International Journal of Food Microbiology 166 (2013) 1–14

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Review

Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods

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ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form 21 May 2013
Accepted 30 May 2013
Available online 6 June 2013

Keywords:
Antifungal
Plant extracts
Essential oils
Food

ABSTRACT

Food decay by spoilage fungi causes considerable economic losses and constitutes a health risk for consumers due to the potential for fungi to produce mycotoxins. The indiscriminate use of synthetic antifungals has led to the development of resistant strains which has necessitated utilization of higher concentrations, with the consequent increase in toxic residues in food products. Numerous studies have demonstrated that plant extracts contain diverse bioactive components that can control mould growth. The metabolites produced by plants are a promising alternative because plants generate a wide variety of compounds, either as part of their development or in response to stress or pathogen attack. The aim of this article is to summarize the results from the literature on *in vitro* and *in vivo* experiments regarding the effects of plant-derived products for controlling fungal growth. Data from research work on the mode of action of these metabolites inside the fungal cell and the influence of abiotic external factors such as pH and temperature are also covered in the present review. Furthermore, an analysis on how the stress factor derived from the presence of plant extracts and essential oils affects secondary metabolism of the fungus, specifically mycotoxin synthesis, is developed. Finally, the effectiveness of using plant-derived compounds in combination with other natural antimicrobials and its application in food using novel technologies is discussed.

Contents

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0168-1605/$ – see front matter
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.05.026

1. Introduction

Numerous pests and diseases attack food crops around the world; most of them are related to pathogenic fungal diseases. Worldwide, post harvest losses have been estimated at 50% and much of this is due to fungal and bacterial infections (Magro et al., 2006). Moulds are ubiquitous biological agents that are able to colonize foods because of their potential to synthesize a wide diversity of hydrolytic enzymes. They cause pathologic disorders in plants bringing considerable economic losses for food producers.

Fruits and vegetables are highly susceptible to fungal spoilage, both in the field and during postharvest storage. Significant genera include *Pythium*, *Phytophthora*, *Fusarium*, *Penicillium*, *Alternaria*, *Botrytis*, *Geotrichum*, *Sclerotinia* and *Rhizoctonia* spp. Fungal growth on fresh fruits and vegetables is responsible for food spoilage and numerous plant diseases, which lead to significant economic losses. Mould growth depends on abiotic factors such as pH, water activity (a_w), solute concentration, temperature, atmosphere, time, *etc.* However, conditions of temperature and a_w are the main variables determining the development of fungi. Grain crops are also vulnerable to fungal contamination, with *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* being the most frequent genera. In this matrix, moulds are responsible for off-flavor formation and contribute to heating and loss in dry matter in grains through the utilization of carbohydrates as an energy source, degradation of lipids and proteins, production of volatile metabolites and production of allergenic compounds. This causes a reduction in the quality of animal feed and seed (Magan and Aldred, 2007). These events can take place even before the fungal growth is evident (Lee et al., 2007).

Apart from causing diseases in plants, many species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* can also synthesize mycotoxins. These compounds are hazardous to animal and human health as they can be lethal, carcinogenic, mutagenic, teratogenic, immunosuppressant, or may mimic estrogens. Their activity depends on the type of toxin and their concentration in the food. Concern about these chemical hazards has been increasing due to the wide range of food types that may be affected and the variability in the severity of symptoms caused. The presence of mycotoxins in food is associated with fungal inoculum on predisposed substrates. Mycotoxins can be produced before and after harvest and levels may increase during postharvest handling and storage. Thus prevention of fungal growth is an effect means of preventing mycotoxin accumulation. Mycotoxins may reach consumers either by direct contamination of plant materials or products thereof, or by 'carry over' of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed. Furthermore, this hazard remains in processed food because these metabolites are not removed by normal industrial processing, and the risk could increase if mouldy fruits or plants are used in processed byproducts.

Inhibition of fungal growth in crops, fresh fruits and vegetables is thus necessary to reduce the risk to human and animal health. However, it is important to note that partial inhibition of fungal growth, such as reduction of fungal growth rate, could enhance mycotoxin production as a response of the mould to stress.

2. Drawbacks of synthetic fungicides

The first step in fighting fungal contamination is the application of fungicides in the field. Fungitoxicants can be applied postharvest, provided they do not adversely affect the appearance or quality of the treated commodities (Amiri et al., 2008). Antimicrobial chemicals such as benzimidazoles (*e.g.* thiabendazole), aromatic hydrocarbons (*e.g.* sodium ortho-phenylphenate) and sterol biosynthesis inhibitors (*e.g.* imazalil, a sterol demethylation inhibitor) have been used for decades in control of plant diseases in agriculture. More recently, two other fungicides, each with different mode of action, have become important in the market: pyrimethanil (anilinopyrimidine) and fludioxonil (phenylpyrrole). The indiscriminate and excessive use of fungicides in crops has been a major cause of the development of resistant pathogen populations, resulting in the use of higher concentrations of these antifungals and the consequent increase in toxic residues in food products. For example, acquired resistance by *Penicillium italicum* and *P. digitatum* to many synthetic fungicides currently used on citrus fruit has been demonstrated (Fogliata et al., 2001). Some of these compounds are not biodegradable, so they can accumulate in soil, plants and water, and consequently affect humans through the food chain. Although chemical treatments have been considered to be the cheapest and most effective way to prevent postharvest diseases, the development of resistant microorganisms has reduced their acceptance. The type and concentration of fungicides allowed for postharvest application are restricted due to their long degradation period and potential effects on food and human health (carcinogenicity, teratogenicity, high and acute residual toxicity, hormonal imbalance and spermatotoxicity). Because of these undesirable effects, recent studies resulted in the revocation of registration of some of the more effective fungicides. Furthermore, public concern about food contamination with fungicidal residues has significantly increased. Considering all these factors, the development of new safe and biodegradable alternatives that are both effective and economically feasible is needed.

3. Low impact chemical preservative agents

In recent years, consumers' preferences are moving towards foods that contain lower levels of chemical preservatives and exhibit more fresh-like and natural characteristics. The salts of weak acids, such as sodium benzoate and potassium sorbate, can inhibit growth of several postharvest fungal pathogens. Using these compounds for fungal inhibition presents several benefits, such as their low mammalian toxicity, a wide spectrum of activity and relatively low cost. However, high concentrations of these compounds are needed to act as fungicides, bringing associated potential organoleptic changes. For example, calcium propionate completely inhibited mycelial growth of *Botrytis cinerea* at a level of 5% (w/v) (Droby et al., 2003). Benzoic acid, one of the most broadly used antimicrobials, is permissible at levels up to 0.1% (Jay, 2000). It is commonly applied in the form of benzoates, mainly as sodium benzoate, due to the higher solubility of the salts. In general, optimal inhibitory activity takes place at low pH since acid conditions favor the undissociated form of the molecule that freely crosses the plasma membrane of the target cell. Inside the cell, the molecule will dissociate due to a higher pH; the preservative action is thought to be due to an accumulation of anions and protons inside the cell (Brul and Coote, 1999; Jay, 2000). Cytoplasmatic pH decrease due to the entry of the undissociated state of the compound may cause the rupture of certain metabolic reactions of the microorganism, leading to the permeabilization of the cytoplasmic membrane and cell death. Other compounds frequently used for their fungistatic activity are the antioxidants butylated hydroxyanisole (BHA), propyl paraben (PP) and butylated hydroxytoluene (BHT). Like benzoic acid, they are considered Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (FDA), which also allows their use as antimicrobial agents in food. The Codex Alimentarius (2006) established the maximum usage level for single or multiple antioxidants as 200 μg/g based on the weight of the fat or oil. Furthermore, nisin, monolaurin, and lactoperoxidase are examples of "natural" preservatives, but they have several limitations, which include limited spectrum of activity, high application costs, the potential emergence of resistant strains and their impact on the organoleptic properties of foods.

The application of the hurdle technology concept, the utilization of several preservative factors (hurdles) together at lower levels, could overcome these constraints. The use of naturally occurring antimicrobial compounds in combination with mild food processing treatments and chemical additives have been widely studied recently. The treated

food product will be microbiologically safe, retaining its sensory, nutritional and economic properties. New techniques such as high pressure, nanotechnology, irradiation, *etc.*, are increasingly being used, while new ingredients with functional properties contribute to improving healthy foods.

4. Plants as natural antifungals

4.1. Plant extracts and essential oils

In order to reduce the utilization of synthetic chemical fungicides in food, several alternative treatments have been studied. The metabolites produced by plants are a promising alternative because plants produce a wide variety of compounds, either as part of their development or in response to stress or pathogen attack. In recent years, they have attracted increasing interest because of their relatively safe status (many of them are considered GRAS by the FDA); they are easily decomposed, environmentally friendly and non phytotoxic. It has been proved that plant extracts obtained with different solvents and essential oils are rich in potentially bioactive compounds, such as phytoalexins. Many are known to have antimicrobial activity for plant protection, including alkaloids, flavonoids, isoflavonoids, tannins, cumarins, glycosides, terpens, phenylpropannes, and organic acids. Essential oils (EOs) are aromatic oily liquids obtained by hydrodistillation from plant material (whole tissues or seed) and are usually mixtures of several components. Essential oils and plant extracts have the potential advantage of being bioactive in their vapor phase, a characteristic that makes them attractive as possible fumigants for the protection of stored products. Their inherent antimicrobial activity is commonly related to the chemical structure of their components, the concentration in which they are present, and their interactions, which can affect their bioactive properties. They also may contain various antioxidant compounds such as polyphenols, phenols, flavonoids, *etc.* which have been thought to be the basis of their antimicrobial properties. The fact that they are constituted by a great variety of compounds confers on them other advantages, such as having different modes of action depending on the compound involved, which gives the ability to attack different fungal genera and hinders the development of resistance by the pathogen.

A lot of research has been done in this area during recent years. *Aspergillus* and *Fusarium* are the fungal genera most commonly used to test EOs and plant extracts, followed by *Penicillium* and other phytopathogenic genera.

