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## Alternatives to conventional fungicides for the control of citrus postharvest green and blue moulds

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### Abstract

**Purpose of review:** This article reviews research based on the evaluation of postharvest control methods alternative to conventional chemical fungicides for the control of citrus green and blue moulds, caused by the pathogens *Penicillium digitatum* and *P. italicum*, respectively. Emphasis is given to advances developed during the last few years. Potential benefits, disadvantages and commercial feasibility of the application of these methods are discussed.

**Findings:** Substantial progress has been accomplished in selecting and characterising new effective physical, chemical and biological control methods. However, their widespread commercial implementation relies, in general, on the integration of different treatments of the same or different nature in a multifaceted approach. For satisfactory penicillium decay control, this postharvest approach should be part of an integrated disease management (IDM) programme in which preharvest and harvest factors are also considered.

**Limitations:** The lack of either curative or preventive activity, low persistence, high variability, inconsistency or excessive specificity are general limitations associated with the use of alternatives to synthetic fungicides as stand-alone treatments. Furthermore, the risk of adverse effects on fruit quality, technological problems for cost-effective application, or the availability of new conventional fungicides for traditional markets are additional reasons that may hinder the broad commercial use of such treatments.

**Directions for future research:** As we learn more about the fundamental basis underlying host-pathogen interactions and how they are influenced by direct or indirect protective effects of existing or new single alternative treatments, more effective methods of applying and combining complementary approaches for additive or synergistic effects will emerge. Research should provide appropriate tools to tailor the application of these nonpolluting postharvest control systems and, further, the complete IDM strategy for each specific situation (ie, citrus species and cultivar, climatic and seasonal conditions, destination market, etc).

**Keywords:** *Penicillium digitatum*; *P. italicum*; physical control; low-toxicity chemical control; biocontrol; integrated disease management

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### Abbreviations

<b>AZX</b>	Azoxystrobin
<b>CA</b>	Controlled Atmosphere
<b>FLU</b>	Fludioxonil
<b>GRAS</b>	Generally Regarded as Safe
<b>IDM</b>	Integrated Disease Management
<b>IZ</b>	Imazalil
<b>PS</b>	Potassium Sorbate
<b>RH</b>	Relative Humidity
<b>SB</b>	Sodium Benzoate
<b>SBC</b>	Sodium Bicarbonate
<b>SC</b>	Sodium Carbonate
<b>SOPP</b>	Sodium ortho-phenyl phenate
<b>TBZ</b>	Thiabendazole
<b>UV-C</b>	Far Ultraviolet Radiation

### Introduction

Green and blue moulds, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively, are the most economically important postharvest diseases of citrus in all production areas that like Spain, California or Israel, are characterised by low summer rainfall [1\*]. Actual losses due to these diseases are quite variable and depend on the area of production, citrus variety, tree age and condition, weather conditions during the growing and harvest season, the extent of physical injury to the fruit during harvest and subsequent handling, the effectiveness of antifungal treatments, and postharvest environment. Both *P. digitatum* and *P. italicum* are strict wound pathogens that can infect the fruit in the grove, the packinghouse, and during distribution and marketing. They reproduce very rapidly, and their spores are ubiquitous in the atmosphere and on fruit surfaces and are readily disseminated by air currents. Therefore, the source of fungal inoculum in citrus groves and packinghouses is practically continuous during the season. The surface of virtually every citrus fruit that arrives at the packinghouse is contaminated with conidia and the inoculum may build up to high levels if appropriate packinghouse sanitation measures are not adopted [2]. Furthermore, citrus fruit can become “soiled” with conidia of the two fungi that are loosened in handling of diseased fruit. The conidia situated in injuries that rupture oil glands or penetrate into the albedo of the peel usually bring irreversible infection within 48 h at 20–25°C [1\*, 3]. The germination of conidia of both *Penicillium* species inside rind wounds requires free water and nutrients [4, 5], and is stimulated by volatiles emitted from the host tissue [6, 7]. Disease development is mediated by complex interactions between pathogen virulence mechanisms and host defence responses. Extensive research work is being conducted to analyse and understand such interactions at either the biochemical or molecular level [8\*–16].

Worldwide, both diseases have been primarily controlled for many years by the application of conventional fungicides such as imazalil (IZ), sodium ortho-phenyl phenate (SOPP), thia-

