Wellspur' recolectadas sanas antes de su madurez comercial desarrollaban *bitter pit* al cabo de 7-10 días. Este hecho coincide con lo que se observa en algunos campos de manzanas 'Golden' en condiciones locales, donde semanas antes de la recolección aparece *bitter pit* en los frutos del suelo.

La precisión de predicción del *bitter pit* obtenida con los métodos basados en la inducción de síntomas se situó en torno al 70-80% y, en general, sin diferencias significativas entre ellos. Los errores cometidos en la predicción se correspondieron, mayoritariamente, a falsos negativos (plantaciones con una alta incidencia real de *bitter pit*, diagnosticadas de bajo riesgo), lo que podría deberse al error intrínseco de muestreo de frutos. Normalmente, en las plantaciones, los frutos con alto potencial a desarrollar *bitter pit* suelen ser menos frecuentes que los frutos que no acaban desarrollando la fisiopatía, por lo que la probabilidad de escoger frutos sanos es mayor. De aquí la importancia de diseñar y realizar un correcto muestreo en función de cada plantación. Aún con todo, los resultados obtenidos se sitúan dentro de la horquilla de precisiones recopiladas por Retamales (1996) para distintos métodos de predicción del *bitter pit*.

Las infiltraciones de Mg o los baños con etefón no mejoraron las predicciones respecto al método pasivo, independientemente del momento de muestreo, por lo que infiltrar con Mg o bañar con etefón tampoco supuso una mejora en la precocidad de predicción del *bitter pit* en las condiciones del estudio. Por ésto y su simplicidad, el método pasivo es el propuesto para complementar el diagnóstico del análisis mineralógico mediante el modelo presentado en el Capítulo 5. Tal como se ha comentado anteriormente, dicho modelo implica una primera predicción a partir del análisis mineralógico temprano a 60 DDPF y, en las plantaciones con contenidos de Ca en fruto por debajo de los 11 mg 100 g^{-1} PF, realizar un seguimiento del desarrollo del *bitter pit* mediante el método pasivo a partir de 40 DDPF con el objetivo de reducir el número de falsos positivos.

Además de la infiltración de Mg, el baño con etefón y el método pasivo, se evaluó el efecto de embolsar muestras de frutos y dejarlas a temperatura ambiente. La hipótesis inicial era ver si era posible avanzar la producción climatérica de etileno al exponer los frutos a una mayor concentración del etileno endógeno al encerrarlos dentro de bolsas (Mattheis, 1996) y de esta manera provocar la aparición de síntomas de forma similar a la que se producen con los tratamientos de etileno (Eksteen et al., 1977). Sin embargo, el embolsado no produjo una mayor aparición de síntomas de *bitter pit*, sino justo lo contrario: las muestras embolsadas registraron un menor porcentaje de frutos afectados y con síntomas más leves que el resto de métodos.

La reducción del *bitter pit* mediante el embolsado podría deberse a una menor disponibilidad de O_2 y, en consecuencia, una disminución de la tasa respiratoria. Sharma et al. (2012) observaron que en frutos afectados por *bitter pit* las tasas de producción de etileno y respiración eran más elevadas. La conservación en atmósferas controladas con bajos niveles de O_2 también ha demostrado reducir significativamente la incidencia de *bitter pit* (Jankovic y Drobnjak, 1994; Khan et al., 2006). Saure (2014) propuso recientemente que la aparición de fisiopatías asociadas con la deficiencia de Ca podría atribuirse a un estrés abiótico, en lugar de a una deficiencia de Ca en el fruto. Según su teoría, la influencia del estrés en la aparición del *bitter pit*, podría basarse en un aumento de la producción de especies reactivas de oxígeno (ROS) en el fruto, como radicales libres y peróxidos de hidrógeno. Según Chen y Zhou (2004), la capacidad de los frutos a eliminar los radicales libres de oxígeno se reduce durante el almacenamiento, siendo una de las principales causas en la aparición de fisiopatías. El embolsado de frutos podría reducir el estrés oxidativo en el fruto y, en consecuencia, disminuir la producción de ROS y la incidencia de *bitter pit*. En línea con todo esto, Pesis et al. (2010) y Val et al. (2009) propusieron, y demostraron su eficacia para reducir el *bitter pit*, tratamientos a corto plazo de bajo O_2 a 20 °C previos al almacenamiento convencional de larga duración en frío.

