The use of essential oils to protect rice from storage fungi

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Abstract

Rice (*Oryza sativa*) is the main food of half of the population of our planet. The growth of fungi closely associated to the eventual occurrence of mycotoxins can be responsible for serious economic losses and public health risks. Knowledge about the origin of the growth of toxigenic fungi is a prerequisite to the establishment of mycotoxin control programs. Socio-economical and environmental factors led to an extreme reduction of rice availability, while the estimated rice production losses increased in all continents what increases the importance to develop new harmless strategies for the control of fungi affecting stored rice. Natural products from plant origin were screened for the control of main pernicious fungi.

In this work we have collected rice samples from different origins (national and imported) and these samples were analysed for fungal infection. Several fungi taxa were isolated: *Absidia, Alternaria, Aspergillus, Bipolaris, Botrytis, Chaetomium, Curvularia, Cunninghamela, Epicoccum, Fusarium, Geotrichum, Helicoma, Nigrospora, Penicillium, Pyricularia, Rhizopus, Scytalidium, Stemphylium, Sordaria, Trichoconiella, Trichoderma, Trichothecium* and *Ulocladium*. Some of the fungi isolated are potentially mycotoxigenic. We also studied a way to control the growth of some of these fungi using plant extracts and essential oils from *Syzyginum aromaticum* and *Laurus nobilis*. Promising results were obtained.

Keywords: Rice, Cereals, Fungi, Bio-pesticides, Plant extracts.

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for over half of the world population and is grown on approximately 146 million ha, i.e. more than 10% of the total available land for agriculture. In the tropics, rice is the primary source of human nutrition, and is one of the cheapest sources of food energy and protein (Cantrell, 2001; Mexia, 2003).

Portugal is the biggest consumer of milled rice in Europe (15 kg per person and per year), with an annual paddy rice production of about 129 000 tonnes of *japonica* variety (short-grain), 26 000 tonnes of *indica* variety (long-grain) distributed mainly by Sado, Tejo and Mondego Valley companies. Besides national production, 98 000 tonnes of rice are imported to satisfy reach consumers needs (Brites et al., 2006; INE, 2007).

Paddy rice is a seasonal crop in Portugal, so storage of paddy and milled rice is of major importance for year-round availability. In storage, the development of fungi, especially *Aspergillus* spp. and *Penicillium* spp., is an unsolved problem. These fungi are responsible for rice quantitative and qualitative losses and are mycotoxins potential producers. Mycotoxins are hazardous to animal and human health and constitute a factor for economic losses in food products worldwide (Omidbeygi et al., 2007; Pitt and Hocking, 2009).

Safety, residual toxicity and resistance to known chemical preservatives led to an increased search on novel strategies of food preservatives. Naturally occurring antimicrobial compounds for food

preservation receive increasing attention due to consumer awareness about natural food products and a growing concern of microbial resistance towards conventional preservatives (Skandamis et al., 2001; Schuenzel and Harrison, 2002). The objectives of this work were: (1) Isolation, identification and evaluation of fungi abundance in rice samples; (2) *in vitro* fungicidal activities of the essential oils of clove (*Syzygium aromaticum* (L.) Merr. & Perry.) and laurel (*Laurus nobilis* L.) on the fungi isolated from the rice samples.

2. Materials and methods

2.1 Mycoflora analysis

Rice samples, namely paddy rice, brown rice and milled rice were collected regularly during the experimental period in a rice mill at different places of the milling process. Five samples were collected in sterilized containers and taken into the laboratory. In the laboratory, the rice samples were sub-divided in samples with 110 grains. These sub-samples were disinfected at surface with 1% sodium hypochlorite, during two minutes, as describe by Pitt and Hocking (1997) and Magro et al. (2008). Ten disinfected grains were placed on Petri dishes with 20 mL of Potato Dextrose Agar (PDA) medium with chloramphenicol (1%) (Fig. 1). For each sample, ten replicates were made.

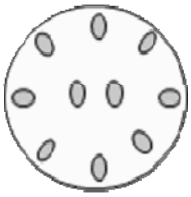

Figure 1 Schematic representation of the grain distribution on a Petri dish with PDA medium supplemented with chloramphenicol.

The grains in Petri dishes were incubated at 28ºC for 8 d and then examined under a stereomicroscope for fungal growth. Isolation of the colonies was made to obtain pure cultures. Slides of fungal growth were prepared and observed under a high magnification microscope for fungal morphology study. The identification was carried out using identification keys (Carmichael et al., 1980; Domsch et al., 1980; Onions et al., 1981; International Mycological Institute, 1991; Hanlin, 1997; Malloch, 1997; Pitt and Hocking, 1997; Barnett and Hunter, 1998; Samson et al., 2004).