benzazole (TBZ) or different mixtures of these compounds. Currently, new active ingredients such as fludioxonil (FLU), pyrimethanil, azoxystrobin (AZX) and trifloxystrobin, most of which are classified by the United States Environmental Protection Agency as “reduced-risk” fungicides, have been extensively assayed in Europe or the USA [2, 17–23]. Postharvest treatments with these synthetic chemicals are typically relatively inexpensive, easy to apply, have curative action against pre-existing or established infections and persistent preventive action against potential new infections that can occur after their application in the packinghouse, and many also inhibit the sporulation from lesions on decaying fruit that reduces airborne inoculum production to break infection cycles. Among fruits treated with conventional fungicides, losses are typically 2–4%, while without postharvest treatment or refrigeration, losses of 15–30% occur within 1–3 weeks after harvest [24, 25\*\*]. However, concerns about environmental contamination and human health risks associated with fungicide residues periodically led to regulatory reviews and potential restrictions or cancellations. Likewise, traditional citrus export markets are increasingly demanding products with lower levels of pesticides in order to satisfy the safety demands from the general public. In addition, new higher-value markets based on organically grown, sustainable, environmentally friendly, ecological or green agricultural produce are currently arising and becoming more popular. Furthermore, the widespread and continuous use of these synthetic compounds has led to the proliferation of resistant biotypes of both *P. digitatum* and *P. italicum* and the build-up of single, double and even triple-resistant isolates in the population of the pathogens in commercial packinghouses seriously compromises the effectiveness of these treatments [26–29]. There is, therefore, a clear and increasing need to find and implement control methods alternative to conventional fungicides for the control of postharvest green and blue moulds of citrus fruit. If conventional chemicals are not used, the goal is to accomplish satisfactory decay control by adopting integrated disease management (IDM) programmes [30, 31\*]. The purpose of such strategy, based on the knowledge of pathogen biology and epidemiology and the consideration of all preharvest, harvest and postharvest factors that may influence disease incidence, is to minimise decay losses with no adverse effects on fruit quality by taking cost-effective action on every one of those factors at the right moment. Besides preharvest, harvest and transport considerations, attention should be devoted during the postharvest phase to three basic aspects when establishing a penicillium decay control programme: effective fruit and packinghouse sanitation to reduce atmospheric and superficial inoculum levels of *P. digitatum* and *P. italicum*; appropriate practices during handling and storage to maintain fruit resistance to infection; and adoption of suitable nonpolluting antifungal treatments to replace the use of conventional fungicides [1\*, 25\*\*]. According to their nature, these alternative decay control methods can be physical, chemical or biological. The purpose of this article is to review significant research work, giving emphasis to that published during the last few years, in which the most important of these control methods have been evaluated for the control of citrus postharvest green and blue

moulds, either alone or in combination with other treatments. Potential benefits, disadvantages and commercial feasibility of the application of these methods are discussed.

### Physical control methods

Major benefits from the use of physical treatments for fungal control are doubtlessly the total absence of residues on/in the treated produce and minimal environmental impact. General disadvantages, however, include limited and variable efficacy and lack of preventive activity and persistence. Nevertheless, it has been observed that the application of heat, far ultraviolet radiation (UV-C) or other physical treatments may, under certain conditions, initiate some defence mechanisms in citrus fruit tissues. Cold storage and controlled atmospheres (CAs) are complementary physical tools to reduce or inhibit the development of the pathogens and maintain fruit resistance to infection.

#### Heat treatments: curing and hot water

Typical procedures for thermal curing treatment of citrus employ exposure of fruit for 2–3 days to an air atmosphere heated to temperatures higher than 30°C at high relative humidity ([RH] > 90%). Since it was first reported by Hopkins and Loucks in 1948 [32], numerous studies demonstrated the elevated curative activity of curing treatments against green mould in a variety of citrus species and cultivars [33–37\*, 38\*, 39\*]. Control of blue mould, however, was less satisfactory when fruit were cold-stored for long periods after treatment [34]. In spite of their good efficacy, commercial implementation of curing treatments for citrus decay control is rare, firstly because of the expense of heating and immobilising large amounts of fruit for relatively long periods and, secondly, because excessive or uncontrolled treatments may harm fruit quality [40, 41]. Fruit weight loss and heat phytotoxicity are major potential risks whose incidence depend not only on treatment conditions but also on the type of fruit and their initial condition. In fact, only early season citrus fruit from Florida, Brazil or other high rainfall areas are nowadays commercially cured because these fruit are degreened with ethylene at temperatures of about 30°C. Besides combination with other control methods, which will be discussed later in this review, new technological approaches for curing treatment include intermittent curing (two 18-h cycles at 38°C) [42], curing at higher temperatures for reduced periods of time (18 h at 40°C) [43] or, in the case of low rainfall areas where early season mandarins are degreened with 5–10 µL/L ethylene at about 20°C for 2–3 days, the integration of curing treatment in the degreening process [44\*]. On the other hand, it has been recently determined that exposure to hot air at 50°C and RH higher than 75% for 1 day effectively killed spores of *P. digitatum* and could be a good sanitation practice for empty storage rooms [45].

Treatments with hot water are a technology easier, cheaper, and more feasible for heat application than curing. Relatively brief immersions (2–5 min) in water at 45–55°C have repeatedly shown value in reducing citrus green and blue moulds

[35, 46–52\*, 53\*]. Likewise, good results have been obtained with packingline machinery where hot water at 55–65°C is applied for 10–30 s over rotating brushes [54–56\*]. However, commercial application of hot water as a stand-alone treatment for citrus decay control is limited to small fruit like kumquat, whose peel is also eaten, or some organically-grown fruit [57\*]. This is primarily because hot water treatments are not fungicidal or very persistent, the range of effective yet non-phytotoxic temperatures is very narrow, and the effectiveness is greatly dependent on type, age, and physical and physiological condition of the fruit [51, 52\*, 58].

