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ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS AGAINST PRE AND POSTHARVEST PATHOGENS

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Abstract

The bio pesticide market is currently expanding in Western Europe and North America. The European market represents a 45% of the total demand, and its importance is shown by the priority given by the Seventh Framework Programme (FP7) of the European Union. Due to environmental side effects and health concerns, many synthetic pesticides have been banned (Council Directive 91/414 EC) or are being under evaluation (Regulation 2009/1107/E and Directive 2009/128/EC). Regarding plant protection, natural extracts represent one of the greatest perspectives. Their impact is efficient, the extraction procedures are not complicated, are safe for environment and people and the degrading process is fast. Therefore, there are already on the market natural fungicides like thyme oil, Thymus zygis (Bio 75®), cinnamon oil (Cinnacoda®), extract of citric seeds (Zytroseed®), extract of Reynoutria sachalinensis, the giant knotweed (Milsana®). An interesting way of searching for bio pesticides, including fungicides is screening naturally occurring compounds in plants. In our study, the antifungal potential of 16 extracts (essential oil, hidrolate, dry ethanolic extract) from various species of Artemisia, Laurus, Argyranthemum, Persea, Euphorbia was investigated in vitro against important pre-and postharvest pathogens: Fusarium oxysporum, F. moniliforme, F. solani, Alternaria alternata, Botrytis cinerea, and Penicillium expansum. The activity of Artemisiaabsinthium essential oil (from stem and leaves) was assesed in vivo on artificially inoculated apple fruits with P. expansum. Our results indicated that the extracts have a variable degree of antifungal activity, depending on plant species, type of extract, fungal isolate and concentration. The present study highlights the fungicidal potential of various extracts from Artemisia species and Argyranthemum frutescens. Extracts from Laurus azorica, Persea indica and Euphorbia azorica did not inhibit mycelial growth. The in vivo test confirmed the high efficacy showed in vitro by Artemisia absinthium essential oil, which was verry effective in controlling disease severity of infected apple fruits by P. expansum. Further studies are in progress to confirm the in vivo efficacy of extracts on different fruits and vegetables.

Key words: *antifungal activity, plant extracts, plant pathogens.*

INTRODUCTION

From the beginning of the 60s, agriculture surface has grown with 11%, from 4.5 billion to 5 billion and the arable land from 1.27 to 1.4 billion has. Directly proportional, the production has increased as well. Being an extensive system, the pesticides consume has also had an ascendant line (Hazell and Wood, 2008). Along with globalization, the consumers preferences have migrated to quality products, healthy, authentic and obtained in hygienic conditions accepted by the society and the environment. The consumer is more and more conscious of the toxic substances present in aliments. Even when the synthetic pesticides are correctly applied, the residues are maintained in aliments, soil and water, by this entering the alimentary chain. Scientific literature abounds in information regarding negative effects versus benefits of these products (Gupta and Dikshit, 2010; Damalas and Eleftherohorinos, 2011).

Necessity of 'cleaner' and without residues products opens new perspectives in bio pesticides use. Unlike synthetic pesticides, bio pesticides have an inexistent risk or a minimum one on environment, accelerated decomposition and they are efficient at low concentrations. Literature studies emphasize the fact that plant extracts represent one of the greatest frames in terms of plant protection: good results, facile extraction procedures, safe to environment and people and their use usually

pays off (Mares et al., 2004; González-Coloma et al., 2009; Al-Samarrai, 2012).

Of more than 500.000 secondary metabolites of plants, only 18.000 have been characterized until 2008 (Ntalli and Menkissoglu-Spiroudi 2011). Principal groups are: phenylpropanoids, phenols, terpenes, steroids, alkaloids and nitrogen compounds (Gonzalez-Coloma et al., 2009). The problems concerning pest resistance, public health risks and environmental damages, have promoted the investigation of natural products with pesticide effects. Thereby are already commercialized natural fungicides like thyme oil, *Thymus zygis* (Bio 75®), cinnamon oil (Cinnacoda®), extract of citric seeds (Zytroseed®), extract of *Reynoutria sachalinensis*, extract of the giant knotweed (Milsana®), natural nematocides like Neem extract (Neemate®), mustard extract (Nemitol®), sesame seeds extract (Dragonfire CCP®).