Chenopodium ambrosioides oil inhibited the mycelial growth of two aflatoxigenic strains of *Aspergillus flavus* at 100 μg/ml. This oil also inhibited the growth of *Aspergillus fumigatus*, *Botryodiplodia theobromae*, *Fusarium oxysporum*, *Phythium debaryanum* and *Sclerotium rolfsii* (Kumar et al., 2007). *Peumus boldus* essential oil was active against *Aspergillus niger*, *A. flavus* and *Fusarium* spp. (Souza et al., 2005). Chamomile (*Anthemis nobilis* L.) and malva (*Malva sylvestris* L.) aqueous extracts at 0.92 and 0.6 g/ml, respectively inhibited the growth of four tested fungi *Aspergillus candidus*, *A. niger*, *Penicillium* sp., and *F. culmorum*, with malva the more effective, since a lower concentration was required for fungal inhibition (Magro et al., 2006). In research work performed by Viuda-Martos et al. (2008) the essential oils of lemon, orange, mandarin and grapefruit obtained by cold-pressing the peel showed, at the concentrations assayed, the capacity to reduce or inhibit the growth of *Penicillium chrysogenum*, *Penicillium verrucosum*, *A. niger* and *A. flavus*. For *A. niger* and *A. flavus* inhibition was achieved when a concentration of 0.94% of any of the EOs was used. The maximum reduction in *P. verrucosum* and *P. chrysogenum* growth was observed with grapefruit EO. Orange EO was the most effective against *A. niger*, while mandarin was the best inhibitor of *A. flavus*. Zabka et al. (2009) reported that EOs obtained from *Carum carvi*, *Cymbopogon nardus*, *Pelargonium roseum*, *Pimenta dioica* and *Thymus vulgaris* were effective against growth of target fungal species *F. oxysporum*, *Fusarium verticillioides*, *Penicillium expansum*, *Penicillium brevicompactum*, *A. flavus* and *A. fumigatus*. Vilela et al. (2009) showed that *Eucalyptus globulus* EO was clearly inhibitory toward the tested fungal species, *A. flavus* and *Aspergillus parasiticus*, both in contact and headspace volatile assays. Ravikumar Patil et al. (2007) reported that among the fungi tested, *A. niger* and *Penicillium* spp. were found to be sensitive to crude ethanolic *Thevetia peruviana* extract, recording a reduction in radial growth of 50%. A considerable reduction in sporulation was also recorded. Cinnamon leaf volatile oil was found by Singh et al. (2007) to be 100% effective against *A. niger*, *A. flavus*, *Fusarium moniliforme*, *Fusarium graminearum*, *Penicillium citrinum* and *Penicillium viridicatum*. Deba et al. (2008) tested the fungitoxic activities of the flower essential oils of *Bidens pilosa* (a plant widely distributed in the subtropical and tropical regions of the world) against *Fusarium* spp. They found that *Fusarium solani* was the most suppressed species, followed by *F. oxysporum*. Naeini et al. (2010) observed varying sensitivities to anti-*Fusarium* properties in five EO from herbs which have long been used as spices or important medicinal sources in Iran emphasizing the variability in the antifungal effects against different *Fusarium* isolates. The highest anti-*Fusarium* activity was found in the EO from *Cuminum cyminum* and *Zataria multiflora* against non-toxigenic (*F. solani* and *F. oxysporum*) and toxigenic (*F. verticillioides*, *F. poae* and *F. equiseti*) isolates studied, respectively (Naeini et al., 2010). Another study against *Fusarium* spp. was carried out by Velluti et al. (2004), who evaluated 37 essential oils. Five of them (lemongrass, cinnamon, clove, palmarosa and oregano) showed antifungal activity. Even though oregano and palmarosa oils were less effective in controlling growth of *Fusarium* species than lemongrass, clove and cinnamon oils, they significantly inhibited growth under the different conditions tested (concentration of oil 0–1000 μg/ml; temperature 20–30 °C and a_w 0.95–0.995). It is important to note that there were no significant interspecific differences in the responses to the essential oils; this suggests that the essential oils may be a good alternative with a broad spectrum of application (Velluti et al., 2004). Boyraz and Özcan (2005) reported that sater (*Satureja hortensis*) hydrosol showed considerable fungistatic activity against the pathogens *Rhizoctonia solani*, *F. oxysporum* f. sp. *tulipae*, *B. cinerea* and *Alternaria citri* followed in efficacy by pickling herb (*Echinophora tenuifolia*) and cumin (*C. cyminum*) hydrosols.

The genus *Alternaria* contains several pathogenic and toxicogenic species and is also frequently used as target for inhibition assays. Feng and Zheng (2007) studied the ability of five essential oils to inhibit *Alternaria alternata*. Cassia oil was most active followed by thyme oil. All the aqueous extracts obtained from leaves of twenty plant species from India at 10% concentration were effective in inhibiting the radial growth of *Alternaria solani*. The leaf extract of Zimmu (*Allium cepa* L. × *Allium sativum* L.) was most effective in inhibiting the mycelial growth of *A. solani* (87.5%), followed by *Datura metel* L., *Ocimum sanctum* L. and *Vinca rosea* L. with 78.5, 76.5 and 76.5% inhibition, respectively (Latha et al., 2009). Parajuli et al. (2005) reported that *Artemisia dubia* showed the highest inhibition (64.44%) followed by *Thymus linearis* (60.00%), *Nardostachys grandiflora* (57.77%), *Juniperus recurva* (42.22%), *Artemisia gmelinii* (33.33%) and *Zanthoxylum armatum* (8.88%) at 10 μl/ml essential oil concentration against *Alternaria brassicicola*.

Jasso de Rodríguez et al. (2007) evaluated the antifungal activity from three *Flourensia* species. *F. microphylla* inhibited *Alternaria* sp. by 42.5% even at 10 μl/l, reaching 76.8% inhibition at 100 μl/l. *R. solani* mycelial growth was inhibited by 20% at 10 μl/l; this behavior was similar for the three extracts. Inhibition reached 100% at 1000 μl/l for *F. cernua* and *F. retinophylla*, but for *F. microphylla* it was reached at 1500 μl/l. *F. oxysporum* was inhibited by 48.5% at 10 μl/l and *F. microphylla* had the highest inhibition at this concentration.

Broad-spectrum fungitoxicity of *Adenocalymma* aqueous extract was reported by Shukla et al. (2008). Most of the fungi treated were

susceptible to 10 mg/ml concentration of the extract amended to Czapek Dox agar medium, with complete inhibition of spore germination after 7 days of incubation at 28 °C. Among them, *A. niger*, *A. flavus* and *Cladosporium cladosporioides* were found to be the most resistant. The most sensitive fungal strains were *Mucor* sp., *Dreschlera* sp. and *Fusarium roseum*.

Other genera widely studied are the pathogens *Phytophthora* and *Botrytis*. Soylu et al. (2006) showed that oregano, thyme and fennel oils at 6.4 μg/ml inhibited growth of *P. infestans* completely whereas mycelial growth was completely inhibited by rosemary and lavender essential oils at 12.8, and 25.6 μg/ml concentrations respectively. The essential oils used in this study also affected sporangial formation, especially the oregano oil. All essential oils derived from aerial parts of origanum, lavender and rosemary were found to inhibit the growth of *B. cinerea* in a dose-dependent manner (Soylu et al., 2010).

Table 1 summarizes the results presented in this section.

4.2. Chemical composition of plant extract and essential oils

As mentioned before, the potential of plant extracts to inhibit fungal growth relies on their composition. In addition, their efficacy varies depending on the nature of the solvent used for the extraction. In general, it is well known that polar solvents have a better penetration capacity than non polar ones, so they are supposed to extract a wider variety of compounds from the plant tissue than non-polar extractants. Cakir et al. (2005) found that acetone and methanol are the best solvents for extraction of antifungal compounds from *Hypericum linarioides*. Likewise, Mahlo et al. (2010) concluded that acetone was the best solvent, extracting a larger quantity of material (between 8 and 12%) from different plants analyzed, of which *Breonadia salicina*

Table 1
Plants extracts and EOs tested for their antifungal capacity. N/A: not available.