Trabajos futuros

El método pasivo ha demostrado ser una herramienta eficaz para predecir el *bitter pit*. Sin embargo, se desconoce el proceso a partir del cual los frutos, una vez son cogidos del árbol, desencadena la aparición de *bitter pit*, mientras que, si son capaces de madurar en el árbol sin incidencias, es posible que no acabe por desarrollar los síntomas. Un mayor conocimiento de los cambios fisiológicos que se producen en los frutos una vez son recolectados o movidos del árbol, especialmente antes de alcanzar su madurez comercial, podrían ayudar a entender el proceso que desencadena el *bitter pit*. La relación del *bitter pit* con el deterioro y disfunción de los vasos xilemáticos durante la maduración de los frutos, tal como indicó Miqueloto et al. (2014), podría ir en esta línea, siendo la deficiencia de Ca una consecuencia de dicho deterioro y no la causa, tal como apuntaba Saure (2014).

El método pasivo, además de representar una nueva oportunidad para el estudio de las causas fisiológicas que provocan la aparición del *bitter pit*, podría ser una herramienta para la evaluación, por un lado, de la eficacia de tratamientos aplicados en precosecha para el control o mitigación del *bitter pit* y, por otro lado, de la sensibilidad al *bitter pit* de nuevas variedades obtenidas en programas de mejora. En ambos casos, la utilización del método pasivo implicaría la obtención de resultados de manera rápida y de bajo coste. Cabe remarcar que los trabajos realizados en la presente tesis fueron desarrollados únicamente en manzanas del cultivar 'Golden Smoothee' por lo que, antes de la utilización del método pasivo para la evaluación de nuevos cultivares, es estrictamente necesario un mayor número de estudios para determinar su eficacia en la aparición de síntomas en cultivares especialmente sensibles al *bitter pit*.

El embolsado de frutos supuso una reducción en la aparición de síntomas en comparación a los demás métodos. De manera contraria al método pasivo, la técnica del embolsado puede suponer una nueva oportunidad de estudio de las causas que pueden ayudar a reducir la aparición del *bitter pit*. Analizando los cambios fisiológicos que se producen mediante ambos métodos se podría llegar a determinar las causas que desencadenan y/o mitigan la aparición del *bitter pit*.

El tratamiento de bajo oxígeno, tanto justo después de recolección como durante todo el proceso de conservación, puede ser una práctica a tener en cuenta para las centrales frutícolas con el objetivo de reducir la incidencia del *bitter pit*. En el caso del tratamiento de bajo oxígeno a 20 °C, propuesto por Val et al. (2009), puede conllevar otras ventajas asociadas como una mejora en la coloración. No obstante, es necesario cuantificar y valorar económicamente la eficacia de dichos tratamientos antes de su implementación.

5.3. Espectroscopía VIS/NIR

En el presente trabajo se evaluó y utilizó la espectofotometría VIS/NIR para la identificación de frutos con *bitter pit* en precosecha, antes de que aparecieran los síntomas, y en poscosecha, una vez los síntomas eran visibles. Para ello, se utilizó un espectrómetro VIS/NIR (UT instrumentos, Lugo, Ravenna, Italia) con un rango de longitud de onda de medición de la absorbancia en los 650-950 nm, focalizando cada medida en un punto de 10 mm^2. Tanto en la predicción en precosecha como en la clasificación en poscosecha, se calculó la precisión de clasificación en función del tipo de escala utilizada (*multiclass* vs. binaria), el número de mediciones realizadas por fruto (1 ó 2 mediciones), la localización de las mediciones (cercana ó alejada de los síntomas) y el tipo de algoritmo utilizado para la obtención del modelo de clasificación (LDA, QDA, SVM).

