Bulgarian Journal of Agricultural Science, 17 (No 1) 2011, 83-92
Agricultural Academy

EFFECT OF *PRUNUS LAUROCERASUS* L. (CHERRY LAUREL) LEAF EXTRACTS ON GROWTH OF BREAD SPOILAGE FUNGI

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Abstract

SAHAN, Yasemin, 2011. Effect of *Prunus laurocerasus* L. (Cherry Laurel) leaf extracts on growth of bread spoilage fungi. *Bulg. J. Agric. Sci.,* 17: 83-92

Six different extracts (4 solvent extracts and 2 water extracts) of *Prunus laurocerasus* L., leaf were used to determine the antifungal effect of it on *Aspergillus chevalieri, A. flavus, A. fumigatus, A. niger, A. oryzae, A. parasiticus, Fusarium oxysporum, Mucor spp., Penicillium commune, P. islandicum, P. roqueforti, P. solitum, P. verrucosum, Rhizopus oligosporus* and *R. stolonifer* by disc diffusion and micro dilution methods. Statistical results showed that the kind and amount of extracts have a significant effect against tested fungi. The maximal inhibition zone, MIC and MFC values for fungal strains, which were sensitive to extracts of *P. laurocerasus* L. leaf, were in range of 2-19 mm, 7, 8-500 µg/ml and 15.6-500 µg/ml, respectively. The highest total antifungal effect was observed from ethanol and acetone extracts. Comparing the sensitivity of the fungi to all *P. laurocerasus* L. leaf extracts, *P. verrucosum* demonstrated higher resistance than the other test fungi while *F. oxysporum* was the most sensitive microorganism ($P < 0.05$).

Key words: Prunus laurocerasus L., cherry laurel, Extracts, Bread Spoilage, MIC, MFC

Introduction

Cereal products, especially bread, have been a major source of food for the human being since the commencement of civilization. Bread consumption has increased with the passage of time, such that it has become an integral and established staple part of the diet of the populace. Worldwide, bread is, and has been a central constituent in the diets of most populations for thousands of years. The bakery sector is also a major contributor to economic growth and employment opportunities. In Europe there are over 120000 enterprises active in bread-making, the vast majority of which are small craft bakers. Given its size, the bakery sector can be considered to be one of the most important sectors of the food industry as a whole(Abdullah, 2008).

Bread, one of the most important staple foods in the world, can be spoiled by many moulds(Legan, 1993). Contaminants of bread are mainly *Penicillium* species, but *Aspergillus, Rhizopus, Fusarium, Mucor* and *Eurotium* species also occur, the latter especially in warmer climates. The most important mould species on bread are *Penicillium commune*, *P. crustosum*, *P. brevicompactum*, *P. chrysogenum*,

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P. roqueforti, *Aspergillus flavus, A. versicolor* and *A. sydowii, A. niger* and *Mucor* species (Lund et al., 1996; Gocmen and Sahin, 1997; Nielsen and Rios, 2000; Suhr and Nielsen, 2004; Hutkins, 2006; Dal Bello et al., 2007). Losses due to mould spoilage vary between 1% and 5% of products depending on season, type of product and method of processing(Legan, 1993; Guynot et al., 2002).

In unpreserved bread a shelf life of 3–4 days may be expected especially if the level of hygiene in the factory is not sufficiently high. Apart from the repelling sight of visible growth, fungi are responsible for off-flavour formation, rendering bread unfit for human consumption by retarding its nutritive value and producing mycotoxins and allergenic compounds (Bluma et al., 2008; Gutiérrez et al., 2009). In addition to the economic losses associated with spoilage of this nature, a further concern is the possibility that mycotoxins produced by the moulds may cause public health problems. A number of methods are applied to prevent or minimize microbial spoilage of bread, use of weak organic acids such as propionic, benzoic, and sorbic, modified atmosphere packaging, irradiation, pasteurization of packaged bread and biopreservation (Cauvain, 2003; Dal Bello et al., 2007; Gutierrez et al., 2009).