Plant (scientific name)	Plant (common name)	Fungi tested	References
Adenocalymma alliaceum	Garlic creeper	*A. niger*, *A. flavus*, *C. cladosporioides*, *Mucor* sp., *Dreschlera* sp. and *F. roseum*	Shukla et al. (2008)
Allium cepa L. × *Allium sativum* L.	Zimmu	*A. solani*	Latha et al. (2009)
Anthemis nobilis L.	Chamomile	*A. candidus*, *A. niger*, *Penicillium* sp., and *F. culmorum*	Magro et al. (2006)
Artemisia dubia	N/A	*Alternaria brassicicola*	Parajuli et al. (2005)
Artemisia gmelinii	Gmelin's wormwood	*A. brassicicola*	Parajuli et al. (2005)
Bidens pilosa	Hairy beggar ticks, sticks tights, and spanish needles	*F. oxysporum* and *F. solani*	Deba et al. (2008)
Carum carvi	Caraway	*F. oxysporum*, *F. verticillioides*, *P. expansum*, *P. brevicompactum*, *A. flavus* and *A. fumigatus*	Zabka et al. (2009)
Cassia fistula	Cassia	*A. alternata*	Feng and Zheng (2007)
Chenopodium ambrosioides	American wormseed/ mexican tea	*A. flavus*, *A. fumigatus*, *Botryodiplodia theobromae*, *F. oxysporum*, *P. debaryanum* and *S. rolfsii*	Kumar et al. (2007)
Cinnamomum zeylanicum	Cinnamon	*A. niger*, *A. flavus*, *F. moniliforme*, *F. graminearum*, *Fusarium* spp, *P. citrinum* and *P. viridicatum*	Singh et al. (2007), Velluti et al. (2004)
Citrus limon L.	Lemon	*P. chrysogenum*, *P. verrucosum*, *A. niger* and *A. flavus*	Viuda-Martos et al. (2008)
Citrus paradisi L.	Grapefruit	*P. chrysogenum*, *P. verrucosum*, *A. niger* and *A. flavus*	Viuda-Martos et al. (2008)
Citrus reticulata L.	Mandarin	*P. chrysogenum*, *P. verrucosum*, *A. niger* and *A. flavus*	Viuda-Martos et al. (2008)
Citrus sinensis L.	Orange	*P. chrysogenum*, *P. verrucosum*, *A. niger* and *A. flavus*	Viuda-Martos et al. (2008)
Cuminum cyminum	Cumin	*F. solani*, *F. oxysporum*, *F. oxysporum* f. sp. *tulipae*, *F. verticillioides*, *F. poae*, *F. equiseti*, *R. solani*, *B. cinerea* and *A. citri*	Naeini et al. (2010), Boyraz and Özcan (2005)
Cymbopogon nardus	Citronella grass	*F. oxysporum*, *F. verticillioides*, *P. expansum*, *P. brevicompactum*, *A. flavus* and *A. fumigatus*	Zabka et al. (2009)
Cymbopogon spp	Lemongrass	*Fusarium* spp	Velluti et al. (2004)
Datura metel L.	Devil's trumpet/metel	*A. solani*	Latha et al. (2009)
Echinophora tenuifolia	Pickling herb	*R. solani*, *Fusarium oxysporum* f. sp. *tulipae*, *B. cinerea* and *A. citri*	Boyraz and Özcan (2005)
Eucalyptus globulus	Eucalyptus	*A. flavus* and *A. parasiticus*	Vilela et al. (2009)
Flourensia cernua	Tarbush	*Alternaria* sp., *F. oxysporum* and *R. solani*	Jasso de Rodríguez et al. (2007)
Flourensia microphylla	N/A	*Alternaria* sp., *F. oxysporum* and *R. solani*	Jasso de Rodríguez et al. (2007)
Flourensia retinophylla	N/A	*Alternaria* sp., *F. oxysporum* and *R. solani*	Jasso de Rodríguez et al. (2007)
Foeniculum vulgare	Fennel	*Phytophthora infestans*	Soylu et al. (2006)
Juniperus recurva	Drooping juniper	*A. brassicicola*	Parajuli et al. (2005)
Lavandula stoechas subsp. *stoechas*	Lavender	*P. infestans* and *B. cinerea*	Soylu et al. (2006), Soylu et al. (2010)
Malva sylvestris L.	Malva	*A. candidus*, *A. niger*, *Penicillium* sp., and *F. culmorum*	Magro et al. (2006)
Nardostachys grandiflora	Spikenard/nard	*A. brassicicola*	Parajuli et al. (2005)
Ocimum sanctum L.	Holy basil	*A. solani*	Latha et al. (2009)
Origanum syriacum var. *bevanii*	Oregano	*P. infestans* and *B. cinerea*	Soylu et al., 2006; Soylu et al., 2010
Pelargonium roseum	Geranium	*F. oxysporum*, *F. verticillioides*, *P. expansum*, *P. brevicompactum*, *A. flavus* and *A. fumigatus*	Zabka et al. (2009)
Peumus boldus	Boldo	*A. niger*, *A. flavus* and *Fusarium* spp	Souza et al. (2005)
Pimenta dioica L.	Allspice/Jamaica pepper	*F. oxysporum*, *F. verticillioides*, *P. expansum*, *P. brevicompactum*, *A. flavus* and *A. fumigatus*	Zabka et al. (2009)
Rosmarinus officinalis	Rosemary	*P. infestans* and *B. cinerea*	Soylu et al. (2006), Soylu et al. (2010)
Satureja hortensis	Sater	*R. solani*, *Fusarium oxysporum* f. sp. *tulipae*, *B. cinerea* and *A. citri*	Boyraz and Özcan (2005)
Syzygium aromaticum	Clove	*Fusarium* spp.	Velluti et al. (2004)
Thevetia peruviana	Digoxin, lucky nut, nerium oleander, yellow oleander	*A. niger* and *Penicillium* spp.	Ravikumar Patil et al. (2007)
Thymbra spicata subsp. *spicata*	Thyme	*P. infestans*	Soylu et al. (2006)
Thymus linearis	Himalayan thyme	*A. brassicicola*	Parajuli et al. (2005)
Thymus vulgaris	Thyme	*F. oxysporum*, *F. verticillioides*, *P. expansum*, *P. brevicompactum*, *A. flavus*, *A. alternata* and *A. fumigatus*	Zabka et al. (2009), Feng and Zheng (2007)
Vinca rosea L.	Madagascar periwinkle	*A. solani*	Latha et al. (2009)
Zanthoxylum armatum	Winged prickly ash	*A. brassicicola*	Parajuli et al. (2005)
Zataria multiflora	Zataria	*F. solani*, *F. oxysporum*, *F. verticillioides*, *F. poae* and *F. equiseti*	Naeini et al. (2010)

and *Olinia ventosa* had the higher yields. This suggests that compounds with intermediate polarity have the highest activity, which could be related to the uptake of compounds by fungal cells. Other reasons to choose acetone as the extraction solvent include its volatility, miscibility with polar and nonpolar solvents and its relatively low toxicity. Besides the extraction solvent, the part of the plant from which the extract is obtained plays an important role. Gamboa-Angulo et al. (2008) presented different results taking these factors into account. In their work, the most active extracts corresponded to the roots of *Acalypha gaumeri* and *Croton chichenensis* (native plants from the Yucatan peninsula) which were active against the four pathogens evaluated (*Alternaria tagetica*, *Colletotrichum gloeosporioides*, *F. oxysporum* and *Rhizopus* sp.); followed by leaves of *Ambrosia hispida*, *Trichilia minutiflora*, *Vitex gaumeri* and the stem of *Randia obcordata*. The overall results indicated a wide activity spectrum only for the ethanolic extract of *C. chichenensis* roots with the greatest inhibitory effect against the plant pathogens tested.

Different techniques applied to fractionate the crude extracts obtained from plants have been studied in order to isolate their active compounds. Ahn et al. (2005) found that the ethyl acetate fraction from a methanolic extract of *Galla rhois* at a concentration of 2000 ppm showed good antifungal activity against six plant disease organisms: *Magnaporthe grisea*, *B. cinerea*, *Phytophthora infestans*, *Puccinia recondita* and *Erysiphe graminis*. No antifungal activity against all the plant disease organisms was observed with the hexane, chloroform, and water fractions. Whole plant bio-guided fractionation of *G. rhois* extract afforded two active principles that were identified as the phenolics methyl gallate and gallic acid. Bio-guided fractionation performed by Nguefack et al. (2009) optimized the activity of extracts from *Cymbopogon citratus*, *Ocimum gratissimum* and *T. vulgaris*. Increments ranging from 70 to 95% were recorded from fractions when compared to the complete essential oil. In general, the most active fractions were obtained at 100% hexane and moderate active fractions at 95–5 (v/v) hexane–ethyl acetate as solvent systems. Fractions obtained with other solvent systems (85–15, 75–25, 50–50, 25–75 hexane–ethyl acetate (v/v) and 100% ethyl acetate) did not show any antifungal activity. Analyzing extracts of *Mimusops elengi*, Satish et al. (2008) observed significant antifungal activity in aqueous and methanol extract, suggesting that the active principle is of polar nature and concluding that the active principle is alkaloid in nature. Alkaloid fraction was highly effective against *Drechslera*, *Aspergillus* and *Penicillium* species with more than 85% inhibition, whereas *Fusarium* recorded varied response. Maximum inhibition was observed at 50 ppm of alkaloid fraction compared to tested synthetic fungicides at the recommended dosage (2000 ppm).

In the same way, essential oils are constituted of many different volatile compounds and their composition varies between species (Feng and Zheng, 2007). Differences in composition related to variety, agronomic practice and processing are also likely to influence antimicrobial properties, since these factors contribute to both the profile and relative concentrations of active ingredients (Delaquis et al., 2002). dos Santos Oliveira and Badiale Furlong (2008) worked with phenolic compounds and concluded that the type of phenolic structure is more important than the concentration. It has been shown that molecules with a hydroxyl group plus a system of delocalized electrons in the phenolic ring structure have high activity. It has also been demonstrated that essential oils may also have antioxidant and antiinflammatory activities. Volatile oils are a complex mixture of compounds, mainly monoterpenes, sesquiterpenes, and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). Other volatile compounds include phenylpropenes and specific sulphur- or nitrogen-containing substances.

Thymol, eugenol, anethole, menthol, citral, pinenes, isothiocyanates, cinnamaldhydes, carvacrol, carvone, benzoic acids, phenolic acids, and flavones are some phytochemicals that have been studied in depth regarding their antimicrobial spectrum on different bacterial and fungal species (Souza et al., 2005). For example, eugenol ($C_{10}H_{12}O_2$) is a phenolic compound extracted mainly from buds and leaves of clove (*Eugenia caryophylata* Thumb) and from cinnamon, constituting the most significant active component of clove oil (85 to 95%) in addition to iso-eugenol and methyleugenol. This compound has been extensively studied for its antimicrobial properties, extracted from various substrates and assayed against a wide variety of microorganisms. Prakash et al. (2010) observed that eugenol, extracted as the major component of *Piper betle* var. *magahi* EO, was more efficacious as fungal growth inhibitor and aflatoxin suppressor than the whole EO. They suggested that the remaining components of the oil synergistically acted in negative direction and reduced the activity of eugenol. On the other hand, Souza et al. (2005) reported that *A. niger* was the single mould strain among all the fungi tested that presented resistance to eugenol on all tested concentrations.

In this field, there have been numerous investigations trying to isolate the compounds responsible for the biological activity. *Eucalyptus teretecornis* EO was subjected to PTLC for isolation of pure molecules by Guleria et al. (2012). Using GC/MS analysis, they found that the bioactive molecules corresponding to two fractions were the oxygenated terpenoids β-fenchol and α-eudesmol, and demonstrated the antifungal potential of these compounds from *E. teretecornis* EO against *A. alternata*.