Los resultados en la predicción del *bitter pit* en precosecha no fueron satisfactorios, con una precisión de clasificación inferior al 50%, independientemente de los factores analizados. Dichos resultados son similares a los que obtuvo Lotze (2005) para su trabajo de tesis, utilizando un rango de longitud de honda similar al utilizado en la presente tesis, planteando como causa de la incapacidad de la espectroscopia VIS/NIR para predecir el *bitter pit* el no realizar las medidas sobre las futuras zonas afectadas. Hay que decir al respecto que esto no fue una limitación en el caso de clasificar manzanas cuando los síntomas eran visibles, obteniendo el mismo nivel de precisión tanto si la medición se realizó sobre un punto alejado o cerca del área afectada, midiendo en cualquiera de los dos casos sobre tejido sano.

En poscosecha, cuando los síntomas eran visibles, la precisión de clasificación de frutos con y sin *bitter pit* fluctuó del 46% al 81%, en función del tipo de escala y/o del algoritmo de clasificación utilizado. Sin embargo, el número de medidas realizadas sobre el fruto y si en los frutos afectados éstas se realizaban próximas o no a la zona afectada, no influyó sobre el grado de precisión de la clasificación, sin diferencias significativas entre realizar 1 o 2 medidas por fruto. Tal como era de esperar, la precisión en la clasificación general *multiclass* fue significativamente inferior que la clasificación general binaria (46% vs. 75%). En el caso de la clasificación *multiclass*, la categoría intermedia, correspondiente a la aparición de síntomas leves (de 1 a 6 manchas), registró los niveles de clasificación más bajos (de 1% al 37%). Para la clasificación binaria se utilizaron únicamente frutos de las categorías 1 (asintomáticos) y 3 (síntomas graves que no dejan duda de la presencia de *bitter pit*), mejorando significativamente la precisión de clasificación y con resultados similares a los obtenidos por otros investigadores (Jarolmasjed et al., 2017; Kafle et al., 2016; Nicolai et al., 2006). En general, el número de frutos utilizados por muestra en el presente trabajo era mayor que el número de frutos utilizados en los trabajos citados anteriormente, lo que da una mayor robustez a los resultados obtenidos.

Lotze (2005) indicó la degradación de la clorofila y/o el cambio en el contenido de agua en el tejido intercelular como las posibles diferencias entre frutos sintomáticos y asintomáticos que permiten la detección de frutos con *bitter pit* mediante la espectroscopía VIS/NIR. Los resultados obtenidos en el presente trabajo soportan dicha teoría, observando las mayores diferencias entre frutos asintomáticos y sintomáticos en la región del visible de los 650 a los 700 nm, región relacionada la clorofila (Abbott et al., 2010), y en la región del infrarrojo de los 840 a los 950 nm, región relacionada con la absorción del agua y el azúcar (ElMasry et al., 2008). Dichos cambios se producirían en el conjunto del fruto por lo que la clasificación sería posible independientemente de la zona medida.

El tipo de algoritmo de clasificación utilizado y la categoría de clasificación tuvieron un efecto significativo en el valor de precisión. Utilizando una clasificación binaria, el algoritmo LDA clasificó mejor los frutos afectados que los frutos sanos, con un mayor número de falsos positivos (manzanas sanas clasificadas como afectadas) en comparación al resto de algoritmos. Por el contrario, los algoritmos QDA y SVM obtuvieron una clasificación mejor de frutos sanos, con un número más elevado de falsos negativos (manzanas afectadas clasificadas como sanas) que el algoritmo LDA. Dichos resultados distan de los obtenidos por Kafle et al. (2016), quienes observaron una mayor precisión para clasificar frutos afectados utilizando los algoritmos QDA y SVM en manzanas 'Honeycrisp'. Sin embargo, la clasificación general binaria fue más precisa mediante el algoritmo LDA, seguido del algoritmo QDA y SVM. Dichos resultados fueron similares a los obtenidos por Kafle et al. (2016) quienes observaron una mayor precisión mediante el algoritmo QDA que con el algoritmo SVM.