The mode of action of heat can be direct on the pathogen by inhibiting spore germination or mycelial growth of *P. digitatum* or *P. italicum* or indirect on the host by inducing different mechanisms of resistance in the rind wounds such as melting of peel waxes, maintenance of the activity of preformed antifungal compounds, and biosynthesis of lignin-like materials, phytoalexins, pathogenesis-related proteins or heat shock proteins [8\*, 57\*, 59, 60\*, 61\*\*, 62\*\*].

#### Irradiation treatments: UV-C and ionising radiation

Exposure to low doses (0.5–8 kJ/m<sup>2</sup>) of UV-C (wavelength from 100 to 280 nm) has significantly reduced the incidence of green or blue moulds in different citrus species and cultivars, although the effectiveness of the treatment and the risk of phytotoxicity varied with irradiation dose and duration, fruit type and maturity, and fruit harvest season and storage conditions [39\*, 63–65\*, 66\*]. Despite the direct germicidal effects of illumination with UV-C at 254 nm on conidia and mycelia of both *P. digitatum* and *P. italicum* [67, 68], the prevalent mode of action of this treatment for penicillium control in citrus fruit is the stimulation of beneficial responses in the host when applied at sublethal doses (hormesis). Responses to UV-C caused, in many ways similar to heat treatment, induction in rind tissues of resistance to fungal infection. Some of these fruit defensive reactions have been identified: alteration of the levels of preformed antifungal flavonoids such as some polymethoxyflavones or flavonones [69], accumulation of pathogenesis-related proteins such as chitinase or β-1,3-endoglucanase [8\*, 70], or induction of the activity of enzymes such as phenylalanine ammonia lyase or peroxidase that are related to the activation of plant defence mechanisms such as the biosynthesis of phytoalexins [65\*, 66\*, 71\*]. Although an on-line UV-C apparatus to treat harvested fresh fruit was developed [72] and currently there is increasing commercial interest to design suitable prototypes for either intact or fresh-cut produce, a number of issues will have to be addressed before realising the practical implementation of UV-C systems in citrus packing-houses. Illumination devices should be appropriately integrated in the packinglines to provide continuous effective treatment of the entire area of the fruit rapidly enough for commercial purposes. At the same time, the system should be flexible enough to change treatment conditions as a function of particular fruit attributes and destination. Currently, considerable attention is on pulsed light (synonyms: pulsed UV

light, pulsed white light), which use short time pulses of intense broad spectrum, rich in UV-C light, and is claimed as an improved technology compared with classic continuous-wave UV-C light delivering [73\*]. To our knowledge, however, this technique has not been specifically tested against citrus penicillium moulds. In any case, besides scaling-up efficacy trials, additional research on the effects of UV-C on fruit physiology, sensory quality and consumer acceptance is also needed before attempting to use this technology at a commercial scale.

Ionising radiation of fresh fruits and vegetables is not permitted at doses exceeding 1,000 Gy (100 krad) [74] and can be performed with radioactive ( $^{60}\text{Co}$  or  $^{137}\text{Cs}$ ,  $\gamma$ -rays) or machine sources (electron beams and X-rays). Conidia of both *P. digitatum* and *P. italicum* were found in early research to be highly sensitive to  $\gamma$ -irradiation [75\*, 76], but effective control of their established infections on oranges or lemons required irradiation doses higher than 1,000 Gy and, in general, such doses induced apparent rind injury [77–79\*]. It is primarily for this reason that ionising radiation as a single treatment for decay suppression cannot be commercially adopted, despite the fact that some beneficial effects have been associated with radiation exposure, including: high penetration power, stimulation of the synthesis of bioactive or functional phenolic components including different antifungal compounds, and extension of shelf-life by delaying ripening and senescence [79\*–81].

#### **Complementary physical methods**

In general, conventional cold storage or storage in controlled or modified atmospheres can be considered as complementary physical tools for postharvest decay control of fresh fruits and vegetables. These systems cannot be used as stand-alone antifungal treatments because typically they only provide fungistatic activity by inhibiting or delaying the growth and development of the pathogens. In addition, they considerably reduce the metabolic activity of the host, delay its senescence, and therefore contribute to the maintenance of fruit resistance to fungal infection.

The optimal growth temperature for both *P. digitatum* and *P. italicum* is 24°C. Green mould is predominant at ambient temperatures, but blue mould becomes more important when citrus fruit are cold-stored for long periods because *P. italicum* grows faster than *P. digitatum* below 10°C [82]. However, the development of both pathogens is greatly suppressed at typical orange or mandarin storage temperatures of 3–5°C. Citrus cold storage in conventional CA (5–10% O<sub>2</sub> + 0–5% CO<sub>2</sub> for oranges and mandarins and 5–10% O<sub>2</sub> + 0–10% CO<sub>2</sub> for lemons, limes and grapefruits) [83] has not been generally adopted because potential benefits do not compensate the high installation and maintenance costs. Results of early research are contradictory and both positive [84] and negative [85, 86] effects of CA on the incidence of postharvest decay have been reported. Other technological options involving CA such as modified atmosphere packaging, storage in either carbon monoxide CA (5% O<sub>2</sub> + 5–10%

CO) [83], low-pressure (hypobaric) CA [87\*], or ethylene removal from storage rooms [88, 89] may have beneficial effects on decay suppression, but they are not economically viable for fresh citrus fruit.