Capsantal, a natural carotenoid mixture, containing capsanthin and capsorubin was tested against some OTA-producing *Aspergillus* isolates. Its addition resulted in increased lag phases at 15 °C for all the tested strains, *A. ochraceus*, *A. westerdijkiae* and *A. tubingensis*, while growth rates remained rather constant. However, at 25 °C the addition of capsantal resulted in reduced growth rates, with rather constant lag phases (Santos et al., 2010).

It is difficult to correlate the fungitoxic activity to single compounds or classes of compounds. As an example, Vilela et al. (2009) tested *E. globulus* EO and its major compound 1,8-cineole against *A. flavus* and *A. parasiticus* and found that the antifungal activity of the isolated molecule was incomplete and only showed effects at the highest concentration tested, which could indicate that the major oil constituent is not the only component responsible for limiting fungal growth. Plant extracts are composed of a great variety of chemical compounds, which makes it difficult to establish a relationship between one compound and a single target in the cell. There is evidence that even minor components have a critical part in antimicrobial activity, and it seems that the inhibitory effects are the result of their synergistic action. Thus, the chances of resistance development in fungi after application of the plant extract would be reduced, and the spectrum of sensitive organisms to its action would be broader. Moreover, steps to purify the compounds would involve higher costs, so whole plant extracts appeared to be more promising in commercial application than single compounds.

4.3. Mode of action of plant-derived extracts

The mechanisms of action of active compounds in plant extracts are not completely understood yet. However, there are three aspects on which most authors agree in attributing their inhibitory function: i) the presence of OH groups able to form hydrogen bonds that have effects on enzymes, modifying a variety of intracellular functions; ii) action on mould morphology due to interactions with membrane enzymes, resulting in the loss of rigidity and integrity of the hypha cell wall; and iii) changes in permeability of cell membranes, granulation of the cytoplasm and cytoplasmic membrane rupture. All three aspects are intimately interrelated. The hydrophobicity of these compounds leads them to cross cell membrane and interact with cell compounds, so they could affect both membrane and intracellular enzymes. It has been suggested that some hydrophobic compounds present in plant extracts could change the permeability of the microbial membranes for cations such as H^+ and K^+, and so could cause a

change in the flow of protons, modifying cell pH and affecting chemical composition of the cells and their activity. The ability of hydrophobic compounds to partition or dissolve in the lipid phase of the cytoplasmic membrane is the key for their activity, but higher solubility does not always mean greater antimicrobial action. The loss of differential permeability of the cytoplasmatic membrane is generally considered the cause of cell death because this may result in an imbalance in intracellular osmotic pressure, subsequent disruption of intracellular organelles, leakage of cytoplasmic contents and finally cell death. The interaction with cell membranes may also lead to the leakage of some cellular components, including ATP, the main energy-storing molecule.

Phenolic compounds are known to alter microbial cell permeability, allowing the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality (Fung et al., 1977).

Other events that may lead to dysfunction of the membrane and subsequent disruption include the following events: dissipation of the two components of the proton motive force (pH gradient and electrical potential); interference with the system of generating energy (ATP) in the cell; inhibition of enzymes; and prevention of substrate utilization for energy production (Coutinho de Oliveira et al., 2011; El-Mogy and Alsanius, 2012).

These actions culminate with inhibition of germination, suppression of mycelial growth, and germ tube elongation, so it has been suggested that the action of plant compound might be related to the perception/transduction of signals involved in the switch from vegetative to reproductive development.

Lee et al. (2007) reported that in the presence of the methanol extracts of *Cinnamomum cassia* the cultures *of A. niger*, *B. cinerea*, *F. moniliforme*, *Glomerella cingulata* and *Phyllosticta caricae* showed an extended lag time and, observing the morphology of the fungal cells under a light microscope, concluded that the inhibitory effect of the herb extracts on fungal growth was due to the inhibition of mycelia growth and spore germination.

Ultra-structural changes were noted under transmission electron microscopy (TEM) (Nogueira et al., 2010) in *A. flavus* cell wall and cytoplasm when fungal materials obtained from 5 days grown culture were treated with *Ageratum conyzoides* oil. The major pathological changes were observed on the endomembrane system, mainly the plasma membrane, and membranous organelles especially the mitochondria. The plasma membrane in fungal treated cells lost its linear aspect, becoming rough and villiform with invaginations of vesicles and sometimes decoupling of plasma membrane from the cell wall was observed. The fibrillar layers gradually lost their constitution, becoming thinner and eventually detaching from the cell wall, and, in cells treated with higher oil concentrations they failed to deposit on the cell wall. The mitochondria suffered a disruption of the internal structure with a decrease in the ridge polarization of the mitochondrial cristae. These results showed that the essential oil of *A. conyzoides* crosses not only the cell wall, but also the plasma membrane, interacting with the membranous structures of cytoplasmic organelles.

TEM observation was also used to analyze the effect of *A. niger* exposure to MIC levels of the *Thymus eriocalyx* and *Thymus x-porlock* oils (Rasooli et al., 2006). Severe damage was demonstrated to cell wall, cell membrane and cellular organelles. The mycelium exposed to the thyme oil showed morphological changes in the hyphae, plasma membrane disruption and mitochondrial destruction. *A. niger* exposed to 250 ppm of essential oil from *T. x-porlock* exhibited folding of the cell, lack of cytoplasm, nuclear membrane damage and loss of integrity and rigidity of the cell wall. The authors concluded that these modifications in cytological structure may be attributable to the interference of the essential oil with the enzymes responsible for wall synthesis.

Soylu et al. (2006) studied the effect of EOs obtained from aerial parts of several aromatic plants, such as oregano (*Origanum syriacum* var. *bevanii*), thyme (*Thymbra spicata* subsp. *spicata*), lavender (*Lavandula stoechas* subsp. *stoechas*), rosemary (*Rosmarinus officinalis*), fennel (*Foeniculum vulgare*) and laurel (*Laurus nobilis*) against tomato late blight disease agent *Phytophthora infestans*. Under the influence of the oils, the growth of the fungus was suppressed and hyphal morphological changes were observed using scanning electron microscopy (SEM). These observations on pathogen hyphae, exposed to both volatile (essential oil vapor) and contact (culture medium amended with the different concentrations of essential oil) phase of the oils, revealed considerable morphological alterations in hyphae such as cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplast leakage. They concluded that general changes in the morphology of the hyphae could be due to the loss of integrity of the cell wall and plasma membrane permeability. A similar experiment was carried out by Soylu et al. (2010), testing the same EOs against tomato grey mould disease agent *B. cinerea*. Again, SEM observations of fungal hyphae exposed to the most effective concentrations of volatile as well as contact phase showed degenerative changes in the hyphal morphology in comparison to thick, elongated, smooth surfaced hyphae in control plates. Complete absence of conidiation was also observed in oil treated Petri plates (Soylu et al., 2010).

According to Tian et al. (2012), at 2 μl/ml of *Cinnamomum jensenianum* EO, the plasma membrane of a strain of *A. flavus* became rough with continuous folding into the cytoplasm and festooned with small lomasomes. A decreased cytoplasmic matrix was also observed. They showed that some mitochondria suffered extensive disruption of the internal structure with a decrease in mitochondrial cristae. The cell ultrastructure damage was aggravated when the oil concentration was doubled. Major alterations were observed, including massive vacuolation of cytoplasm with vacuole fusion, appearance of numerous lomasomes with folding, and detachment of plasma membrane from the cell wall. The fibrillar layers gradually lost their integrity, becoming thinner, and eventually failing to deposit on the cell wall. The plasma membrane was also folded at many sites. The cytoplasmic matrix and some cytoplasmic organelles, such as the electron dense granules, were absent. In addition, the mitochondria suffered a severe disruption of the internal structure with complete lysis. The results demonstrated that the ergosterol content (measured at 282 nm) in the plasma membrane of *A. flavus* was significantly reduced by the different concentrations of essential oil. A dose-dependent decrease in ergosterol production was observed when isolates were grown in the presence of the EO. Therefore, the authors concluded that this essential oil exerts its effect directly on the plasma membrane without any obvious damage to the cell wall, unlike the research works discussed earlier.

Tolouee et al. (2010) found that the major morphological alterations in *A. niger* after being exposed to *Matricaria chamomilla* L. flower essential oil occurred on endo-membrane system mainly affecting plasma membrane and membranous organelles such as mitochondria. Early changes in fungal compartments in the presence of the lowest concentration of *M. chamomilla* essential oil tested (15.62 μg/ml) were vacuolation of cytoplasm, early degradation of electron-dense granules and folding and detachment of plasma membrane from the cell wall. In the presence of 250 μg/ml of the plant oil, where the fungal growth was inhibited 41%, morphological changes advanced affecting membrane structures of the cytoplasm. At this concentration, the major alterations were disrupting of plasma membrane accompanied by the formation of small vesicles (lomasomes) between the plasma membrane and cell wall. Complete autolysis, disorganization and depletion of hyphal cytoplasm and membranous organelles including nuclei, endoplasmic reticulum and mitochondria seemed to be responsible for cell death. TEM data revealed that plasma membrane and membranous structures of the hyphal cytoplasm, especially mitochondria, were the main targets of the plant oil. From the SEM observations, for the fungus exposed to 1000 μg/ml of plant oil, collapse and folding of whole hyphae and complete

inhibition of conidia production were the prominent morphological changes observed.

In conclusion, there is evidence that plant compounds are able to collapse cell walls and plasma membranes, penetrate through them and affect several intracellular functions with consequent disruption of the normal mycelium development.

4.4. Influence of environmental factors

In the control of fungal growth, environmental factors are very important issues to bear in mind. As was mentioned before, temperature and water activity (a_w) affect fungal development, either enhancing or inhibiting it, depending on medium conditions and the microorganism involved. These factors may also influence the stability of the antifungal activities of plant extracts. Some other environmental factors may influence their activity, such as pH, lipids that could decrease activity of hydrophobic compounds, and proteins that may cause binding of some compounds reducing their activity.