2.2 Extraction of essential oils

The essential oil of laurel used in this study was obtained by hydro-distillation of air-dried leaves in a modified Clevenger-type apparatus for 3 h. The extracted oil was dried over anhydrous sodium sulfate and stored in a sterilized amber bottle at 4ºC until used. The essential oil of clove was supplied by the Portuguese company Segredo da Planta, reference No. 127005582.

2.3 Fungi selection for growth inhibition test

The fungi used for the bioactivity tests were *Aspergillus candidus*, *A. niger*, *Fusarium culmorum* and *Penicillium islandicum*. These fungi were obtained from samples of rice grains collected in a Portuguese rice processing factory.

2.4. Determination of effect of essential oils in solid media (Potato Dextrose Agar -PDA)

For the determination of effect of essential oils on the growth of the fungi tested, different amounts of essential oils were deposited on the surface of PDA, namely, 10, 25, 50, 100, 250, 500 and 750 µL. The Petri dishes were inoculated with a 5 mm diameter disk of fungi grown on potato dextrose agar (PDA) medium for 8 days at 28ºC. This disk was placed on the agar surface and incubated at 28ºC. Inhibition of fungal growth according to the effects of essential oils were determined by a periodic measurement of the fungal colony diameter change with time carried out during 25 weeks. In the control, equal amounts of sterilized water were placed on the surface of PDA. The mean radial mycelia growth of the fungi was determined by measuring the diameter of the colony in two directions when the plate surface of the control Petri was covered by fungus, 7 days after inoculation. For each concentration, four replicate dishes were used.

3. Results and discussion

3.1. Mycoflora analysis

Field and storage fungi were detected and identified in all samples (Table 1). The field genera isolated from rice grain samples were: *Absidia, Alternaria, Bipolaris, Botrytis, Chaetomium, Cunninghamella, Curvularia, Epicoccum, Geotrichum, Helicoma, Nigrospora, Pyricularia, Rhizopus, Scytalidium, Sordaria, Stemphylium, Trichoconiella, Trichoderma, Trichothecium* and *Ulocladium.*

Table 1 Fungi *taxa* identified on samples of paddy, brown and long grain rice from different origins.

	Step of Processing		
	Paddy	**Brown**	**Long grain**
National rice	*Absidia corymbifera*	*Alternaria* sp.	*Aspergillus* spp.
	Alternaria sp.	*Aspergillus* spp.	*A. fumigatus*
	Aspergillus spp.	*A. flavus*	*A. penicillioides*
	A. candidus	*A. fumigatus*	*Penicillium* sp.
	A. flavus	*A. niger*	*Trichoconiella padwickii*
	A. fumigatus	*A. terreus*	
	A. niger	*Bipolaris* sp.	
	A. terreus	*Chaetomium* sp	
	Bipolaris sp.	*Epicoccum* sp.	
	Botrytis sp.	*Fusarium* spp.	
	Chaetomium sp.	*Geotrichum* sp.	
	Curvularia sp.	*Nigrospora oryzae*	
	Cunninghamella sp.	*Penicillium* spp.	
	Fusarium culmorum	*Pyricularia* sp.	
	Geotrichum sp.	*Scytalidium* sp.	
	Nigrospora oryzae	*Stemphylium botryosum*	
	Penicillium spp.	*Trichoconiella padwickii*	
	P. islandicum	*Trichoderma harzianum*	
	Rhizopus oryzae	*Trichothecium roseum*	
	Scytalidium sp.	*Ulocladium atrum*	
	Sordaria fimicola		
	Trichoconiella padwickii		
	Trichoderma harzianum		
	Trichothecium roseum		
Imported rice		*Aspergillus* spp.	*Alternaria* sp.
		A. flavus	*Aspergillus* spp.
		A. fumigatus	*A. flavus*
		A. niger	*A. fumigatus*
		Bipolaris sp.	*Stemphylium botryosum*
		Curvularia sp.	*Trichoconiella padwickii*
		Fusarium spp.	
		Helicoma sp.	
		Nigrospora oryzae	
		Penicillium sp.	
		Trichoconiella padwickii	
		Rhizopus oryzae	