Storage in ozonated atmospheres and general ozone applications for sanitation and control of postharvest diseases of fresh fruits and vegetables have been recently reviewed [90\*]. Ozone (O<sub>3</sub>) is a highly reactive, potent biocide that has recently received regulatory approval for many food contact applications. It is a residue-free effective sanitiser, but its efficacy in controlling postharvest diseases cannot be predicted by its toxicity against free fungal spores and hyphae. Continuous or intermittent exposure to ozone gas at non-phytotoxic concentrations of 0.3–1.0  $\mu\text{L/L}$  does not control infection of fruit by *P. digitatum* and *P. italicum* in wounds and consequently does not reduce final disease incidence after storage. Gaseous ozone, however, inhibits aerial mycelial growth and sporulation of these fungi, which can help to reduce the proliferation of fungicide-resistant strains of the pathogens [91]. Nevertheless, these effects are transitory and limited to infected citrus fruit stored in highly vented packages or open-top containers that allow direct contact with the gas [92, 93]. Ozone, like other strong oxidant sanitisers such as hypochlorite or chlorine dioxide, readily kills free *Penicillium* conidia when they are immersed in ozonated water, but it fails to control infections in wounds already established in citrus fruit [90\*, 94]. Like all oxidising agents, ozone can harm humans if there is exposure to high concentrations for a sufficient duration. Therefore, issues related to the safety of workers and personnel must be addressed before the installation of ozone in air or water application systems in fresh citrus packinghouses.

#### **Chemical control methods**

Chemical alternatives to conventional fungicides for postharvest disease control should be natural or synthetic compounds with known and minimal toxicological effects on mammals and the environment. The origin of these alternatives includes classifications such as food additives and substances listed as GRAS (Generally Regarded as Safe) by the United States Food and Drug Administration, natural compounds obtained from plants, animals or microorganisms including some volatiles and essential oils, phenolic compounds, plant extracts, peptides, alkaloids, lectins, antibiotics, propolis, latex or chitosan [95\*\*, 96\*], and other chemicals such as calcium polysulfide or ammonium molybdate.

#### **Food additives and GRAS compounds**

In California for over 75 years, the standard method of cleaning oranges or lemons was to soak fruit for 2–4 min in a heated (43°C) solution of 4% borax (sodium tetraborate decahydrate) and 2% boric acid or 3% sodium carbonate (SC) within a day or two after harvest [97–99]. Soap or a detergent was usually added and the fruit were rinsed with a fresh water spray to remove salt residues from the surface. The borax bath treatment was abandoned because of residue issues and disposal of rinse water containing boron. SC (soda ash,

Na<sub>2</sub>CO<sub>3</sub>) or sodium bicarbonate (SBC, baking soda, NaHCO<sub>3</sub>) treatments remain in common use to today because they are effective and inexpensive food additives allowed with no restrictions for many applications including organic agriculture [100–102\*\*]. While they can also be applied effectively through high-pressure washer nozzles [103], low-volume spray applications over rotating brushes are avoided because their efficacy is lower and calcium carbonate scale accumulates on the brushes. Although their effectiveness is lower in mandarins than lemons or oranges, good control of penicillium moulds and fair control of sour rot, caused by *Geotrichum citri-aurantii*, is obtained with these treatments, especially if heated solutions and prolonged immersion times are used [51, 52\*, 101, 102\*\*, 104, 105]. The mechanism of action of carbonate salts against penicillium decay is unclear. It appears to be due in part to the presence of an alkaline residue in wounds [3, 106, 107], although equimolar solutions of the same pH prepared from SC or SBC were more effective than those prepared from potassium or ammonium salts, which suggested that the sodium cation and other factors may be important [102\*\*]. In contrast, it was found in other work [108] that the effectiveness of potassium bicarbonate against green mould was equivalent to that of SBC at the same concentration.

Besides carbonates, other common food preservatives have been evaluated for the control of citrus green or blue moulds. Some short-chain organic acids such as formic, acetic or propionic acid have been assayed as fumigants [109–111\*] and some organic acid salts such as sodium propionate, sodium benzoate (SB) or potassium sorbate (PS) have been applied as aqueous solutions [112, 113\*\*]. Among more than forty food additives and low-toxicity chemicals tested, PS and SB were the most effective on oranges and lemons [113\*\*]. They were about equal in activity to each other and to SC. PS (C<sub>6</sub>H<sub>7</sub>O<sub>2</sub>K) was firstly evaluated against fungicide-resistant strains of *Penicillium* spp. [114, 115] and it has been applied to citrus fruit in commercial packinghouses to control decay, although its use for this purpose is rare and some regulatory approvals may not be current [116\*]. Immersion of fruit in heated solutions is the most effective method of application [116\*–118]. Advantages of PS are that *P. digitatum* and *P. italicum* developed little or no tolerance after prolonged and repeated exposure to it [119] and that disposal of used solutions would have fewer regulatory issues than the sodium salts SC or SBC [116\*].