The genus *Artemisia* is one of the largest (over 400 species) and widely distributed genera of the family *Asteraceae*. The most common species in the world are: *Artemisia absinthium* L., *A. vulgaris* L., *A. maritime* L., *A. dracunculus* L., *A. abrotanum* L., *A. annua* L., *A. pontica* L., *A. cina* O. Berg & C.F. Schmidt.. In Romania there are known 15 species: *Artemisia annua* L., *A. pontica* L., *A. dracunculus* L., *A. vulgaris* L., *A. austriaca* Jacq., *A. lerchiana* Weber, *A. pontica* L., *A. abrotanum* L., *A. santonicum* L., *A. eriantha* Ten., *A. absinthium* L., *A. tschernieviana* Besser., *A. alba* Turra, *A. campestris* L., *A. scoparia* Waldst. & Kit. (Badea, 2011). *Artemisia dracunculus* is a well-known spice and *A. absinthium* is extremely cultivated for the preparation of absinth and vermouth, as for medical purposes also (antidote for opium, tonic and antifebrile) or for its insecticidal properties against aphids, mites and caterpillars (Chiasson et al., 2001; Brudea 2008; Dancewicz and Gabrys 2008). In the Canary Islands, an endemic species, *Artemisia thuscula* Cav., is traditionally used in medicine and in plant protection as repellent for insects. Other genera of plants which have been found as bioactive are: *Argyranthemum, Persea* (Gonzalez-Coloma et al., 1993), *Laurus* (Rodilla et al., 2008) and *Euphorbia* (Kamba and Hassan 2010). The objectives of our study were to investigate, *in vitro,* the fungicidal potential of 16 extracts (as essential oils, hydrolates and ethanol dry extracts) from 3 species of *Artemisia*, *Laurus novocanariensis* Rivas Mart., *Argyranthemum frutescens* (L.) Sch.Bip., *Persea indica* (L.) Spreng. and *Euphorbia azorica* Hochst. Nine fungal isolates, from important pre and postharvest pathogens as *Fusarium oxysporum fs. Lycopersici* Scheldt,*Fusarium moniliforme* Sheldon, *Fusarium solani* Mart), *Alternaria alternata* Keissl, *Botrytis cinerea Pers.: Fr*and *Penicillium expansum* Link were tested. Further, the activity of *Artemisia* absinthium essential oil was assesed *in vivo* on artificially inoculated apple fruits with *P. expansum*.

MATERIALS AND METHODS

Preparation of plant extracts

Recollection of samples of *Artemisia thuscula*, *Artemisia spp., Laurus azorica*, *Argyranthemum frutescens*, *Persea indica*, *Euphorbia azorica* took place in different locations (Table 1). The plant samples (root, stem, leaves and seeds) were air dried at room temperature in order to prepare the extracts. Plants vouchers were carried out to authentication and conservation. Extracts in liquid and solid form were used. In the case of solid extracts, after 48 hours of maceration in ethanol, the solvent was removed by low-pressure distillation on a rotary vacuum evaporator. Ultimately the extracts were introduced in the stove to be dried. As for the liquid extracts distillation process has been used to obtain essential oils and hydrolates.

Fungal isolates

The fungal isolates used in this study are listed in Table 2. All the isolates were purified by monospores isolation and maintained in tubes of malt agar medium (malt extract 20 g, agar 20 g in 1 L distilled water) at 4^0C. Fresh subcultures were made by transferring hyphae plugs to Petri dishes containing potato dextrose agar (PDA Sigma®) medium to obtain inoculum for sensitivity tests.