Since industrialization, urbanization and change of life style started to make demands for longer shelf life of bread; the use of chemical preservatives has been the main choice. However, today consumers show preferences for products without preservatives but kept free from microbial growth, toxins and other quality deteriorating factors while maintaining freshness and sensorial qualities. The problem for the food industry is to fulfill the demands of minimum changes in food quality and maximum security, without using chemical preservatives (Nielsen and Rios, 2000; Guynot et al., 2002; Gutierrez et al., 2009).

For the many years, there has been a traditional application for delaying the mold spoilage in breads in the eastern Black Sea region of Turkey. For this purpose, after fermentation, breads are put into the fresh *Prunus laurocerasus* L. leaf and baked at oven. Thus, fungal spoilage can be prevented and the shelf life of bread can be prolonged. As a result of these, the economical losses can be reduced. *Prunus laurocerasus* L. (syn: *Laurocerasus officinalis* Roem, *Cerasus laurocerasus* (L.) Lois, *Laurocerasus* V*ulgaris* Carr.), also known as Taflan is in the Rosaceae family and Prunoideae subfamily (Yesilada et al., 1999). It is grown in the eastern Black Sea region of Turkey, some of the Balkans, Northern Ireland, Western Europe, southern and western Caucasus, Iran, eastern Marmara, and some Mediterranean countries and is widely consumed in the eastern Black Sea region. The cherry laurel tree is an evergreen plant of 6 m height with ovoid dark purple to blackish fruits 8-20 mm in diameter. Plantings of *P. laurocerasus* L. contribute to reduction of the risk of desertification due to its evergreen nature, and its leaves present an alternative use in landscape architecture. *P. laurocerasus* L., as many plants do, can synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Kolayli et al., 2003; Calisir and Aydin, 2004). The leaves of *P. laurocerasus* L. are used for asthma, coughs, indigestion and dyspepsia. The fresh leaves of this plant, moreover, are used in Turkish folk medicine as analgesic for headache and as antipyretic externally. Fresh leaves are applied on the forehead, after being wilted over fire (Erdemoglu et al., 2003).

Despite the high number of publications that document the antimicrobial activity of plants extracts against different fungal species, none of reports have concerned with *Prunus laurocerasus* L. leaf. Because of their antimicrobial effects, spices and herbals extracts are of interest regarding their possible use as alternatives to food preservatives currently in use. The objective of this work was to determine the inhibitory effect of selected *Prunus laurocerasus* L. leaf extracts against the fungi associated with stale bread. For each strain, the minimal fungicidal or inhibitory concentration was determined.

Materials and Methods

Preparation of plant extracts

Prunus laurocerasus L. leaves were collected from Bursa province in Turkey in the Autumn of 2007. Collected leaves were rinsed with sterile distilled water and the leaves were separated from the stem, and cut into pieces with 2 mm diameter mesh. Extracts were prepared as described below.

Solvents extracts: The homogenized material (60 g) was extracted for 8 h with methanol (MeOH), ethanol (EtOH), acetone ($OC(CH_3)_2$) or chloroform ($CHCl_3$) by using a Soxhlet extractor (Fisher Sci., Germany) at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whatman filter paper (No. 1) and then concentrated in vacuum at 40°C using a rotary evaporator (Bibby RE100, UK). The residues obtained were stored in a freezer at -18°C until further tests (Sahin et al., 2004).

Water extracts: The water extracts were prepared by two different methods. One of these was same as for the solvent extracts. In the other, homogenized leaves were extracted in water at a 60% (w/v) concentration and autoclaved at 121°C for 20 min. The leaves were then separated and the resulting extract was autoclaved again (Markin et al., 2003). The extracts were filtered using Whatman filter paper (No. 1) and then concentrated in rotary evaporator (Bibby RE100, U.K). The crude extract yield were calculated as gram extract/gram plant material (w/w, %) and displayed in Table 1.

Test Fungi

The activity of *P. laurocerasus* L leaf extracts was tested against 15 fungal strains. Test fungi are shown in Table 2. Fungi were obtained from the Faculty of Agriculture (UUAF) and Arts and Sciences(UUAS), University of Uludag and the American Type Culture Collection(ATCC) strain from Oxoid(Wesel, Germany).