In general, handicaps associated with the use of GRAS salt solutions include lack of preventive activity, limited persistence [51, 52\*, 102\*\*, 113\*\*, 116\*, 118], risk of fruit injury or weight and firmness losses during long-term storage if treated fruit have not been rinsed, reduction of treatment effectiveness by high-pressure water washing or rinsing, and disposal issues associated with high pH and sodium or potassium content [102\*\*, 120\*, 121]. Moreover, chlorine (200 µg/mL) should be added and maintained to kill conidia of *Penicillium* spp. in the solutions and on fruit surface [102\*\*,

122]. Some of these problems could be solved by the development of new technologies such as the incorporation of anti-fungal GRAS compounds as ingredients of new edible coatings or synthetic waxes.

## Natural compounds

### Volatiles and essential oils

A large variety of volatile compounds with antifungal activity have been isolated from plants: acetaldehyde, benzaldehyde, benzyl alcohol, ethanol, methyl salicylate, ethyl benzoate, ethyl formate, hexanal, (E)-2-hexenal, lipoxygenases, jasmonates, allicin, glucosinolates and isothiocyanates, etc [123]. Among them, jasmonates [124\*] and some aroma components like acetaldehyde, benzaldehyde, ethanol, ethyl formate, nerolidol and 2-nonanone [100, 125\*, 126] have been specifically tested against *P. digitatum* or *P. italicum*. A method based on the use of allyl-isothiocyanate on citrus fruit has been patented in Japan [127].

Aromatic plants, such as citrus, produce essential oils that basically contain volatile C<sub>10</sub> and C<sub>15</sub> terpenes derived from isoprene units. Caccioni *et al.* [128\*] stated that citral was the most potent monoterpene in citrus essential oils, although its two isomers geranial and neral were similarly toxic [129]. Citral has been described as a preformed antifungal component in the flavedo of citrus fruit associated with a first line of resistance to infection by *P. digitatum* [130, 131\*]. Other constitutive components present in oil glands are phenolic compounds such as flavanones (eg, narirutin, didymine, hesperidin), polymethoxylated flavones (eg, nobiletin, tangeretin, sinensetin) or *p*-coumaric acid (a precursor of coumarins) [132, 133\*\*]. A second line of defence would include the synthesis of phytoalexins (mainly coumarins such as scoparone, scopoletin, scopolin) in the fruit rind as a response to fungal challenge. Stress triggered by certain physical, chemical or biological postharvest treatments can induce the retention or biosynthesis of both preformed and induced volatile antifungal compounds with subsequent maintenance or induction of fruit resistance to disease [60\*, 66\*, 69, 71\*, 133\*\*–135]. Products to control green or blue moulds with components of essential oil from citrus peel as active ingredients have been described. The efficacy of citral against *P. digitatum* and *P. italicum* *in vitro* depended on the method of application [136], but exogenous application *in vivo* was phytotoxic and not promising [130]. Angioni *et al.* [137\*] isolated 7-geranoxycoumarin from grapefruit peel, a phenolic compound that effectively reduced decay and was not phytotoxic. Recently, a product containing essential oils and limonene hydroperoxides from citrus flavedo was developed that controlled green mould after either natural or artificial inoculation with *P. digitatum* [138].

Inhibitory activity of essential oils from plants other than citrus against *P. digitatum* and *P. italicum* has also been reported. Compounds from species of thyme, oregano, cinnamon, clove, dictamnus or mint were very effective *in vitro*, but

results from *in vivo* experiments were contradictory and applications to citrus fruit were often ineffective or phytotoxic [139–143\*, 144\*, 145\*\*, 146\*]. In fact, despite their potent antifungal activity, commercial implementation of treatments with essential oils is strongly restricted in citrus because of problems related to potential phytotoxicity, intense sensory attributes or technological application as fumigants or in aqueous solutions. The mode of action of essential oils on *P. digitatum* and *P. italicum* and other fungi has not been determined, and many aspects of essential oil toxicity remain unresolved [138]. It has been shown that their antimicrobial activity is dependent on their hydrophobicity and partition in microbial membranes [147]. Compounds with saturated carbonyl groups had less antifungal activity than their corresponding alcohols [138].

#### **Plant extracts**

Strictly, most of the volatiles, essential oils or phenolic compounds that have been mentioned are included in this section because they are active phytochemical components that can be isolated from certain extracts of plant tissues. Powders, gels and aqueous or organic solvent extracts of plants from different origins are reported to have activity against *P. digitatum* or *P. italicum* under different experimental conditions. These plants include *Aloe vera* [148], garlic [149], *Huamuchil* [150], *Thymus* sp., *Eucaliptus* sp., *Cistus* sp., *Juglans* sp., *Myrtus* sp. [146\*], *Acacia* sp., *Whitania* sp. [151\*] and a variety of weeds from Jordania [152].

#### **Peptides and proteins**

Plants and animals produce a variety of peptides and small proteins with antimicrobial activity that are presumed to be part of constitutive or inducible defence mechanisms against fungal infection [153, 154]. Their mechanism of action is presumed to involve the interaction of the amphipathic cationic peptide with the target cell membrane, followed by membrane disruption [155]. Several peptides from different origins have been identified, characterised and tested for activity against *P. digitatum* or *P. italicum*, and some have shown promise for the control of the diseases caused by these fungi [156–159\*, 160\*\*]. These researchers identified PAF26, a tryptophan-rich, cationic hexapeptide, which moderately controlled penicillium decay even caused by fungicide-resistant strains of the pathogens. Strategies envisioned to be feasible employ peptide synthesis by transgenic plants, either to protect the plant or to economically produce the peptides, since at present the high cost of synthetic peptides is a barrier to their practical application. Some new peptide derivatives of PAF26 with broader spectrum activity have also been recently obtained [161].