Table 1. Plant species and type of extract

Species	Extract code [EC]	Plant organ	Date and place of recollection	Extract type [ET]
Artemisia absinthium	718	Stems and leaves	Loc1, 2010	Essential oil
	720	Stems and leaves	Loc2, 2010	Hydrolate
	749	Stems and leaves	Loc 1, 2009	Essential oil
Artemisia absinthium	759	Leaves	Cape Verde; 2011	Dry EtOH extract
	760	Leaves	Cape Verde; 2011	Dry EtOH extract
Laurus azorica	766	Leaves	Terceira, Azore; 2011	Dry EtOH extract
Artemisia thuscula	775	Leaves	Las Aguas, Tenerife; 2012	Dry EtOH extract
	776	Seeds	Las Aguas, Tenerife; 2012	Dry EtOH extract
	777	Leaves	Taganana, Tenerife; 2012	Dry EtOH extract
	778	Stems	Las Aguas, Tenerife; 2012	Dry EtOH extract
	181	Roots	La Matanza, Tenerife; 2000	Dry EtOH extract
	182	Stems	La Matanza, Tenerife; 2000	Dry EtOH extract
	183	Leaves	La Matanza, Tenerife; 2000	Dry EtOH extract
Argyranthemum frutescens	359	Stems	La Matanza, 2011 Tenerife; 2002	Dry EtOH extract
Persea indica	406	Leaves	Mercedes, Tenerife; 2002	Dry EtOH extract
Euphorbia azorica	444	Stems and leaves	Azore; Terceira; 2011	Dry EtOH extract

Table 2. Fungal isolates used

Fungal species	Isolate code	Provenience	Origin
Alternaria alternata	Aa 2207	Pepper	Bulgaria
Alternaria alternata	Aa 100	Unknown	Tenerife
Botrytis cinerea	Bc 2107	Grapes	Romania
Botrytis cinerea	Bc 0510	Grapes	Tenerife
Penicillium expansum	Pe 2712	Apple	Romania
Fusarium oxysporum	Fo 809	Cucumber	Romania
Fusarium solani	Fs 810	Cucumber	Romania
F. o. f. sp. lycopersici	CECT 2715	-	Valencia
Fusarium moniliforme	CECT 2152	-	Valencia

***In vitro* tests-assay on mycelium**

Tests were carried out to determine the biological activity of extracts using biometric agar dilution method. The extracts were incorporated into the culture media (PDA) as follows: 0.1-0.5 – 1 mg/ml for solid extracts and 0.1 – 0.5 – 1% for hydrolytes and essential oils. For a better solubility of the essential oils, Tween 20 (polyoxyethylene derivative of sorbitol fatty acid ester) was used in ethanol solution (40% Tween 20 and 60% ethanol). The final percentage of ethanol in the media was adjusted to a concentration of 2% (v/v). Plates containing the solvent (ethanol) were used as negative control.

Each pathogen was spot-inoculated at 8 equidistant points to PDA media amended with the plant extracts at tested concentrations. Three replicates were used per treatment. For each extract and concentration, inhibition of radial growth compared with the untreated control was calculated after 48 hours of incubation at 27^0C, in the dark. The radial growth was measured with an image-processing program ImageJ-Wayne Rasband (NIH).

Results were expressed as effective concentration EC50 (the concentration which reduced mycelial growth by 50%) determined by regressing the inhibition of radial growth values (%) against the log 10 values of the fungicide concentrations (GraphPad Software).

***In vivo* tests on apple fruits**

Based on the *in vitro* tests, *Penicilium expansum* isolate was selected for the *in vivo* study conducted on apple fruits, variety 'Idared'. *Artemisia absinthium* essential oil (from stems and leaves, code 749) was tested at the concentration of 1%. Apples were superficially surface disinfected by soaking in 80% ethanol for 3 minutes, rinsed with distilled water and left to dry. Then, they were wounded to 2 mm depth with a needle (4 wounds per each apple, equidistant at 2 mm, on the side of the apple half way between stem and calyx). All wounds were artificial inoculated by pipetting 7 µl *P. expansum* spore suspension (10^4 spores/ml). Apples were divided into five lots/variants (Table 3).

Lot no. 1 (V1) was considered as inoculated and non treated control. Fruits of lot no. 2 and no. 3 (V2 and V3) considered as controls, also, were artificially inoculated and then treated (V2), respectively treated and artificially inoculated (V3) with a solution of Tween 20 and ethanol.