Table 1

Extracts yields of *P. laurocerasus* L. leaf

Extracts	Yields, %
MeOH	20.56 ± 1.22*
EtOH	25.28 ± 1.35
$OC(CH_3)_2$	28.65 ± 1.18
$CHCl_3$	4.93 ± 0.74
H_2O-Soxhelet	8.83 ± 0.86
H_2O-Autoclaved	10.98 ± 0.96

MeOH=Methanol Extract; EtOH= Ethanol Extract; $OC(CH_3)_2$=Acetone Extract; $CHCl_3$= Chloroform Extract; H_2O-S= Water Extract- Soxhelet; H_2O-A= Water Extract-Autoclaved

* Mean value of ± SD (n = 3)

Production of Conidia

All of the test fungi were cultivated on Sabouraud Dextrose Agar (Difco, Detroit, MI) slants at 30°C for 7 days and spores were harvested with 10 ml of 1% Tween 80(Merck, Darmstadt, Germany) solution sterilized by membrane filtration. Conidia were harvested by centrifugation and washed with 10 ml of sterile distilled water. This step was repeated three times and the spore suspension was stored in sterile distilled water (30 ml) at 4°C until used. The concentration of spores in the suspension was determined by a viable spore count on Sabouraud Dextrose Agar plates using the spread plate, surface count technique (Yin and Tsao, 1999; Korukluoglu et al., 2008; Korukluoglu et al., 2009).

Disc-diffusion assay

The dried extracts were dissolved in the methanol to a final concentration of 500 µg/ml and sterilized by filtration by 0.45 µm Millipore filters. Antimicrobial tests were then carried out by the disc diffusion method (Sokmen et al., 2004) using 100 µl of suspension containing 10^4 spore/ ml of fungi spread on Sabouraud dextrose agar (SDA).

Table 2
Fungi used in the *P. laurocerasus* L. leaf extracts experiments

Fungi	Origin	Source
Aspergillus chevalieri	Country bread	UUAF Food Engineering Department
Aspergillus flavus	Country bread	UUAF Food Engineering Department
Aspergillus fumigatus	Country bread	UUAF Food Engineering Department
Aspergillus niger	ATCC 16604	
Aspergillus oryzae	Sliced bread	UUAF Food Engineering Department
Aspergillus parasiticus	Sliced bread	UUAF Food Engineering Department
Fusarium oxysporum		UUAF Plant Protection Department
Mucor spp.		UUAF Plant Protection Department
Penicillium commune		UUAS Biology Department
Penicillium islandicum	Sliced bread	UUAF Food Engineering Department
Penicillium roqueforti	Sliced bread	UUAF Food Engineering Department
Penicillium solitum	German bread	UUAF Food Engineering Department
Penicillium verrucosum	Ordinary bread	UUAF Food Engineering Department
Rhizopus oligosporus	Sliced bread	UUAF Food Engineering Department
Rhizopus stolonifer	Maize bread	UUAF Food Engineering Department

UUAF Uludag University, Agriculture Faculty
UUAS Uludag University, Uludag University

The discs (6 mm in diameter) were impregnated with the 500 µg/ml extracts and placed on the inoculated agar. Negative controls were prepared using the methanol employed to dissolve the plant extracts. The antibiotic Ketaconazole (Sigma-10 µg/disc) was used as a positive reference standard to determine the sensitivity of strain/isolate in each fungal species tested.

The inoculated plates were incubated at 30°C for 72 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test fungi.

Microdilution Assay

The Minimal Inhibitory Concentrations (MIC) values of plant extracts against pathogenic strains were determined based on a micro dilution method (Zgoda and Porter, 2001) with some modifications. The plant extracts dissolved in 10% dimethyl sulfoxide (DMSO) were first dilution to the highest concentration (500 µg/ml) to be tested, and then serial two fold dilution was made in concentration range 7.8–500 µg/ml in 10ml sterile test tubes.