#### **Chitosan and derivatives**

Chitin is a primary constituent of crustacean shells, insect cuticles and fungal cell walls [162]. Chitosan, its deacetylated soluble form, has wide antifungal properties and, at low concentrations, can elicit defensive responses in fresh fruit against phytopathogenic fungi. Chitosan and its derivatives

such as glycolchitosan can be used in solution, powder form or as wettable coatings [96\*]. Antifungal activity against *P. digitatum* or *P. italicum*, *in vivo* significant reduction of citrus penicillium decay, and fruit senescence retardation during long-term cold storage of different citrus species and cultivars have been observed after application of certain chitosan formulations [163–166\*].

#### **Other chemicals**

Liquid lime sulphur solution, an inexpensive and widely available fungicide that contains calcium polysulfide, is often used by organic growers on many crops before harvest. As a postharvest treatment, it was approved for use on citrus fruit in California and Arizona because, if heated, it is equal or superior in effectiveness to SCs for the control of green mould and sour rot. However, it has not become popular because of the objectionable sulphide odour it emits and its corrosiveness to some packinghouse equipment [104]. The fertiliser ammonium molybdate [113\*\*] and the inducer of disease resistance  $\beta$ -aminobutyric acid [167\*] have also shown activity against citrus penicillium moulds. Schirra *et al.* [168] developed a new effective postharvest antifungal product by complexation of IZ with beta-cyclodextrin. On the other hand, it has been repeatedly observed that fumigation with the ethylene inhibitor 1-methylcyclopropene to prolong postharvest life of stored citrus fruit increased the incidence of postharvest decay [13, 169, 170].

#### **Biological control methods**

In this review, this category will be restricted to the utilisation of microbial antagonists. Substantial progress has been made in developing antagonistic microorganisms for the control of postharvest diseases [171–175\*, 176\*, 177\*\*]. During the last two decades, numerous strains of yeasts (eg, *Candida oleophila* [178\*\*–180], *Candida guilliermondii* (syn.: *Pichia guilliermondii*, *Debaryomyces hansenii*) [181–185\*\*), *Candida saitoana* [163, 186\*, 187\*], *Candida famata* [188\*, 189], *Metschnikowia fructicola* [190\*, 191], *Metschnikowia mulcherrima* [192\*], *Rhodotorula glutinis* [193], *Cryptococcus laurentii* [194\*], *Kloeckera apiculata* [195, 196\*], *Pichia anomala* [197]), bacteria (eg, *Pseudomonas syringae* [198\*\*–201], *Pseudomonas cepacia* [202–204], *Pseudomonas glathei* [205\*], *Pantoea agglomerans* [206–209\*], *Bacillus subtilis* [210–214\*, 215\*], *Bacillus pumilus* [216], *Serratia plymuthica* [217]) and filamentous fungi (eg, *Trichoderma viride* [218], *Verticillium lecanii* [164], *Aureobasidium pullulans* [219, 220]) have been selected, identified and characterised because of their biocontrol activity against citrus green or blue moulds. However, by the early 2000s, there were only two postharvest biological products registered for use against postharvest rots of citrus fruit that were available on the market: Aspire™ (*C. oleophila*, limited to the USA and Israel) and BioSave™ (*P. syringae*, limited to the USA). Other products (Biocure, Bio-Coat) were developed with *C. saitoana* [221], but have not reached the marketplace yet. Another recently developed product is based on the use of a heat-tolerant strain of *M. fructicola* and is marketed under the name Shemer™ in Israel by the company AgroGreen Ltd.

(Ashdod, Israel). Besides *Penicillium* spp., it has been shown to be also effective against rots caused by *Botrytis cinerea*, *Rhizopus* spp., and *Aspergillus* spp. on citrus, strawberries and grapes [190\*, 222–224]. Depending on the antagonist, the pathogen and the fruit host, different modes of action might explain the biocontrol activity of antagonistic microorganisms: competition for nutrients and space, secretion of antibiotics, direct effects of the antagonist on the pathogen or induction of host defence mechanisms [8\*, 175\*, 225, 226\*, 227\*\*]. In general, microbial antagonists are used as aqueous cell suspensions in postharvest spray, drench or dip applications. On citrus fruit, some of them have been tested as preharvest treatments [225] and others as active ingredients in fruit coatings [163, 185\*\*, 187\*, 221, 228–230]. An unusual case is the control of citrus penicillium decay by biofumigation with volatile compounds produced by grain cultures of the fungus *Muscodor albus* [231\*\*].