López-Malo et al. (2005) tested several antimicrobials (potassium sorbate, sodium benzoate, sodium bisulfite, carvacrol, citral, eugenol, thymol and vanillin), and the statistical analysis showed that antimicrobial concentration, a_w, pH and their interactions significantly affected the rate of *A. flavus* radial growth. Depending on a_w and pH, the increase in antimicrobial concentration slightly reduced (or did not affect) the fungal development until a critical concentration, where radial growth was drastically reduced or mould growth was arrested. Important differences were observed among antimicrobials, with the natural antimicrobials being, in general, less pH-dependent than chemical preservatives.

Bluma and Etcheverry (2008) found that all concentrations assayed of boldus (*P. boldus* Mol), mountain thyme (*Hedeoma multiflora* Benth) and poleo (*Lippia turbinate* var. *integrifolia*) EOs inhibited fungal growth of the four isolates of *Aspergillus* section *Flavi* studied at 0.955 and 0.900 a_w, whereas anise (*Pimpinella anisum* L.) and clove (*Syzygium aromaticum* L.) showed minor inhibitory activity at these conditions. At higher substrate a_w (0.982), the increments in the inhibition rate of the EOs were more pronounced with higher concentrations tested (2000 and 3000). At 0.955 a_w, only anise and clove EOs increased their antifungal activity as the EO concentration augmented. A lower concentration was studied at 0.900 a_w, and growth rate was reduced between 33–54 and 31–100% by anise and clove EO, respectively.

Passone et al. (2012) studied the efficacy of boldo, poleo, clove, anise and thyme EOs against *A. niger* aggregate and *A. carbonarius* growth on peanut meal extract agar under different a_w conditions (0.98, 0.95, 0.93). They found that a decrease of the medium a_w, in combination with sub-inhibitory doses of anise EO stimulated the growth rate in most of the strains studied, whereas poleo EO produced the lowest inhibition rates at the highest a_w. In the headspace volatile assay (EO not in direct contact with the culture medium) it was found that the combination of EO volatile fractions and low a_w increased the antifungal effects on the growth rate of all ochratoxigenic strains, with boldo EO being most effective.

Garcia et al. (2011) studied the effect of two plant extracts on growth of several *Fusarium* and *Aspergillus* strains, using temperature and a_w conditions similar to those that occur in cereals in pre and post-harvest stages. In general, no growth was observed when applying *Equisetum arvense* ethanolic extract at 3% by any of the isolates at all studied conditions. When *E. arvense* extract at 2–3% was added to the medium, growth of *A. flavus* and *A. parasiticus* was significantly retarded. With 3% *Stevia rebaudiana* in the medium, growth decreased significantly except at 0.90–0.93 a_w/30 °C and also at 0.93a_w/25 °C where growth was similar to the control, whereas *S. rebaudiana* at 2% had little or no effect. When *A. carbonarius* and *A. westerdijkae* were inoculated into medium containing 3% *E. arvense* extract, growth was significantly decreased under all studied conditions. With *S. rebaudiana* extract, growth was promoted for all conditions assayed, except for 0.85 a_w at all temperatures assayed. Growth of *A. westerdijkiae* in the presence of *E. arvense* extract at 2% also decreased between 37 and 100%.

On the other hand, Velluti et al. (2004) concluded that variation of temperature showed very little effect on the activity of the essential oils tested against *Fusarium* isolates. Neither did a_w show a significant effect in the effectiveness of these oils, with only a slight increase in their activity at the lowest a_w tested.

The influence of pH was investigated by Nguefack et al. (2009). Essential oil from *O. gratissimum* was more active at pH 9, where growth of *A. ochraceus, P. expansum* and *P. verrucosum* was significantly reduced. The activity of the same oil at pH 3 was higher than that recorded at pH 6 but not statistically significant. The authors related the highest antifungal activity of *O. gratissimum* at high pH to its thymol and γ-terpinene content.

5. Effects on mycotoxin production

In regard to human health, the main issue to consider is the capability of the fungi to synthesize mycotoxins. Contact with plant extracts or EOs may enhance the production of secondary metabolites by fungi, thus it is crucial to analyze each individual case. Partial inhibition of

Table 2
Plant extracts and EO reported as inhibitors of mycotoxin synthesis. ND: not determined.

Toxin	Fungal species	Plant material	Active compounds	References
Aflatoxin B_1	*A. flavus*	*Chenopodium ambrosioides* EO	ND	Kumar et al. (2007)
		Ocimum sanctum EO	Eugenol	Reddy et al. (2009), Kumar et al. (2010)
		Cicuta virosa EO	ND	Tian et al. (2011)
		Cinnamomum jensenianum EO	ND	Tian et al. (2012)
	A. parasiticus	*Ephedra major* metanolic extract	Flavonoid compounds (p-coumaric acid, quercetin)	Bagheri-Gavkosh et al. (2009)
		Thymus eriocalyx and *T. x-porlock* EO	Thymol	Rasooli and Abyaneh (2004)
		Rosmarinus officinalis and *Trachyspermum copticum* EO	Thymol, γ-terpinene, p-cymene, limonene	Rasooli et al. (2008)
	A. flavus and *A. parasiticus*	*Eucalyptus globulus* EO	ND	Vilela et al. (2009)
		Pëumus boldus, *Lippia turbinate*, *Syzygium aromaticum* and *Hedeoma multiflora* EO	ρ-Cimene, α-terpinolene, carvacrol, anethol, eugenol	Bluma and Etcheverry (2008)
Aflatoxin B_1 and B_2	*A. flavus*	Banana pulp and peel; orange, eggplant and potato pulp methanolic extracts	Phenolic compounds	dos Santos Oliveira and Badiale Furlong (2008)
Aflatoxin B_1 and G_1	*A. parasiticus* and *A. flavus*	*Betula alba* EO	Methyl syringate	Jermnak et al. (2012)
Ochratoxin A	*Aspergillus* section *Nigri*	*P. boldus* and *S. aromaticum* EO	α-Terpinolene, α-terperpine, eugenol, α-cariofilene	Passone et al. (2012)
Zearalenone and deoxynivalenol	*Fusarium graminearum*	*S. aromaticum* EO	Eugenol plus minor components	Marín et al. (2004)
Fumonisin B_1	*F. verticillioides*	–	Thymol, carvacrol, and isoeugenol	Dambolena et al. (2011)

fungal growth cannot be correlated with inhibition of mycotoxin production because this fungistatic activity could trigger secondary metabolism as a response to stress. The most relevant results from research work on mycotoxin inhibition by plant compounds are shown in Table 2.

Aflatoxins are the most harmful fungal toxins known; aflatoxin B_1 is included in group 1 (considered to have enough evidence for carcinogenicity in humans) by the International Agency for Research on Cancer (IARC, 2002). As a consequence, much research has investigated the potential of natural antifungals either to inhibit growth of aflatoxigenic fungi or to repress mycotoxin biosynthesis. Bagheri-Gavkosh et al. (2009) showed that methanolic extracts of *Ephedra major* aerial parts and roots inhibited the AFB_1 production by *A. parasiticus*, whereas the EO of the plant aerial parts did not exhibit any effect on its biosynthesis. The authors attributed the inhibition of *A. parasiticus* growth and AFB_1 production to the presence of flavonoid compounds such as p-coumaric acid and quercetin in plant extracts. dos Santos Oliveira and Badiale Furlong (2008) noted that aflatoxin B_1 and B_2 production by *A. flavus* was inhibited in the presence of methanolic extracts from banana pulp and peel, orange, eggplant and potato pulp. However, these authors found that in the presence of banana pulp and potato pulp extracts, *A. flavus* produced aflatoxin B_2, which was not detected in the control. As was mentioned before, such secondary production may be a defense response of the fungus. *C. ambrosioides* oil completely inhibited aflatoxin B_1 production by *A. flavus* at 10 μg/ml (Kumar et al., 2007). The oils from *Thymus eriocalyx* and *T. x-porlock* severely inhibited *A. parasiticus* growth while aflatoxin production was significantly inhibited at fungistatic dilutions of both oils. However, *T. eriocalyx* oil exerted greater antifungal as well as antitoxigenic effects than that of *T. x-porlock*. The authors suggested that the presence of thymol in the oils may be responsible, at least in part, for the obtained results (Rasooli and Abyaneh, 2004). Vilela et al. (2009) found that inhibition of aflatoxin B_1 production by *A. flavus* and *A. parasiticus* in the presence of *E. globulus* EO required a higher oil dose than was required for inhibition of fungal growth. Reddy et al. (2007) reported that on treated rice grains, aflatoxin B_1 biosynthesis by *A. flavus* was not inhibited by eugenol obtained from clove, except when fungal growth was completely inhibited. As the concentration of the antifungal increased, growth and mycotoxin inhibition also increased. In a later work, Reddy et al. (2009) found that *Syzigium aromaticum* completely inhibited *A. flavus* growth and consequently AFB_1 production at 5 g/kg concentration. They also found that *Curcuma longa*, *Allium sativum* and *O. sanctum* were effective in reducing fungal growth and AFB_1 production at the same concentration. EO of *O. sanctum* and its major component eugenol showed potent fungitoxicity against *A. flavus*. Also Kumar et al. (2010), reported that reduction in fungal growth and AFB_1 production was due to the presence of some phenolic compounds in the *O. sanctum* EO, mainly its major component, eugenol. Rasooli et al. (2008) found that both *A. parasiticus* growth and aflatoxin biosynthesis were suppressed by EO of *R. officinalis* and *Trachyspermum copticum* L. The inhibitory effect of the oils increased in proportion to their concentrations.

In general, essential oil-related retardation of mycelial growth was observed to significantly decrease aflatoxin production. However, Tian et al. (2011) reported that although mycelial growth was observed at 4 μl/ml of *Cicuta virosa* L. var. *latisecta* Celak EO, AFB_1 production was completely inhibited. The AFB_1 content was reduced to about half that of the control at 2 μl/ml. Similar results were obtained by Tian et al. (2012) with *C. jensenianum* essential oil, which reduced AFB_1 content to about half that of the control at 2 μl/ml. Both EO exhibited antiaflatoxigenic properties at concentrations lower than their fungitoxic concentration. The inhibition of AFB_1 production cannot be completely attributed to reduced fungal growth, but it was considered a consequence of the inhibition of carbohydrate catabolism in *A. flavus* by acting on some key enzymes, thus reducing its ability to produce the toxin (Tatsadjieu et al., 2009).