En definitiva, la espectroscopía VIS/NIR mostró resultados erráticos en la predicción del *bitter pit* en precosecha cuando los síntomas no son visibles, pero mostró potencial para discriminar frutos con *bitter pit* cuando los síntomas son visibles. Los resultados obtenidos en la presente tesis demuestran que el número de medidas realizadas por fruto y la zona en la que se realiza la medida no afecta a la precisión de clasificación, a diferencia de lo que cabe esperar de una cámara visible. Esto hace suponer que dicha tecnología es capaz de detectar cambios que se producen en el conjunto de la composición del fruto que podrían estar relacionados con la aparición del *bitter pit*, como la degradación de clorofila o una deshidratación.

Trabajos futuros

Los futuros trabajos en la utilización de la espectroscopía VIS/NIR para la predicción y clasificación de frutos con *bitter pit*, en general, deberían ir enfocadas, por un lado, en la realización de estudios con grandes conjuntos de datos para dar robustez a los modelos obtenidos y estos puedan ser implementados de manera comercial y, por otro lado, en la utilización de aparatos con un mayor rango de medición de longitud de onda.

Una mejora de los resultados obtenidos en la predicción del *bitter pit* mediante la espectroscopía VIS/NIR podría venir por ampliar el rango de medición de longitud de onda. Mediciones por encima de los 935 nm han mostrado ser eficientes en la discriminación de compuestos orgánicos y complejos químicos de los principales minerales asociados al *bitter pit*, como son el Ca, Mg y K (Jarolmasjed et al., 2017). Dicha tecnología podría utilizarse para determinar el estatus nutricional de los frutos en desarrollo, de forma rápida y sencilla, permitiendo indicar el riesgo de aparición de desórdenes fisiológicos en estadios de desarrollo tempranos.

Además de la espectroscopía VIS/NIR, otras técnicas espectroscópicas, basadas principalmente en la fluorescencia y/o tomografía por computadora de rayos X, están siendo actualmente evaluadas por distintos autores para la detección del *bitter pit* y/o la determinación del estado nutricional de los frutos de manera no destructiva con resultados prometedores (Jarolmasjed et al., 2016; Kalcsits, 2016; Si y Sankaran, 2016; Zúñiga et al., 2017). El avance de la tecnología permitirá en breve disponer de un amplio surtido de aparatos portátiles que permitirán la utilización de dichas tecnologías a pie de campo, permitiendo la obtención de un amplio abanico de resultados por fruto mediante una sola medición (estado nutricional, estado de madurez, contenido de sólidos solubles, materia seca, desordenes fisiológicos, etc.). Sin embargo, antes de que dicha tecnología pueda ser utilizada a nivel comercial, es necesario la realización de estudios con grandes conjuntos de datos que permitan la obtención de modelos robustos que pueden ser incorporados en los mismos aparatos, así como la validación de resultados en diferentes condiciones de cultivo.

5.4. Factores de muestreo

Todos los métodos analizados en el presente trabajo conllevan un error intrínseco de muestreo o de estimación. De aquí la importancia de realizar un correcto muestreo para minimizar al máximo dicho error en el diagnóstico. A modo de ejemplo, en relación a los métodos por inducción de síntomas, los resultados obtenidos en el presente trabajo fueron bastante similares a los conseguidos en Chile utilizando una metodología similar de muestreo (Retamales y Valdes, 1996), mientras que Lotze et al. (2010) reportaron peores resultados, apuntando como posible causa las diferencias en el tipo de muestreo.