The sterile empty tubes were prepared by dispensing into each tube 950 µl of Sabouraud dextrose broth (SDB) and 50 µl of the conidial suspension containing about 10^4 conidia. 1 ml from the stock solution of *L. officinalis* extracts initially prepared at the concentration of 500 µg /ml were added into the first tubes. Then, 1 ml from their serial dilutions was transferred into other tubes. Sabouraud dextrose broth (SDB) without compound and 50 µl of the inoculums on each strip was used as negative control. Then all the tubes were incubated at 30ºC for 7 days. Microbial growth was determined by visible growth of fungi. The highest dilution showing no visible growth was regarded as MIC during 7 days. Cells

from the tubes showing no growth were subcultured on SDA plates and incubated at 30ºC for 5 days to determine if the inhibition was reversible or permanent. Minimal Fungicidal Concentration (MFC) was determined as the highest dilution at which no growth occurred on the plates. All the tests were done in three replicates.

Statistical Analysis

Statistical analyses were carried out using SPSS software (SPSS 13.0 SPSS Inc, Chicago, IL). The standard deviation was calculated by analysis of variance Minitab 14.0 software (State College, PA) and/or SAS software (Cary, NC). Cluster analysis was carried out to establish the inhibition rate of similar groups on selected fungi. Duncan's multiple-range test was used to determine the differences between variances by using an MSTAT statistical package.

Results and Discussion

The *in vitro* antifungal activities of the *P. laurocerasus* L. leaf extracts against the fungi and their activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameters, MIC and MFC (Tables 3 and 4).

Statistical results showed that the kind and amount of extracts have a significant effect against tested fungi ($P < 0.05$). In addition, for every extract tested, an analysis of variance demonstrated that MIC, MFC, and zone significantly ($P < 0.05$) affected by fungi.

Results clearly demonstrated that the plant investigated exhibited significant antifungal activity ($P < 0.05$). The results showed that out of all the six solvents used for extraction, the ethanol and acetone extracts displayed a broader spectrum of antifungal activity, followed by the chloroform and methanol extracts.

The highest inhibition zone, MIC and MFC values for fungal strains, which were sensitive to extracts of *P. laurocerasus* L. leaf, were in range of 2-19 mm, 7.8-500 µg/ml and 15.6-500 µg/ml, respectively (Tables 3 and 4).

Zone evaluation

Among all the *P. laurocerasus* L. leaf extracts, the ethanol and acetone extracts showed maximum antifungal activity according to zone diameters ($P < 0.05$). Ethanol extract was found most effective against *F. oxysporum, P. solitum* and *R. oligosporus* while acetone extract was most effective against *A. chevalieri, A. paraciticus, R. stolonifer* and *P. verrucosum* (Table 3). Methanol extract generally indicated weak inhibitory activity against the fungi used ($P< 0.05$). On the other hand, this extract was found the most effective (13 mm diameter) against *A. fumigatus*, which is a mycotoxigenic fungus. Chloroform extract of *P. laurocerasus* L. leaf showed low antifungal activity compared with other solvent extracts. However, *A. niger, Mucor* spp. and *P. roqueforti* were most affected by this extracts with 12, 11 and 10 mm, respectively.

The water extract had no or limited inhibiting effect (average 0.6 mm diameter) on any of the tested fungi. This could be explained due to the excessive heat and the resulting chemical change from the high boiling temperature during extraction with water (Kim et al., 2004). In addition, it might be explained that water weakly solved phenolic compounds, non-polar fraction in *P. laurocerasus* L. leaf. The crude aqueous extracts of some of the plants showed limited antifungal activity against mycotoxigenic molds for extracting antifungal compounds from medicinal plants (Afifi et al., 1991; Vlachos et al., 1996; Chandrasekaran and Venkatesalu, 2004).

Comparing the sensitivity of the fungi to all *P. laurocerasus* L. leaf extracts, *P. verrucosum* demonstrated higher resistance than the other test fungi while *F. oxysporum* was the most sensitive microorganism ($P < 0.05$).