Prakash et al. (2010) reported that for *P. betle* var. *magahi* EO at low concentration (0.1 μl/ml), AFB_1 production by a strain of *A. flavus* was higher than the control. At higher concentration, aflatoxin inhibitory effect of the oil was observed, and at 0.6 μl/ml aflatoxin production by this isolate was completely inhibited. In view of these results the authors suggested that low fungicide doses create some stress condition which was responsible for the production of more secondary metabolites as a defense mechanism by the fungus.

Although the crude essential oil of *Betula alba* inhibited both aflatoxin production and fungal growth in parallel, Jermnak et al. (2012) after roughly purifying the oil by silica gel column chromatography obtained an active fraction that inhibited only aflatoxin production. The fraction was further purified and identified as methyl syringate. This compound inhibited production of aflatoxins B_1 and G_1 by *A. parasiticus* in liquid medium in a dose-dependent manner and also inhibited aflatoxin B_1 production by *A. flavus*. It was shown that it strongly inhibits norsolorinic acid production, an early step of the aflatoxin biosynthetic pathway. Methyl syringate was shown to inhibit aflatoxin production in a model infection system consisting of *A. flavus* on raw peanuts.

Bluma and Etcheverry (2008) reported that AFB_1 inhibition was closely dependent on the water activity, concentration and incubation periods and their interactions. They showed that boldus, poleo, and mountain thyme EO completely inhibited AFB_1 at 2000 and 3000 μg/g at a_w 0.981 and inhibition was also observed at all concentrations assayed at lower a_w (0.955 and 0.900). Moreover, clove EO proved to have an important inhibitory effect on AFB_1 accumulation. At a_w 0.982, the AFB_1 inhibition percentages for all *Aspergillus* strains tested were higher than 98% at all clove EO concentrations investigated. On the other hand, Garcia et al. (2011) found stimulation of aflatoxigenesis by *A. flavus* and *A. parasiticus* in some of the analyzed a_w and temperature conditions in the presence of both *E. arvense* and *S. rebaudiana* plant extracts, even when no aflatoxins were found in the control medium. These authors also studied the effects of plant extracts on the production of ochratoxin A (OTA), another toxin of high relevance. As with AFB_1, they found that OTA production by *A. carbonarius* and *A. westerdijkae* was higher than in the control, and this increment was higher with *S. rebaudiana* than with the *E. arvense* extract medium.

Passone et al. (2012) showed that OTA accumulation by six ochratoxigenic *Aspergillus* section *Nigri* isolates was completely inhibited in the presence of 2000 μl/l of boldo EO. However, at a_w 0.98, most of the strains of *A. niger* aggregate and *A. carbonarius* showed OTA stimulation in the presence of 1000 μl/l of poleo EO. The antiochratoxigenic effect of clove EO was more significant at a_w 0.93, resulting in total inhibition at 5000 μl/l. In this work inhibition of OTA required a lower oil dose than was required for inhibition of fungal growth, regardless of medium a_w.

Inhibition of toxigenesis in *Fusarium* has also been studied. Marín et al. (2004) explored the efficacy of treating maize grain with cinnamon, clove, lemongrass, oregano and palmarosa essential oils in order to prevent zearalenone (ZEA) and deoxynivalenol (DON) accumulation when inoculated with *F. graminearum*. This assay was based on non-sterilized, naturally contaminated maize grain. Two different studies were carried out. In the first, *Fusarium* species were inoculated 24 h before the different essential oils were added to maize grain, while in the other test, the essential oils were added 24 h before inoculation. All factors (essential oils, temperature, a_w, treatment timing) as well as their interactions had a significant impact on ZEA production. ZEA accumulation was only inhibited at 0.950 a_w and mainly at 30 °C; under these conditions ZEA production in the controls was maximum. At 30 °C, total prevention of DON accumulation by all essential oils occurred at 0.995 a_w; while at 0.950 a_w, cinnamon, clove and lemongrass essential oils were shown to inhibit the accumulation, and oregano and palmarosa stimulated it when added 24 h after inoculation. The authors concluded that clove essential oil was the best candidate

for simultaneous inhibition of both ZEA and DON production; although its efficacy was poor. Dambolena et al. (2011) studied the capacity of ten natural phenolic compounds to inhibit fumonisin B_1 (FB_1) synthesis by *F. verticillioides* and revealed that thymol, carvacrol, and isoeugenol followed by eugenol were the most active. These phenolic compounds also had the highest antifungal activity. On the other hand, m-cresol, creosol and guaiacol did not show any significant effects on reducing FB_1 production or biomass accumulation. These findings highlighted that, in addition to the functional group, other properties such as slight structural differences may affect the physical or chemical properties of such compounds, altering their bioactivity. Given that the phenolic compounds with the greatest antifumonisin activity also presented the highest rates in growth inhibition, the authors suggested that the antifumonisin activity of the phenolic compounds may be due to an increase in the lag phase and/or result from an effect of the growth rate. Hence, a delay can arise in the onset of the stationary growth phase, which is the moment when mycotoxins are produced. Another hypothesis suggested was that, because of the stress induced by the EO, the fungi respond by limiting secondary metabolite production.

On the contrary, Garcia et al. (2011) found that production of fumonisins B_1 and B_2 by *F. verticillioides*, and ZEA and DON by *F. graminearum* was stimulated or similar to the controls in most of the conditions tested using *E. arvense* and *S. rebaudiana* extracts.

6. *In vivo* assays

In the search of alternative antifungals to apply in food, an important issue to take into account is their performance *in vivo*. Although *in vitro* screening of plant extracts is an important first step in identifying potential plants for this purpose, *in vivo* confirmation of activity is essential because food matrices may interact with the bioactive compounds, decreasing their efficacy. In general, to obtain the same effect in food products as those observed in *in vitro* assays, higher concentrations of EOs or plant extracts must be utilized. A possible explanation for this is that when they are in contact with the food surface, highly hydrophobic and volatile active substances are bound by food components (carbohydrates, fat and proteins), while other components are partitioned through the product according to their affinity with water. If this is so, undesirable flavor and sensory changes may occur. It has been suggested that lipids in food could form a coating around the microorganism, protecting them from antimicrobial agents. Furthermore, the lower water content in food compared to laboratory media could hinder the transfer of the antimicrobial molecules to the active site within the microbial cell. Recent *in vivo* assays reported in literature are summarized in Table 3.

Based on *in vitro* assays, Costa Carvalho et al. (2011) selected five plant extracts (*Anadenanthera colubrina*, *Artemisia annua*, *Cariniana estrelensis*, *Ficus carica* and *Ruta graveolens*) to be evaluated against *A. alternata* in Murcott tangor fruits. Only that from *A. colubrina* showed suppression of lesions caused by this pathogen. The authors suggested polyphenols as the active substances in the *A. colubrina* extract, since these compounds could form complexes with proteins and polysaccharides, inactivating enzymes essential for fungal growth. Furthermore, the bark of *A. colubrina* is rich in tannins, which may confer antimicrobial properties to the plant extract. The results obtained in the test with fruits inoculated with *A. alternata* suggested consistency between *in vitro* and *in vivo* assays, since the extract of *A. colubrina* was the most active in all experiments, reducing the effects of the fungus on fruits to levels comparable to those observed for the commercial fungicides.

El-Mogy and Alsanius (2012) studied the *in vivo* efficacy of cassia oil against *B. cinerea* in strawberries. This oil completely inhibited *in vitro* growth of the fungus, having fungistatic activity at low concentrations and long-term exposure, or fungicidal effects at high concentrations. The percentage decay of fruit artificially inoculated with *B. cinerea* and unwounded fruit was significantly reduced by cassia oil application. The authors found that this treatment did not affect organoleptic quality parameters and that weight loss of strawberries was decreased by all concentrations. Similar effects of this oil on *A. alternata* decay of cherry tomatoes have been reported by Feng and Zheng (2007). Both works highlighted that, as expected, the inhibitory effects of cassia oil concentrations were more effective *in vitro* than *in vivo*.

The study of *Origanum vulgare* extract to control rots on "Rocha" pears inoculated with *B. cinerea* and *P. expansum* showed that the extract was less active against both fungi than observed in the *in vitro* tests. The authors suggested that this was probably due to low solubility in water of some of its components (Matos and Barreiro, 2004).

Tomato fruit (*Lycopersicon esculentum*) is a crop widely commercialized around the world, and this plant is highly susceptible to the attack of several fungal pathogens, therefore, much research is devoted

Table 3
In vivo antifungal effects of plant-derived extracts.

Plant extract/EO	Fungal species	Food matrix	Effect	References
Anadenanthera colubrina methanol extract	*Alternaria alternata*	Murcott tangor fruits	Suppression of lesions due to polyphenols and tannins.	Costa Carvalho et al. (2011)
Cassia EO	*A. alternata*	Cherry tomatoes	34.2% reduction of decayed tomatoes at 500 ppm.	Feng and Zheng (2007)
	Botrytis cinerea	Strawberries	40% reduction of decayed fruits at 800 ppm.	El-Mogy and Alsanius (2012)
Origanum vulgare methanol extract	*B. cinerea* and *P. expansum*	"Rocha" pears	Reduction in lesion diameter at 20 μl.	Matos and Barreiro (2004)
O. vulgare EO	*Colletotrichum coccodes*	Tomato fruit	22% suppression of spore viability with vapour-treatment.	Tzortzakis (2010)
	R. stolonifer and *A. niger*	Table grape cultivar Isabella	Delay on fungal development and reduction of infected fruits (coating of chitosan and EO).	dos Santos et al. (2012)
O. syriacum EO	*B. cinerea*	Tomato leaves	Protection at 75 mg/l: 77% (curative activity) and 33% (protective activity).	Soylu et al. (2010)
Cicuta virosa EO	*A. flavus*, *A. oryzae*, *A. niger* and *A. alternata*	Cherry tomatoes	<10% of infected fruits at 200 μl/ml for all the strains.	Tian et al. (2011)
Thyme (*Thymus vulgaris*), summer savory (*Satureja hortensis*) and clove (*Syzygium aromaticum*) EO	*A. flavus*	Tomato paste	Maximum inhibitory concentrations: 350 ppm (*T. vulgaris*), 500 ppm (*S. hortensis*).	Omidbeygi et al. (2007)
Cabbage (*Brassica oleracea*) isothiocyanates	*A. alternata*	Green bell pepper	Maximum fungicidal effect at 0.56 mg/ml.	Troncoso et al. (2005)
Chenopodium ambrosioides EO	*A. flavus*	Wheat	91% protection at 100 μg/ml.	Kumar et al. (2007)
50:50 mix of orange: bergamot EO	*P. chrysogenum* and *A. niger*	Grain	Reduction of 70.8% (*A. niger*) and 57.8% (*P. chrysogenum*) growth.	Phillips et al. (2012)

to studying how to control infections in this plant. Phillips et al. (2012) showed that exposure to a 50:50 mix of orange:bergamot essential oils (Citri-V™®) is not an effective antifungal treatment against *A. alternata* on tomatoes because all wounds of both the control and treated tomatoes were found to be infected after 7 days regardless of the number of times they had been treated.