En el momento de diseñar la metodología de muestreo en una plantación, es esencial conocer los diferentes factores que pueden afectar a la aparición del *bitter pit*. Visser y Pienaar (1976) contabilizaron que la variabilidad de *bitter pit* en cajas procedentes de una misma plantación puede fluctuar del 9% al 80% (citado en Lotze y Theron, 2006). Los autores del estudio atribuyeron las causas de esta variabilidad a diferencias en el estado de madurez, el tamaño y la posición de los frutos dentro del árbol. Fergusson and Triggs (1990) comprobaron que, en una misma plantación, la variabilidad era mucho mayor entre frutos de un mismo árbol que entre frutos de árboles diferentes, llegando a la conclusión de que, para la obtención de muestras con una mayor representación del conjunto de la plantación, era mejor disponer de menos frutos pero de un mayor número de árboles distintos.

En la metodología de muestreo es importante definir el tipo de árboles que se van a muestrear. Éstos deben mantener unos niveles de vigor, cuajado y carga representativos de la plantación, teniendo en cuenta que dichos factores afectan directamente a la incidencia del *bitter pit*. Además del estado vegetativo y de la carga, hay que considerar la localización de los árboles dentro de la parcela. Árboles localizados en posiciones con una mayor insolación, por ejemplo en los márgenes de la plantación o en los extremos de las filas, o próximos a los árboles polinizadores, presentan mejores condiciones para el cuajado y, por lo tanto, pueden producir un mayor número de semillas y, en consecuencia, mostrar una mayor resistencia a la aparición del *bitter pit* (Marcelle, 1990).

La posición del fruto en el árbol es otro de los factores que influye en la incidencia del *bitter pit* (Ferguson y Triggs, 1990; Lotze y Theron, 2006; Marcelle, 1990). Frutos en la parte superior del árbol o en la cara con mayores horas de insolación, son más susceptibles al *bitter pit*. En plantaciones envejecidas, con un mayor porcentaje de la producción en las partes altas del árbol y/o en las caras más soleadas, sería recomendable incluir una mayor proporción de frutos de dichas zonas del árbol. El tamaño del fruto es otro de los factores a considerar en el momento del muestreo. Ferguson y Triggs (1990) cuantificaron un aumento del 5% de la incidencia de *bitter pit* por cada 10 g de incremento de peso en frutos del cultivar 'Orange Pippin de Cox', por lo tanto, con el objetivo de obtener una muestra representativa, es importante realizar una inspección al conjunto de la parcela para definir el rango del tamaño del fruto a muestrear.

En los muestreos para inducir síntomas, muestreos dirigidos a frutos más vulnerables incrementaría la probabilidad de la aparición de frutos sintomáticos con riesgo de incrementar los erroes debidos al diagnóstico de falsos positivos. Aún así, en futuros trabajos, sería interesante evaluar el efecto de dirigir los muestreos para inducir síntomas hacia frutos con, supuestamente, mayor sensibilidad al *bitter pit* con el objetivo de reducir los errores de predicción debidos a falsos negativos.

5.5. Estrategias de mitigación

Una mayor precocidad en la predicción del *bitter pit* supone un mayor tiempo para la toma de medidas correctoras para la mitigación de su incidencia. Las medidas correctoras, generalmente, corresponden a prácticas culturales para controlar el vigor de los árboles (correcciones en la fertilización, aplicaciones de fitorreguladores, poda en verde, etc.) y/o a la realización de aportaciones de Ca. En este trabajo se pretendió cuantificar la eficacia de distintas estrategias de mitigación basadas en la aportación de Ca con el objetivo de poder recomendar, en el caso de predicciones de riesgo, actuaciones validadas en condiciones locales.