MIC and MFC evaluation

MIC and MFC values obtained in microdilution assays using solvent extracts were in range of

Table 3

Antimicrobial activity of *P. laurocerasus* L. leaf extracts against the fungi tested based on disc diffusion method

Fungi	Inhibition zone in diameter (mm) [a] around the discs impregnated with 10 µl of extracts, 500 µg/disc							
	MeOH	EtOH	$OC(CH_3)_2$	$CHCl_3$	H_2O-S	H_2O-A	Negative control Methanol	Positive control KET, 10µg/disc
A. chevalieri	11 ± 0.18	9 ± 0.10	15 ± 0.13	14 ± 0.11	2 ± 0.13	3 ± 0.21	-	21 ± 0.35
A. flavus	9 ± 0.09	9 ± 0.13	8 ± 0.11	7± 0.05	-	-	-	17 ± 0.56
A. fumigatus	13 ± 0.11	10 ± 0.11	12 ± 0.11	11 ± 0.13	-	-	-	16 ± 0.38
A. niger	6 ± 0.15	8 ± 0.09	9 ± 0.15	12 ± 0.11	-	-	-	17 ± 0.42
A. oryzae	8 ± 0.11	9 ± 0.08	8 ± 0.10	8 ± 0.11	-	-	-	20 ± 0.63
A. parasiticus	9 ± 0.13	8 ± 0.11	10 ± 0.10	9 ± 0.14	-	-	-	12 ± 0.49
F. oxysporum	16 ± 0.23	19 ± 0.14	13 ± 0.16	12 ± 0.13	4 ± 0.17	4 ± 0.11	-	24 ± 0.19
Mucor spp.	9 ± 0.14	9 ± 0.12	10 ± 0.11	11 ± 0.09	-	-	-	13 ± 0.26
P. commune	7 ± 0.08	10 ± 0.17	9 ± 0.07	10 ± 0.10	3 ± 0.19	2 ± 0.23	-	18 ± 0.75
P. islandicum	9 ± 0.10	9 ± 0.10	8 ± 0.09	7 ± 0.13	-	-	-	17 ± 0.31
P. roqueforti	9 ± 0.10	9 ± 0.15	8 ± 0.10	10 ± 0.15	-	-	-	15 ± 0.22
P. solitum	7 ± 0.13	11 ± 0.12	9 ± 0.11	8 ± 0.11	-	-	-	17 ± 0.45
P. verrucosum	6 ± 0.11	8 ± 0.11	9 ± 0.10	8 ± 0.09	-	-	-	16 ± 0.33
R. oligosporus	8 ± 0.16	10 ± 0.22	9 ± 0.09	9 ± 0.11	-	-	-	14 ± 0.72
R. stolonifer	8 ± 0.15	9 ± 0.14	10 ± 10	7 ± 0.09	-	-	-	13 ± 0.52

MeOH=Methanol Extract; EtOH= Ethanol Extract; $OC(CH_3)_2$=Acetone Extract; $CHCl_3$= Chloroform Extract; H_2O-S= Water Extract- Soxhelet; H_2O-A= Water Extract-Autoclaved; KET= Ketoconazole antifungal control

[a] Mean of three assays

± = Standard deviation

7.8- 250 µg/mLand 15.6- 250 µg/ml, respectively, while the values recorded of water extracts were 125- 500 µg/ml. *A. niger* was found the most resistant fungi to solvent extracts followed by *A. oryzae* and *P. verrucosum* according to MIC values(Table 4).

The lowest minimum inhibitory concentration recorded for the water extract that used soxhelet apparatus against eight fungi were breakpoint (500 µg/ml) recommended while autocloved water extract were four fungi. This situation explained as crude extracts of uncertain composition with components that can have synergistic or antagonistic effects (Fabry et al., 1998).

Hierarchical cluster analysis from Figure 1 permitted us to establish two main groups for the MIC values. In figure, below main group was more effected by all extracts tested, except *A. niger*, while upper main group was less influenced.