In spite of the large volume of research published about postharvest biocontrol of citrus rots, the commercial use of these products was and remains limited and accounts for only a very small fraction of the potential market. As discussed in several reviews [173, 174, 176\*, 177\*\*, 232], the main shortcoming of the use of postharvest biocontrol products has been inconsistency in their performance, especially when used as a stand-alone product to replace synthetic fungicides. Furthermore, another important handicap for current commercial adoption in EU countries of such products is the strict regulatory issues that prevent registration [233]. The combination of biological control with other control methods is one of the most promising means of establishing effective nonpolluting integrated control systems [173, 234–236\*] and will be later discussed in this review. Other approaches to enhance the biocontrol activity of antagonistic microorganisms include the addition of nutrients such as certain nitrogenous compounds [172] or genetic manipulation of the antagonists. Efforts to identify genetic traits of the yeast *C. oleophila* and determine its potential to enhance biocontrol activity showed that both chitinase and glucanase activities are constitutively produced by the yeast in culture and in planta. *CoEXG1*, a  $\alpha$ -1,3-glucanase gene of the yeast biocontrol agent *C. oleophila* was cloned from a partial genomic library as a segment containing the open reading frame and the promoter [237\*]. Transformants with double copy of *CoEXG1* exhibited two fold  $\alpha$ -1,3-glucanase activity compared with the wild type. When tested on citrus fruit against *P. digitatum*, biocontrol efficacy of the transformant overexpressing glucanase gene was not significantly enhanced [238\*]. Another important aspect to improve the commercial performance and generalise the use of biocontrol agents is the development of stable, reliable and economically acceptable product formulations [207, 239, 240].

### **Combination of treatments for integrated disease management**

Successful commercial control of postharvest diseases of fruits and vegetables must be extremely efficient, in the range

of 95–98%, unlike the control of tree, field crop or soil borne diseases. Consistent performance to such levels of control cannot presently be achieved by alternatives to fungicides as stand-alone treatments, so strategies where they are combined are needed to attain commercially acceptable performance. Therefore, researchers have devoted considerable attention to the integration of different treatments in order to overcome the variable performance and augment the efficacy of existing alternative approaches. In general, three objectives may be pursued by the integration of two or more treatments: additive or synergistic effects to increase the effectiveness or the persistence of individual treatments; complementary effects to combine preventive and curative activities; and potential commercial implementation of effective treatments that are too impractical, costly or risky as single treatments. For example, combinations of treatments can be made to reduce the length and cost of curing treatments or reduce the dose and phytotoxicity risk of irradiation treatments.

Most of the research on the combination of alternative treatments to control citrus green and blue moulds included postharvest heat or biocontrol treatments as components of an integrated strategy, so particular subsections will focus on these combinations. Ionising radiation at low doses combined with reduced levels of either conventional fungicides (eg, SOPP, diphenyl) [241, 242] or GRAS compounds (eg, SC) [121], and conventional fungicides at low doses combined with GRAS compounds or sanitisers (eg, SBC, PS, chlorine) [20, 22, 116, 243–245\*] are other options that have been assessed.

On the other hand, there is an increasing interest in the application of antifungal preharvest treatments to reduce field populations of *Penicillium* spp. or induce fruit resistance as part of IDM programs. Therefore, fungicides such as benomyl, cyprodinil, thiophanate methyl, pyraclostrobin, AZX, FLU and phosphorous acid, and other chemicals such as several carbonates, calcium chloride ( $\text{CaCl}_2$ ), dichlorophenoxyacetic acid, gibberellic acid and a mannaoligosaccharide (ISR 2000®) have been recently evaluated for these purposes [39\*, 192\*, 244, 246–249\*].

### **Combination of heat with other control methods**

#### *Combination with other physical control methods*

In order to reduce potential negative impacts of antifungal treatments on citrus fruit quality, curing or hot water treatments have been combined with variable results with individual plastic packaging of fruit [33, 54], ionising radiation at low doses [47, 78, 250\*], UV-C treatments [39\*, 251, 252] or brief  $\text{CO}_2$  shocks [253\*].

#### *Combination with chemical control methods*

It has been repeatedly reported that heating aqueous solutions of either conventional fungicides [17, 20, 58, 243–245\*, 254–257] or low-toxicity alternative chemicals such as SC, SBC [51, 52\*, 100–102\*\*, 105, 245\*, 258\*\*], PS [113\*\*,

116\*–118, 259], SB, sodium and ammonium molybdates [113\*\*], ethanol, sulphur dioxide [100] or calcium polysulfide [104] significantly enhanced their effectiveness against penicillium moulds and other citrus postharvest diseases. Heat probably facilitates the uptake of the active ingredient through the fruit cuticle [61\*\*] in a similar way that it is facilitated by dip treatments in comparison to spray or drench applications [257, 260]. The most appropriate solution temperature should be specifically determined for each combination of active ingredient and fruit species and cultivar, but in general, if compared with hot water alone, similar effectiveness is obtained at much lower solution temperatures, which considerably reduces the risk of heat injury to the fruit. The combination of curing treatments with conventional fungicides [36, 39\*], GRAS compounds such as SC [38\*, 261\*] or ethanol [38\*], or postharvest surfactants such as dodecylbenzenesulfonate [37\*] also resulted in improved control of citrus green or blue moulds.

#### **Combination with biocontrol antagonists**

Heat treatments and the application of antagonistic microorganisms are complementary treatments that often show synergistic effects for the control of postharvest diseases. In some cases, both are components of complex integrated control strategies that also include other control means [234–236\*].