Soylu et al. (2010) sprayed *Origanum syriacum* essential oil on the tomato leaves before or after *B. cinerea* inoculation in order to reveal whether essential oil has curative or protective activities. Although both treatments were effective in reducing fungal infection, the most effective control was consistently achieved when essential oil applications were made 24 h after inoculation (curative activity). This would suggest that the whole oils exerted their greatest effect on early fungal development on the leaf surface, *e.g.* spore germination, germ tube growth and/or appressorium formation.

Tzortzakis (2010) evaluated oregano oil in the gas phase against *Colletotrichum coccodes* spoilage in tomato fruit, showing that this vapor-treatment suppressed spore viability by 22%. This research revealed that volatile-enrichment markedly reduced spoilage by anthracnose rot during the reproductive phase (spore germination/production) of the fungus, which is of great importance for the disease cycle and spread.

According to Tian et al. (2011), application of *C. virosa* EO resulted in the lowest percentages of infection in inoculated cherry tomatoes compared with the non-treated control at 200 μl/ml for *A. flavus* (11.1%), *A. oryzae* (11.1%), *A. niger* (8.6%), and *A. alternata* (2.8%). In healthy fruits, the results indicated that the percentage of infected fruits was significantly reduced by the essential oil at 18 °C for 21 days. In conclusion, the oil significantly reduced decay both in artificially inoculated and unwounded cherry tomatoes.

Omidbeygi et al. (2007) evaluated the antifungal activity of essential oils of thyme (*T. vulgaris*), summer savory (*S. hortensis*) and clove (*Syzygium aromaticum*) *in vitro* and in tomato paste. They observed that the addition of the EO inhibited the development of the test fungus, *A. flavus*, with thyme oil at 350 ppm and summer savory at 500 ppm being the most effective. Results from sensory evaluation showed that there were no significant differences between samples with 500 ppm of thyme oil and the control (without essential oil), but significant organoleptic differences were observed between samples with 500 ppm of summer savory and clove oil and the control. Thus, the use of plant essential oils at the concentrations required to be effective in tomato paste could raise concerns regarding changes in the organoleptic properties of the food.

dos Santos et al. (2012) evaluated the efficacy of the combined application of chitosan and *O. vulgare* essential oil to inhibit *Rhizopus stolonifer* and *A. niger* in the table grape cultivar Isabella and its effect on the physical, physicochemical and sensory characteristics of the fruits during storage at room and cold temperatures. The mixture showed antifungal activity when it was applied as a coating on grapes artificially contaminated with both moulds, as well as against the native fungal microbiota of grape. In general, grapes treated or not treated did not differ in weight at any of the assessed storage intervals at both tested storage temperatures, nor did their firmness, level of titratable acidity, soluble solids content, levels of anthocyanin and color. The results of the sensory characteristics of the fruits revealed that those not coated had the highest scores for all analyzed attributes when compared to coated fruits, especially for the attributes aroma, flavor, aftertaste and firmness. These scores decreased throughout the storage time for treated fruits.

Troncoso et al. (2005) treated green bell pepper with isothiocyanates extracted from cabbage (*Brassica oleracea* var. *capitata*) leaves and found that a higher concentration was needed to completely inhibit the disease caused by *A. alternata* in the pods than that which was inhibitory in *in vitro* tests. The authors proposed several theories to explain this behavior: (a) the more complex composition of the pepper fruit compared with the PDA media used in the *in vitro* test, (b) the stability throughout time and volatility of the compounds studied, (c) the diffusive effect of the active compound inside the fruit, and (d) the possible leak of the volatile compounds through the low density polyethylene used in the experiments. However, this treatment was better than the commercial fungicide to control fungi rot on bell pepper without any effect on fruit quality.

Other than in fruits and vegetables, some studies have also been carried out in grains as a food matrix because of their common contamination by fungi. Kumar et al. (2007) found that *C. ambrosioides* oil was efficient in control of fungal contamination of wheat samples when it was tested as fumigant. Phillips et al. (2012) showed that treatment with Citri-V™® reduces growth of *P. chrysogenum* and *A. niger* on grain. As this mixture of EOs has been shown not to affect the organoleptic properties of foodstuffs (Fisher et al., 2009) it was suggested that it may be used in industrial situations such as the storage of grain.

7. Combined treatments

The major limitation of antifungal compounds derived from plants is the strong flavor they may impart, thus restricting their applicability only to products with compatible flavor. Researchers have proposed different ideas, such as using the plant extract not only as a preservative but also as a flavor component. Alternatively, if the product in which the extracts are incorporated already has strong flavor, it may mask that of the natural antifungal. Another option is to apply some of the most active components, rather than the whole extract; though, as it was mentioned before, it is thought that the antimicrobial activity of any plant essential oil is likely to be a result of the synergistic interaction between different components, which provides different modes of inhibition and decreases the risk of resistance development in target microbial cells. Finally, the most promising alternative appears to be the development of synergistic combinations, either as two plant extracts or one antimicrobial agent combined with a stress factor such as reduced a_w or low pH.

Amiri et al. (2008) showed that treatment of conidia from apple postharvest pathogens *B. cinerea*, *M. fructigena*, *P. expansum* and *P. vagabunda* with eugenol oil extracted from buds and leaves of clove (*E. caryophylata*) at 2 mg/ml combined with 50 mg/ml lecithin at 50 °C for 2 min significantly reduced their germination in MEA medium. During *in vivo* assays on two apple cultivars, significant disease incidence reductions were observed on fruit treated with the same conditions. SEM observations revealed damage to the cuticle on fruit treated with eugenol solution without lecithin. This may have permitted significant penetration of the pathogens, consequently increasing disease incidence. The addition of lecithin to the eugenol solution eliminated the phytotoxic effect of eugenol at high temperatures and no damage was caused to the cuticle. Thus, it was demonstrated that the combination of lecithin–eugenol formulation with heat treatment significantly decreased the natural infections caused by the different pathogens. High temperature improved the emulsification of the formulation, rendering it more active against the pathogens. The authors also concluded that eugenol did not affect the flavor and the appearance of the apples.

Feng and Zheng (2006) reported that the inhibitory effect of cassia oil on mycelial growth of *A. alternata* was enhanced by amending with KCl on PDA. The combined inhibitory effects of cassia oil and KCl were higher than that of cassia oil alone at the same concentration. The oil combination with NaCl gave similar results. The results in *in vivo* assays showed that cassia oil at 500 ppm significantly reduced decay incidence in tomato fruits. The combination of 500 ppm cassia oil with 1% of NaCl or KCl exhibited significant inhibitory effect against *A. alternata* on the fruits. The enhanced effects of KCl or NaCl with cassia oil *in vivo* appeared to be lost as KCl or NaCl concentration reached 3%. The authors suggested that this high concentration of the

salts may cause damage to the cells of the tomatoes, resulting in decreased resistance to pathogens.

Matan et al. (2006) studied the inhibitory effects of cinnamon and clove oils, added singly and in various combinations under MAP conditions (modified atmosphere of low O_2 and high CO_2), on growth of fungi frequently found on intermediate moisture foods (IMF, a_w =0.65–0.90), such as *P. roqueforti*, *A. flavus*, *Eurotium* sp. and *M. plumbeus*. The results showed that the gas composition has a synergistic influence on the inhibitory effects of the oils; they were more effective in inhibiting the growth of all microorganisms when used in combination with low oxygen levels (<0.05%) and high CO_2 concentrations (40%).