In the literature, antifungal studies of plant extracts in bakery products are very limited. Guynot et al. (2003) investigated the volatile fractions of 16 essential oils for activity against the more common fungi, *Eurotium amstelodami, E. herbariorum, E. repens, E. rubrum, Aspergillus flavus, A. niger* and *Penicillium corylophilum,* causing spoilage of bakery products. They reported on the antifungal activity of cinnamon leaf, bay,

Table 4
MIC and MFC values of *P. laurocerasus* L. leaf extracts against some bread spoilage fungi, µg/ml

Fungi	µg/ml	MeOH	EtOH	$OC(CH_3)_2$	$CHCl_3$	H_2O-S	H_2O-A	Antibiotic KET
A. chevalieri	MIC	31.5	31.5	15.6	31.5	250	250	7.8
	MFC	62.5	62.5	31.5	31.5	500	500	
A. flavus	MIC	62.5	62.5	125	125	500	500	15.6
	MFC	62.5	125	250	125	500	500	
A. fumigatus	MIC	15.6	62.5	31.5	62.5	500	500	15.6
	MFC	31.5	62.5	62.5	125	500	500	
A. niger	MIC	250	125	125	31.5	250	250	15.6
	MFC	250	250	250	62.5	250	500	
A. oryzae	MIC	125	62.5	125	125	500	500	7.8
	MFC	125	125	250	125	500	500	
A. parasiticus	MIC	125	62.5	62.5	62.5	500	250	31.5
	MFC	250	125	125	125	500	500	
F. oxysporum	MIC	15.6	7.8	31.5	31.5	125	125	7.8
	MFC	31.5	15.6	31.5	62.5	250	250	
Mucor spp.	MIC	62.5	31.5	62.5	31.5	250	250	31.5
	MFC	62.5	62.5	125	62.5	250	250	
P. commune	MIC	31.5	31.5	31.5	31.5	125	250	7.8
	MFC	62.5	31.5	62.5	62.5	250	250	
P. islandicum	MIC	62.5	31.5	62.5	125	250	250	7.8
	MFC	62.5	62.5	62.5	125	250	500	
P. roqueforti	MIC	62.5	31.5	62.5	31.5	500	250	15.6
	MFC	125	31.5	125	62.5	500	250	
P. solitum	MIC	125	62.5	62.5	62.5	500	250	15.6
	MFC	125	125	125	125	500	250	
P. verrucosum	MIC	125	125	62.5	125	500	500	15.6
	MFC	125	125	125	250	500	500	
R. oligosporus	MIC	62.5	62.5	62.5	62.5	500	250	31.5
	MFC	125	125	125	62.5	500	500	
R. stolonifer	MIC	125	62.5	62.5	125	250	250	31.5
	MFC	125	125	125	250	250	250	

MeOH=Methanol Extract; EtOH= Ethanol Extract; $OC(CH_3)_2$=Acetone Extract; $CHCl_3$= Chloroform Extract; H_2O-S= Water Extract- Soxhelet; H_2O-A= Water Extract-Autoclaved

MIC= Minimal Inhibitory Concentrations(µg/ml); MFC= Minimal Fungicidal Concentrations(µg/ml)