Thermal curing or hot water treatments have been successfully combined with microbial antagonists for citrus penicillium decay control, including *C. oleophila* [38\*, 258\*\*], *C. famata* [189], *M. mulcherrima* [192\*], *P. glathei* [205\*], *B. subtilis* [214\*] and *P. agglomerans* [262\*].

#### **Combination of biocontrol antagonists with other control methods**

##### **Combination with physical control methods**

Besides heat treatments, other physical control means that have been combined with the application of antagonistic microorganisms to control of citrus green or blue moulds include UV-C illumination and storage in CAs. The application of UV-C in combination with the yeast antagonist *D. hansenii* completely inhibited the development of *P. digitatum* on Dancy tangerines [263\*]. While similar results were obtained on navel oranges with the combination of UV-C and the yeast *C. oleophila*, no synergistic effects were observed when UV-C was combined with the bacterium *B. subtilis* [264]. Satisfactory decay control was found on clementine mandarins previously treated with the bacterium *P. agglomerans* and stored for 60 days at 3.5°C in 5 kPa O<sub>2</sub> + 3 kPa CO<sub>2</sub>. These storage conditions did not adversely affect the viability of the antagonist on fruit surface wounds (Palou, Usall, and Viñas, unpublished results).

##### **Combination with low levels of conventional fungicides**

In laboratory and large scale tests, biocontrol products such as Aspire™ and BioSave™ often provide a level of control equivalent to synthetic fungicides only when combined with

low doses of these fungicides [178\*\*, 192\*, 265–268\*]. For instance, *C. oleophila* in combination with 200 µg/mL of TBZ controlled citrus decay at the level equivalent to a commercial fungicide treatment, where TBZ is often used at 10 to 20 times this concentration, and reduced the variability often observed when using the antagonistic yeast alone [178\*\*].

##### **Combination with food additives and other chemicals**

Among the low toxicity chemicals examined to enhance biocontrol efficacy against *P. digitatum* or *P. italicum* were ethanol [38\*], peracetic acid [191] and oxalic acid [180]. However, SCs (especially SBC) are the additives that have been most widely evaluated for synergistic activity with microbial antagonists. Their combination with *P. syringae* [102\*\*], *P. agglomerans* [209\*, 269\*\*, 270], *C. oleophila* [38\*, 258\*\*], *B. subtilis* [214\*] or *C. laurentii* [194\*] was superior to either treatment alone in controlling green or blue moulds on different citrus species and cultivars.

The addition of calcium chloride to citrus fruit increased the protective effect of the antagonist *P. guilliermondii* and also greatly reduced the populations of yeasts required to give effective control [271\*\*]. The combination of *C. saitoana* with a low dose of 0.2% (w/v) of the sugar analog 2-deoxy-D-glucose applied to fruit wounds before inoculation was more effective in controlling decay of orange and lemon caused by *P. digitatum* than either *C. saitoana* or 2-deoxy-D-glucose alone [186\*]. These results were confirmed in semi-commercial trials [272].

##### **Combination with chitosan and derivatives**

A biocontrol preparation termed “bioactive coating” that consists of a unique combination of the antagonistic yeast *C. saitoana* with chemically-modified chitosan (0.2% glycolchitosan) was evaluated in laboratory and semicommercial studies against *P. digitatum* on oranges and lemons. The biocontrol activity of *C. saitoana* was markedly enhanced by the addition of glycolchitosan and the combination made it possible to synergistically exploit the antifungal properties of both treatment components [163, 187\*].

## **Conclusion**

As this review makes evident, extensive research work has been conducted worldwide for many years and continues today to identify, evaluate, select, characterise and eventually implement alternative means to conventional synthetic fungicides for the control of postharvest penicillium moulds of citrus fruit. These sustained efforts are warranted by the economical importance of postharvest losses caused by *P. digitatum* and *P. italicum* in all citrus growing areas and the increasing market and social pressure to adopt safe nonpolluting technologies for fresh fruit production. Particularly, consumer safety concerns are more important for postharvest pesticide treatments than for field applications, because the residues are likely to be present on the fruit at the time of consumption.

Despite the evident substantial progress that has been accom-



plished, the commercial use of available alternative postharvest antifungal treatments has been rather limited given the potential market. The lack of either curative or preventive activity, low persistence, high variability, cost, inconsistency or excessive specificity are general limitations associated with the nature of alternative physical, chemical or biological control methods. As stated once by a student: "... it is not going to be easy to kill with no poison...". Furthermore, the risk of adverse effects on fruit quality, technological problems for cost-effective application or the availability of new conventional fungicides for traditional markets are additional reasons that may hinder the broad commercial use of such treatments. As we learn more about the fundamental basis underlying host-pathogen interactions and how they are influenced by direct or indirect protective effects of existing or new alternative treatments, more effective methods of applying and combining complementary approaches for additive or synergistic effects will emerge. So far the results obtained with combinations of antifungal treatments demonstrate the promise of this multifaceted integrated approach to become a viable alternative to the use of synthetic fungicides. Once developed, these alternatives should prove durable and valuable. The complexity of the mode of action associated with combined alternative treatments should make the development of pathogen resistance unlikely and provide higher levels of stability and effectiveness than approaches relying on single mode of action treatments.

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\* Marginal importance

\*\* Essential reading

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