The storage species (or field fungi species remaining alive during storage) isolated were: *Aspergillus* spp., *A. candidus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Fusarium* spp., *F. culmorum*, *Penicillium islandicum* and *Penicillium* spp. The samples of paddy rice presented a higher number of *taxa* (Table 1), that may be the result of not having been processed and of storage conditions. On the other hand, the long grain rice, presented a lower number of *taxa* in relation to the other samples studied, probably due to the processing of the rice from paddy to long grain rice. This reduction of the number of taxa observed on polished rice was probably related to the effect of the abrasive process (Lima et al., 2000). In the brown rice samples 20 and 12 *taxa* (Table 1) were detected and identified in domestic production and imported rice, respectively. These results are in accordance with the results obtained by Mourato (1984)

and Manabe and Tsuruta (1991). According to these authors, the field fungi are gradually replaced by storage fungi as the storage period increases. However, with imported rice the storage period is not always well defined.

Field fungi colonize rice grains only when the water activity (a_w), temperature and relative humidity are high. However, as a result of an adaptation to low a_w, the fungi belonging to *Aspergillus* spp., and *Penicillium* spp., also designated as storage fungi, are able to invade the rice grains stored at levels of a_w considered as safe. They are frequently responsible for causing serious losses, even before they were visually detected. They affect negatively the product's appearance, flavor, odour and nutritional content. They also may produce mycotoxins with high impact in public health (Magro, 2001). It is important to emphasize that *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., resist to the processing of rice. Since these fungi are potential mycotoxins producers, it is fundamental to improve and control the rice storage conditions as well as the cleaning process before processing. We can concluded that the processing of the rice grains is responsible for the reduction of the number of fungi *taxa*, and that storage duration should be reduced to less than one year, when possible.

3.2. Fungicide activity of essential oils in PDA

The ability of the essential oils of clove and laurel to inhibit the growth of rice fungi was evaluated (Tables 2, 3). Since the storage durability is an important factor in the storage of food products, in this work it was stipulated that the assays would have the durability of 40 weeks for the clove essential oil and 25 weeks for the laurel essential oil.

Table 2 Determination of the effect of clove essential oil on the growth of storage fungi in solid media (*in vitro* bioassay).

Fungi	Volume µL)	Incubation time (weeks)								
		1	5	10	15	20	25	30	35	40
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Fusarium culmorum	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Penicillium islandicum	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Aspergillus candidus	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Aspergillus niger	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	●	●
	Control	●	●	●	●	●	●	●	●	●

Note: (○) – without growth (●) – with growth.

Table 3 Determination of the effect of laurel essential oil on the growth of storage fungi in solid media.

Fungi	Volume µL)	Incubation time (weeks) 1	2	3	4	5	6	7	8	9	10	11	12	13
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Fusarium culmorum	250	○	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Penicillium islandicum	250	○	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	●	●	●
Aspergillus candidus	250	○	○	○	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Aspergillus niger	250	○	○	○	○	○	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●

Continue

Fungi	Volume µL)	Incubation time (weeks) 14	15	16	17	18	19	20	21	22	23	24	25
	750	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○
Fusarium culmorum	250	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○
	Control	○	○	○	○	○	○	○	○	○	○	○	○
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	○	○
Penicillium islandicum	250	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●	●	●	●
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	●	●
Aspergillus candidus	250	●	●	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	○	○
Aspergillus niger	250	●	●	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●

Note: (○) – without growth (●) – with growth

The clove essential oil showed a strong antifungal potential along the 40 weeks, being active at the lowest dose of the extract against *F. culmorum*, *P. islandicum* and *A. niger* and at a dose of 25 µL against to *A. candidus*. For the essential oil of laurel, the total control of *F. culmorum* was reached with the dose of 100 µL, of *P. islandicum* with the dose of 250 µL, while *A. niger* and *A. candidus* were controlled only when the dose of 750 µL was applied and only for 13 and 10 weeks, respectively. The results obtained in this study are similar to these obtained by Nielsen and Rios (2000), Guynot et al. (2003), Lopez et al. (2005), and Matan et al. (2006). But our results are relevant, because we used different species of fungi and we get no fungi growth for several months, which is extremely important when we deal with stored grains. The essential oils of clove and laurel showed inhibitory effects on the four tested fungi, *Aspergillus candidus*, *A. niger*, *Fusarium culmorum* and *Penicillium islandicum*, at all concentrations but the clove essential oil was more efficient in the control of fungi that laurel essential oil. These findings

clearly indicate that the essential oils used should be more studied in order to better characterize their potential for future use as a substitute for chemical fungicides.

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