Las estrategias evaluadas consistieron en aplicaciones de Ca en precosecha y poscosecha. Las aplicaciones en precosecha consistieron en aplicaciones radiculares y/o foliares de $CaCl_2$, y las aplicaciones de poscosecha en baños en soluciones de $CaCl_2$. Las aplicaciones foliares, radiculares y de poscosecha se evaluaron por separado y en combinación. Además, en el caso de las aplicaciones foliares, se evaluó el momento de iniciar el programa de aplicaciones (30 vs. 60 DDPF), así como cambiar el número de aplicaciones realizadas (7 vs. 13 aplicaciones). En los 4 años de estudio se observó cómo el efecto año tuvo un mayor efecto sobre la incidencia del *bitter pit* que la aplicación de los tratamientos, obteniendo 2 años con una alta incidencia de *bitter pit* (valores superiores al 20% en la mayoría de parcelas) y 2 años sin apenas *bitter pit*.

El contenido de Ca en el fruto en el conjunto del ensayo no sirvió para predecir el efecto año en la incidencia del *bitter pit*. Aunque el año con el mayor contenido de Ca (5,1 mg 100 g^{-1} PF) se asoció con una incidencia prácticamente nula de *bitter pit*, el resto de años se obtuvo una gran variabilidad, con años con más del 20% de los frutos afectados y años sin afectación, mientras que el contenido de Ca se mantuvo con valores similares entre años (3,9 mg 100 g^{-1} PF) y por debajo del umbral considerado óptimo. Estos resultados coinciden con los obtenidos en los capítulos 1 y 5 en referencia al alto número de falsos positivos que puede proporcionar el análisis de Ca para la predicción del *bitter pit*.

Los resultados obtenidos respecto a la eficacia de los distintos tipos de aplicaciones de Ca están en línea con los resultados encontrados en la literatura. En general, las aplicaciones foliares registraron una mayor eficacia que las aplicaciones radiculares, tanto en el incremento del contenido de Ca en el fruto como en la mitigación del *bitter pit*. Wilsdorf et al. (2012) también obtuvo mejores resultados mediante aplicaciones foliares que con aplicaciones radiculares en el incremento de Ca en el fruto. La aplicación de Ca radicular por sí sola no mostró una disminución significativa del *bitter pit* y la combinación de aplicaciones radiculares y foliares no se tradujo en una mejora significativa en comparación a las aplicaciones foliares por sí solas. Por lo tanto, se puede concluir que, en las condiciones del ensayo, las aplicaciones radiculares de Ca no aportan una mejora en el control del *bitter pit*. Debemos tener en cuenta que el estudio se realizó sobre suelo calcáreo y, por tanto, con altos niveles de Ca disponibles en el suelo (Ca asimilable > 3000 ppm), por lo que los bajos niveles de Ca detectados en el fruto podrían deberse a un problema de distribución en el interior del árbol y no a la falta de su disponibilidad en las raíces.

Por lo tanto, de los tipos de aplicaciones evaluados en precosecha, las aplicaciones foliares serían las recomendadas para mitigar la incidencia de *bitter pit*. El inicio del programa de aplicaciones foliares no afectó a la incidencia del *bitter pit*, obteniendo resultados similares tanto si iniciamos las aplicaciones a media estación (30 DDPF) o al final del periodo de crecimiento del fruto (60 DDPF). Estos resultados coinciden con los obtenidos por Casero et al. (2002), Peryea et al. (2007), Lotze y Theron (2007) y Lotze et al. (2008), con una tendencia a mejorar la eficacia de las aplicaciones cuando éstas se concentran más próximas a la recolección. Sin embargo, existen estudios en donde la tendencia es inversa, lo que ha propiciado que, en los últimos años, se haya extendido entre los productores la creencia de que las aplicaciones tempranas son más eficientes que las aplicaciones tardías (Neilsen et al., 2005; Wilsdorf et al., 2012). Igualmente, el tratamiento con 13 aplicaciones foliares mostró una tendencia a mejorar la eficacia en comparación a 7 aplicaciones, siendo el único tratamiento con una incidencia significativamente inferior al testigo sin aplicaciones.