Table 3. Tested variants

V1	Control	Contamination	Sterile water
V2	Control PT	Ethanol + Tween 20	Contamination
V3	Control CT	contamination	Ethanol + Tween 20
V4	Treatment PT	*Artemisia absinthium* extract	Contamination
V5	Treatment CT	contamination	*Artemisia absinthium* extract

Treatment with *A. absinthium* extract has been applied with a micro pulverization dispenser, forming an uniform layer on apples, before artificial contamination, as preventive treatment (PT) in variant V4 and after, as curative treatment (CT), in variant V5. Time between spore inoculation and treatment was 10 minutes to avoid simultaneous contact. Ten apples and three repetitions were used for each variant. The apples were placed in sterile plastic boxes and incubated at 25^0C and 78,8% RH. To maintain the air condition in the container while the apples respire, the containers were opened during 5 minutes in a sterile environment (biosafety cabinet) every day. Subsequently diameters of lesions were monitored and recorded. The efficacy [%] of the treatment to reduce disease severity was calculated using Abbot Formula (Efficacy = (lesion size control – lesion size test) /lesion size control x 100).

RESULTS AND DISCUSSIONS

In vitro tests – effects on mycelial growth

The effect of essential oil, hidrolate or dry ethanolic extracts from species of *Artemisia, Laurus, Argyranthemum, Persea, Euphorbia* has been variable, depending on plant species, type of extract, fungal isolate and concentration. Some of the tested extracts were highly effective. In the presence of the essential oil (718 and 749) and hydrolate (720) from *A. absinthium*, all the tested isolates were sensitive (EC50 by 0.3%). Dry ethanolic extracts from leaves and seeds of *Artemisia thuscula* (775, 776, 777) were very effective against *Fusarium oxysporum* isolate (EC50 between 0.06-0.15%). Also, dry ethanolic extract from leaves of *A. absinthium* (760) showed fungicidal activity against *A. alternata* isolate (EC50 = 0.9%).

The ethanolic extracts from *Argyranthemum frutescens* (359) inhibated the mycelial growth of all tested isolates (Table 4).

Table 4. In vitro sensitivity of fungal pathogens to plant extracts

Extract code	Isolate code	Concentration (%)			EC50
		0.1	0.5	1	
718	*Bc 0510*	81.8	100	100	0,08 (0.03 – 0.14)
	Aa 100	5322	100	100	0.07 (0.03 – 0.1)
	Fo 2715	100	100	100	< 0.1
720	*Bc 0510*	90.4	100	100	< 0.1
749	*Bc 0510*	38.8			0.04 (0 – 0.6)
	Aa100	41.9	89.2		0.02 (0 – 2.9)
	Bc 2107	60	100	100	
	Pe 2712	0	100	100	0.3 (0.3-0.3)
ExtractCode	Fungal species	Concentration (mg/ml)			EC50
		0.1	0.5	1	
760	*Aa 2207*	40	30	40	0.9
	Fs 810	15	20	20	2.9 (2-2)
775	*Fo 2715*	18. 7	36.1	51.4	0.09 (0-0.11)
776	*Fo 2715*	19.9	31.3	38.6	0.15 (0-0.21)
777	*Fo 2715*	23.5	47.5	63.9	0.06 (0-0.21)

The fungicidal activity of the extracts from *Artemisia* species and *Argyranthemum frutescens* could be attributed to different compounds as the main active encountered are essential oils and polyphenols (Gonzalez et al., 1997; Soylu et al., 2005; Ahameethunisa and Hopper, 2010; Umpierrez et al., 2012;). Recent studies provided data on antibacterial, antifungal and antioxidant activities of the essential oil of *A. annua* and the chemical composition of this essential oil was also described (Cavar et al., 2012).

A series of extracts did not present activity against tested isolates (Table 5). No antifungal activity on mycelial growth has been observed for *Laurus azorica*, *Persea indica* and *Euphorbia azorica* extracts.