KET= Ketoconazole antifungal control

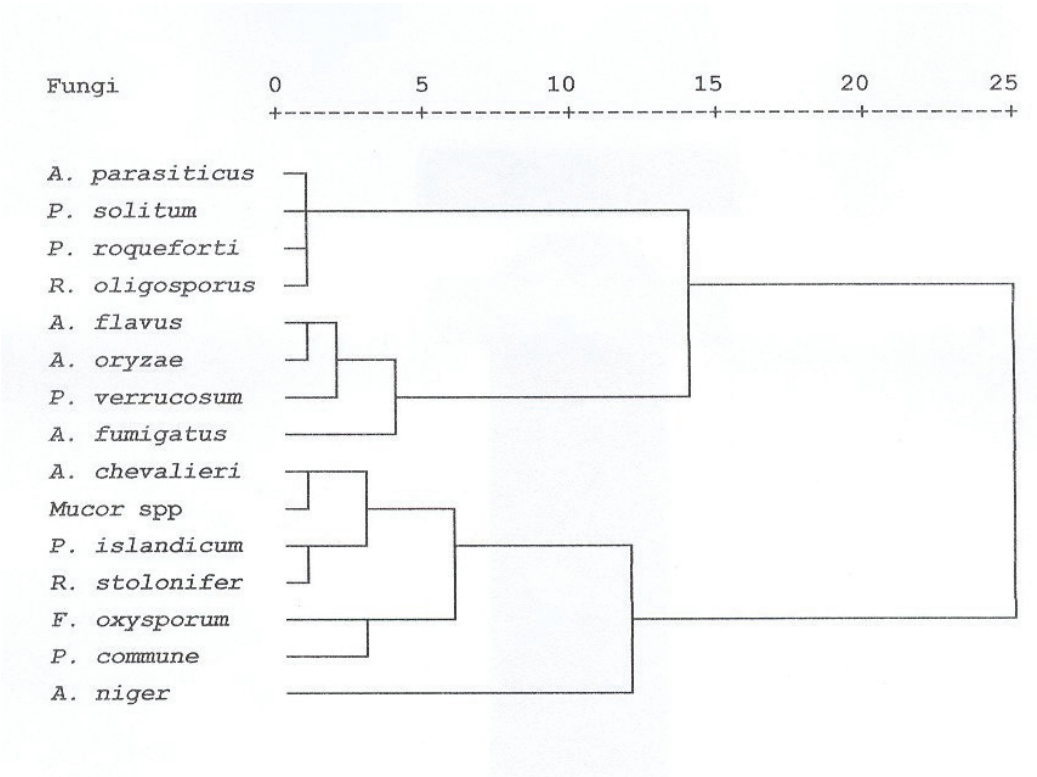

Fig. 1. Dentogram from cluster analysis grouping the fungi studied of different extracts of *P. laurocerasus* L.

lemongrass, clove and thyme essential oils; they prevented or delayed mycelium growth of tested fungi. A study by Nielsen and Rios (2000) showed that the volatile substances from mustard, cinnamon, garlic and clove essential oils and oleoresins were efficient in the control of common bread spoilage fungi, *P. commune, P. roqueforti, A. flavus* and *Endomyces fibuliger*. However, comparison of the data obtained by different studies is difficult, because of differences in the extract composition of plants, in methodologies followed to assess antimicrobial activity and in microorganisms chosen to be tested.

Several studies have been conducted to understand the mechanism of action of plant extracts and essential oils. In the literature it is reported that compounds penetrate inside the cell, where they interfere with cellular metabolism (Marino et al., 2001), and also that they disturb the cellular membrane and react with active sites of enzymes or act as a H+ carrier, depleting adenosine triphosphate pool (Farag et al., 1989).

Conclusion

Fungal growth is the most frequent cause of spoilage in baked goods. With the increase in resistance of microorganisms to the currently used preservatives and the high cost of production of these synthetic compounds, producers are now looking for alternatives. Plants could be that alternative because most of them are safe with few side effects if any, they cost less and affect a wide range of resistant microorganisms. Our results displayed the differences of microorganism strains on antimicrobial activity importance. The antifungal resistance may depend on genus, species, strain and source of isolation, as well

as on the active components in the leaf extracts. Moreover, leaf composition and the solvents used in extracts, origin and cultivars of plant, harvesting time and climate, may affect the consequent antifungal properties.

It is concluded that the antifungal activity of *P. laurocerasus* L. leaf extracts against bread spoilage fungi has been observed. Overall, these results indicate that *P. laurocerasus* L. extracts can be considered a potential source of antifungal agents for food. Further research is needed in order to obtain information regarding the practical effectiveness of *P. laurocerasus* L. extracts to prevent the growth of a broad spectrum of fungi under specific conditionals of application, as well as to investigate the biologically active components in the *P. laurocerasus* L. leaf and their application in bakery products.

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Received January, 12, 2010; accepted for printing December, 2, 2010.