Los baños en poscosecha mostraron una eficacia significativa en la mitigación del *bitter pit* e incluso mayor que las aplicaciones foliares por sí solas. Aún con todo, los mejores resultados se observaron al combinar los tratamientos de precosecha con las aplicaciones de poscosecha, sin observarse una interacción significativa entre los dos momentos. Por lo tanto, los baños de Ca en poscosecha contribuyen de manera significativa en la mitigación del *bitter pit*, tanto en combinación, como si no, con los tratamientos de precosecha. La eficacia de los baños con soluciones de Ca en poscosecha ya se había demostrado anteriormente por distintos autores (Guerra y Casquero, 2010; Guerra et al., 2011; Mignani et al., 1983). Sin embargo, esta es la primera vez que se compara y se combina con aplicaciones de Ca en precosecha. Los resultados obtenidos indican que en años con incidencias de alrededor de un 20%, se puede reducir entorno a un 8%, un 12% o un 17% mediante aplicaciones foliares, baños en poscosecha o la combinación de ambas, respectivamente.

Los resultados obtenidos indican que los programas de aplicaciones foliares podrían iniciarse una vez conocido el primer diagnóstico proporcionado por el análisis de Ca en fruto temprano a 60 DDPF (principios de junio). En las plantaciones de alto riesgo (< 11 mg Ca 100 g^{-1} PF), el programa de aplicaciones podría iniciarse con una frecuencia de cada 10-14 días, lo que supondría realizar entre 5 y 7 aplicaciones antes de la siguiente predicción. Posteriormente, en el caso de predicciones de alto riesgo mediante el método pasivo, se recomendaría incrementar la frecuencia de las aplicaciones, llegando a realizar más de 10 aplicaciones foliares antes de recolección. En el caso de diagnósticos de bajo riesgo mediante el método pasivo se mantendría la frecuencia a cada 14 días.

Trabajos futuros

Trabajos futuros en la mitigación del *bitter pit* deberían enfocarse en cuantificar la eficacia de las recomendaciones habituales para el control del *bitter pit*, como la poda en verde, cambios en la fertilización o el retraso de la recolección, con el objetivo de poder definir un protocolo de actuaciones en función del diagnóstico obtenido en cada caso.

En el presente trabajo se evaluaron distintas estrategias a base de una dosis única de aplicación, sin embargo, el incremento de la concentración de Ca en la solución de aplicación podría mejorar la eficacia de los tratamientos foliares. En este sentido, Yuri et al. (2002) lograron una eficacia significativa del 81% respecto al testigo realizando sólo 6 aplicaciones foliares aplicando un 40% más de unidades de CaO por hectárea (18 vs. 11 kg CaO ha $^{-1}$). Por lo tanto, incrementar la concentración de Ca en la solución de aplicación mediante el incremento de la dosis del producto utilizado o utilizando productos con una mayor concentración de Ca podría suponer una reducción del número de tratamientos necesarios para obtener resultados significativos. El desarrollo de nuevas formulaciones que favorezcan la absorción y/o penetración del Ca podría suponer también una mejora.

Para explicar la falta de relación entre el contenido de Ca en el fruto y la incidencia de *bitter pit*, tal como se ha comentado anteriormente, algunos investigadores han sugerido causas distintas a la deficiencia de Ca como desencadenantes del *bitter pit*. Dichas causas podrían estar asociadas a situaciones de estrés oxidativo y la disminución de Ca en el fruto sería una consecuencia más de dicho estrés (Saure, 2014). Sin embargo, las aplicaciones de Ca muestran una eficacia significativa en la mitigación de la fisiopatía. Tradicionalmente, la efectividad de las aplicaciones de Ca se ha asociado con un incremento de Ca los tejidos y, en consecuencia, una mayor estabilidad de las paredes celulares, aunque son numerosos los trabajos que demuestran que la presencia de Ca puede tener efectos en los procesos endógenos de la propia planta para la mitigación de diversas situaciones de estrés, más allá de la de formar parte de la estructura de las paredes celulares (An et al., 2016; Hocking et al., 2016). Las futuras investigaciones deberían ir dirigidas a determinar los mecanismos por los que las aplicaciones de Ca son efectivas en la mitigación del *bitter pit*.