Table 5. Extracts without bioactivity on tested isolates

Isolate code	Extract code
Bc 2107	182, 183, 406, 444, 759, 760, 766, 775, 776, 777, 778
Bc 0510	181, 183, 775, 776
Aa 100	181, 182, 183, 444, 406, 766, 775, 776, 777, 778
Aa 2207	182, 183, 775, 776
Fs 810	182, 183, 444, 406, 759, 766, 810, 777, 778
Pe 2712	182, 183, 444, 406, 759, 760, 766, 775, 776, 777, 778

'In vivo' tests

After 7 days, lesion size of apples inoculated and non treated (V1 control) was 1.3 cm. For the apples treated with the solvent used in preparation of the extract (ethanol + Tween) and inoculated (V2, PT) the size of the lesions was 0.63 cm. The differences between the variant where the solvent was applied after inoculation (V3) and the control (V1) were almost inexistent (lesion size 1.27 cm, respectively 1.3 cm). We have observed that when the solvent was applied before artificial contamination (V2) it has determined a slower growth of the pathogen by 50% compared to control in sterile water (V1), the lesions size measured being 0.63 cm, respectively 1.30 cm. One of the probabilities we may consider is that Tween 20 is forming a layer which may act as a barrier against spores germination.

After 17 days, we have noticed an evolution of lesions size, in control, where symptomatic areas were developed on 3.58 cm. For the variants where apples were treated with the solvent and inoculated (V2) or inoculated and treated (V3), the size of the lesions was 1.75 cm, respectively 3.0 cm (Table 6).

The efficacy of *Artemisia* extract treatment was confirmed by very small symptomatic lesions with a diameter of only 0.3 cm (V4, preventive treatment) and 0.08 cm (V5, curative treatment). An inhibition (or efficacy) of this treatment was recorded, at 7 days: 76.9% (V4) and 93.8 (V5) compared to control (Figure 1).

After 17 days, we have noticed an efficacy of 76.8% (V4) and 88.5% (V5); evolutions of the symptomatic areas were only 0.33 cm and 0.53 cm.

Table 6. Lesion size evolution in time (days after inoculation and treatment)

Variants		Lesion size [cm]	
		7 days	17 days
V1	Control	1.30	3.58
V2	Control (solvent) PT	0.63	1.75
V3	Control (solvent) CT	1.27	3.00
V4	Treatment *A. Absinthium* PT	0.30	0.83
V5	Treatment *A. Absinthium* CT	0.08	0.41

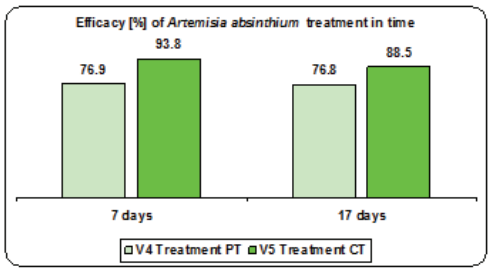

Figure 1. Efficacy of *Artemisia absinthium* extracts treatment on apples

CONCLUSIONS

The present study concludes that some essential oils, hydrolates or dry ethanolic extracts from various species of *Artemisia* and *Argyranthemum frutescens* showed antifungal activity against important plant pathogens. Our results indicated that the extracts have a variable degree of antifungal activity, depending on plant species, type of extract, fungal isolate and concentration.

No antifungal activity on mycelial growth was been noticed for *Laurus azorica*, *Persea indica* and *Euphorbia azorica* extracts.

The *in vivo* test confirmed the high efficacy showed *in vitro* by *Artemisia absinthium* essential oil, which was very effective in controlling disease severity of infected apple fruits by *P. expansum*, applied as curative treatment. Further studies are in progress to confirm the *in vivo* efficacy of extracts on different fruits and vegetables.

Exploring new plant species for their antifungal activity would bring more resource base for use in eco-friendly and sustainable agriculture, especially in organic farms.

To summarize, it is our strong belief that the study of plants with traditional uses as 'plant protectors' is essential for understand more

about the inner value of flora. Therefore, different kinds of studies should be followed as metabolic interactions, physiological reactions and biochemical profile which may lead to the real understanding of fungicide mechanisms.

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