Nguefack et al. (2012) reported the synergy observed by mixing fractions of EO from *Cymbopogon citrates*, *T. vulgaris* and *O. gratissimum* against *P. expansum*, a post-harvest fungus responsible for the deterioration of fruits and vegetables and the causal agent of "blue rot" in apples. This synergy was explained by the combined effect of the different components of each individual fraction. The major constituents of one fraction from *O. gratissimum*, thymol and carvacrol, are much more active than terpene hydrocarbons, such as p-cymene, the major component of another fraction of the same EO. However, the latter swells cell membranes to a greater extent than citrals, carvacrol and thymol. The synergistic effect varied according to the proportion of each fraction in the mixture, being higher when these fractions were mixed in a 50/50 v/v proportion. Furthermore, a synergistic effect was observed with mixtures of non-active fractions. A possible explanation is an increase of the concentration of oxygenated terpenes present in traces in each of the individual fractions, and whose transmembrane transportation could be facilitated by terpene hydrocarbons. On the other hand, mixtures of some of the most active fractions from the three plants revealed antagonistic effect. These observations might result from the chemical reaction that could occur between the constituents of the fractions, such as the reaction in neutral or acidic medium between the hydroxyl group (of alcohols and phenols) with a carbonyl function to form acetals that are compounds with blocked hydroxyl group and carbonyl function.

dos Santos et al. (2012) evaluated the efficacy of the combined application of chitosan, a linear polysaccharide derived from chitin deacetylation with antimicrobial properties, and *O. vulgare* EO to inhibit the post-harvest fungal pathogens *R. stolonifer* and *A. niger* in laboratory media and in the table grape cultivar Isabella. The combined application of both antimicrobials at the different tested concentrations strongly inhibited spore germination of the fungi. SEM analyses of spores revealed changes in their form and structure and morphological changes of the mycelia. The authors suggested that the strong, rapid and consistent effect observed from the combined application of chitosan and the EO at sub-inhibitory concentrations was due to synergism. The mixture also possessed antifungal activity when it was coated on grapes artificially contaminated with *R. stolonifer* and *A. niger*, as well as against the native fungal microbiota of grape throughout storage at room (12 days) and cold temperatures (24 days). Fruits coated with the mix stored at low temperature and contaminated with *R. stolonifer* showed no visible fungal growth throughout the storage period, while the growth of *A. niger* was visible from the tenth day of storage (25% infected fruits), and 33% of fruits were infected at the twelfth day of storage. Application of both antimicrobials also inhibited the native fungal population of fruits since there were no visible signs of infection throughout the assessed storage intervals at both tested temperatures. Fruits not coated with chitosan and *O. vulgare* EO combinations showed visible signs of fungal infection from the second day of storage at room temperature (41% infected fruits) and from the tenth day of storage at cold temperature (36% infected fruits).

In conclusion, the combination of two or more plant extracts or other environmentally friendly antimicrobial agents could be a viable option for application in food, achieving the desired antimicrobial effects with minor changes in the organoleptic properties of the product and reducing the risk of developing resistance by fungi as the mixture contains a greater variety of compounds with different target sites within the cell than the extract alone.

8. Recent developments

8.1. Supercritical fluid extraction (SFE)

At present, extraction of bioactive compounds requires the utilization of large amounts of solvent and technologies that are not environmentally friendly. SFE is an alternative extraction technique for solid materials with better selectivity and efficiency. Recently, it has been studied for the separation of active compounds from plants in order to eliminate solvents, to avoid degradation of sensitive substances and to reduce the high energy consumed and the residues generated in conventional processes. CO_2 is the compound generally used for this application because its critical pressure and temperature are low and easily achievable (Tc = 31.04 ºC, Pc = 73.8 bar). Besides, its other benefits include that it is a non-toxic compound, it is nonflammable and it is available in high purity at relatively low cost. Supercritical CO_2 has good solvent properties for extraction of non-polar components such as hydrocarbons. To facilitate the extraction of polar components, an entrainer (*e.g.* ethanol, methanol) is frequently used to raise the solvent power of supercritical CO_2.

Vági et al. (2005) reported that the ethanolic and supercritical extracts of *Origanum majorana* showed a similar composition, although in the SFE extract the content of terpinen-4-ol was almost twice that in the ethanolic extract. According to the results of the fungal growth test, the strongest inhibition activity against *A. niger*, *T. viride* and *P. cyclopium* was observed in the presence of the SFE extract. The authors explained this behavior by the fact that the SFE extract contained a higher concentration of volatile compounds and higher molecular weight biologically active compounds. The interactions between these constituents could be responsible for the increased activity.

8.2. Edible films and encapsulation

The use of edible coatings is one of the most important methods applied for preserving quality. They are useful to improve food appearance and to delay transmission of moisture, oxygen, aroma and solutes during processing, handling and storage, thus enhancing product shelf life. They are commonly made of proteins or polysaccharides, so they are considered environmentally friendly. Furthermore, they can retard food spoilage by inhibiting the growth of microorganisms, due to their natural intrinsic activity or to the incorporation of antimicrobial compounds. The incorporation of plant compounds (purified, the whole extract or the essential oil) to the coating may prolong its antimicrobial activity. Films can reduce diffusion into the product since the essential oil forms part of the chemical structure of the film and interacts with the polymer and the emulsifying agent, which is generally required to ensure dispersion and formation of a homogeneous coating. Moreover, the plant compounds are gradually released on the product surface over time, maintaining a proper concentration of antimicrobial components during the storage period and allowing the use of smaller amounts compared with direct application. Antimicrobial release from the edible film depends on several factors, such as electrostatic interactions between the antimicrobial agent and the polymer chains, osmosis, structural changes induced by the presence of antimicrobial and environmental conditions (T, pH). Starch is a universal encapsulating medium for food products; it is also used for encapsulating herbicide and pesticide products because of its biodegradability and versatility in processing.

Avila-Sosa et al. (2012) studied the degree of fungal inhibition against *A. niger* and *P. digitatum* by different EOs (cinnamon, Mexican oregano and lemongrass) incorporated into three edible films:

amaranth, chitosan and starch. They concluded that the level of inhibition depended directly on the type of polymer used to form the film because different polymers retain EOs to different degrees. Amaranth films were the least effective as carriers for the tested EOs. The main compounds of the tested EOs (thymol, carvacrol, eugenol and geranial) could interact with the components of the film, potentially affecting the antimicrobial activity. In order to cancel out this effect, higher concentrations of EOs must be added to the coating, promoting saturation of the system and thus allowing the presence of free active molecules. Chitosan and starch films, having a single type of polymer, retained biocompounds less effectively and therefore allowed more molecules into the vapor phase with resultant increase in antimicrobial activities. Chitosan edible films incorporating Mexican oregano or cinnamon EO resulted to be the most effective in inhibiting *A. niger* and *P. digitatum* growth at lower EO concentrations than those required for amaranth and starch edible films.

Vu et al. (2011) reported the use of EOs incorporated in modified chitosan (N-acylated chitosan) films for coating strawberries in order to improve their shelf life. The bioactive coating suspensions were prepared by dispersing the EO limonene or peppermint (*Mentha piperita*) in the presence of Tween 80, an emulsifier to improve the stability of the coating suspension. These two EOs were chosen because they showed the highest antifungal activity against total mycota isolated from strawberries, mainly *R. stolonifer* and *B. cinerea* during *in vitro* studies. Polymer suspensions were adjusted to pH 3.5 (pH of strawberries) by 10% acetic acid solution before spraying over the fruit. Generally the percentage of strawberries showing decay was lower in the coatings with limonene at most time points during the experiment in comparison with chitosan alone or with peppermint coatings and with uncoated strawberries. Moreover, they noticed that the limonene coating did not cause phytotoxicity and did not affect the appearance of strawberries, probably due to the presence of the emulsifier and to pH conditions (pH 3.5–4.0) that improved the solubilization of modified chitosan.

8.3. Active packaging

Recently, a new concept focused on the idea that active interactions between the package and the product may have positive effects has been increasing in relevance as a response to the consumers' demands for safe, high quality, fresh-like, and extended shelf-life foods. Active packaging is one of the most innovative food packaging concepts that implies the interaction between the package and the product or the headspace between the package and the food system to limit growth of microorganisms and reduce other quality deterioration processes. Applications of active packaging include using oxygen and carbon dioxide absorbers to manipulate headspace gas concentrations and adding the bioactive agents, as plant extracts, to the packaging system. It is expected that the headspace volatiles will increase in concentration following their release, which might be triggered by a change in environment (*e.g.* increased temperature or humidity), then decline during storage (depending on the permeability of the package and length of storage) and be dispersed when the packaged is opened by the consumer (Matan et al., 2006). The main advantages of using this technology, as well as edible films, for the application of natural antimicrobials in foods, are the controlled release of the bioactive compounds into the product during storage time and the lower possibility of development of undesirable flavors than its direct addition into food could cause. Polyolefin-based films are usually used for the development of active packaging systems, combining the polymer good general properties (mechanical, barrier, optical and thermal) and the antimicrobial or antioxidant efficiency given by the additives (Ramos et al., 2012).

Manso et al. (2013) analyzed the influence of the substrate of several packaging materials containing cinnamon (*Cinnamomun zeylanicum*) EO as active agent on the antifungal activity against *A. flavus*. They demonstrated that plastic films (PET, PP and PE/EVOH) require much less EO load than active paper to inhibit the fungus. This indicated that the release of the cinnamon essential oil from the polymer of plastic material to the microorganism occurs in the same manner, without a strong dependence on the film substrate and that the interaction with the polymer surface (absorption, degradation) is negligible. However, both substrates feature excellent long-term effectiveness and stability.

9. Final conclusions

The search of alternative antifungals is of great concern for food industry, mainly due to the substantial post-harvest losses that occur due to fungal contamination. Meanwhile, the environmental protection agencies and organizations express their concern about the widespread use of synthetic fungicides that contaminate soil and water, and leave toxic residues that might affect crops as well as consumers. From a health point of view, the greatest hazard associated with the development of fungi is their ability to produce mycotoxins. The possibility of using compounds extracted from plants or their whole extracts to control mycotoxin contamination is a promising alternative and has been extensively studied as has been reviewed in this article. The fact of employing plants that have historically been used in alternative medicine, whose safety is proved, builds confidence in consumers, who are increasingly interested in obtaining “green products”. It also raises additional economic benefit due to the possibility of providing a use for a wide range of plants or weeds. Even though some are already used as aromatic herbs or spices, they might be added in greater concentrations than in normal practice, hence further studies on animal and human toxicity must be carried out, when they are intended to be added as natural antifungals to foods or when they are used in combination with other substances.

The field of research on natural antifungals is wide and there are still a great number of possibilities to explore. It is our belief that further systematic studies are needed to broaden the knowledge on this area. For future studies, tested plant species must be thoroughly described and identified; including location and season where they grow. Similarly, the type and number of microorganisms to be evaluated should be appropriately chosen, using both collection strains and isolates from the substrate in which the plant extract would be applied. Moreover, the application of new preservatives needs the approval of governmental authorities. Finally, it is important to keep in mind the complexity of food matrixes and the microbial ecology of foods when a new antifungal is to be tested, taking into account the behavior of microorganisms and the physiological and molecular mechanisms within cells that cause these microbial responses.

Acknowledgments

Financial support from the Universidad de Buenos Aires is acknowledged. A. Patriarca is a member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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