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CONCLUSIONES

6. CONCLUSIONES

Capítulo 1: Predicción del bitter pit mediante análisis mineralógico de fruto temprano

1. El contenido de Ca en fruto a 60 días después de plena floración fue el indicador mineralógico con las correlaciones más altas con el *bitter pit* de poscosecha. Modelos multivariantes basados en el contenido en fruto de K, Mg, N, B y Ca no mostraron una mejora significativa en la precisión de predicción en comparación al contenido de Ca en fruto por si sólo.

2. Valores de Ca a 60 días después de floración superiores a 11 mg 100 g^{-1} de peso fresco se relacionaron con una baja incidencia de *bitter pit*. Sin embargo, valores inferiores se relacionaron con un alto porcentaje de falsos positivos, por lo que no siempre implicó una alta incidencia de *bitter pit*.

Capítulo 2: Métodos de predicción del bitter pit mediante la inducción de síntomas

3. Se desarrolló un nuevo método para predecir el *bitter pit*, denominado método pasivo, más secillo, económico y sostenible, y con la misma precisión, que los métodos de referencia mediante la infiltración con sal de Mg o el baño en solución de etefón. Al igual que los métodos de referencia, el método pasivo mostró eficacia a partir de los 40 días antes de la recolección y hasta la recolección, con correlaciones respecto a la incidencia del *bitter pit* en poscosecha del 70-80%.

4. El embolsado de frutos en precosecha resultó en una reducción de la aparición de síntomas, tanto en incidencia como en severidad, en comparación a los métodos de referencia.

Capítulo 3: Identificación y predicción del bitter pit mediante espectofotometría VIS/NIR

5. La espectrofotometría VIS/NIR mostró resultados erráticos en la predicción del *bitter pit* en precosecha antes de que los síntomas fueran visibles. Sin embargo, mostró una alta precisión en la discriminación de frutos sintomáticos y asintomáticos en poscosecha, cuando los síntomas eran visibles.

6. La precisión de la espectrofotometría VIS/NIR para clasificar frutos con *bitter pit* no mostró diferencias significativas en función del número de mediciones realizadas por fruto, ni de la posición de las mediciones en relación a la zona afectada en los frutos sintomáticos, lo que refleja cambios en el fruto, debidos a la aparición del *bitter pit*, en todo su conjunto y no sólo en las zonas afectadas.

Capítulo 4: Estrategias de mitigación del bitter pit mediante aplicaciones de $CaCl_2$

7. Las aplicaciones radiculares de Ca no mostraron eficacia en la mitigación del *bitter pit*, mientras que las aplicaciones foliares de $CaCl_2$, los baños en poscosecha y la combinación de ambas

mostraron reducciones significativas de la incidencia del 8%, 12% y 17%, respectivamente, en años con incidencias entorno al 20%.

Capítulo 5: Modelo de predicción del bitter pit para plantaciones de 'Golden Smoothee' basado en el contenido de Ca en fruto y la inducción de síntomas

8. Para la predicción del *bitter pit* en plantaciones de 'Golden Smoothee', se propone la combinación del análisis del contenido de Ca en fruto a 60 días después de plena floración, para la detección de fincas de bajo riesgo, con el método pasivo a partir de 40 días antes de recolección, para la confirmación de fincas de alto riesgo. El modelo propuesto permite adaptar la estrategia de mitigación del *bitter pit* basada en aplicaciones de $CaCl_2$ a partir de los diagnósticos obtenidos en cada momento.

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