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Study of thiabendazole resistance and volatile organic compounds production of *Penicillium expansum* strains

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A mes très chers parents Ali et Jalila,
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pour faire de moi une femme modèle ;
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Pour votre assistance durant ma vie estudiantine ;
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Pour ma sœur et mes freres pour tant d amour et de comprehension
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I will never regret this experience that I will tell about it my sons and grandchildren ☺

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List of abbreviations

℃: degree Celsus
µg: microgram
µm: micrometer
BLAST: Basic local alignment search tool
bp: base pair
BSA: Bovine serum albumin
CFU: Colony forming unti
CO_2: Dioxyde of carbon
DMI: Demethylation inhibitor
DNA: Deoxyribonucleic Acid
DPI: Day post inoculation
DWM: Dry weight mycelium
EDTA: Ethylenediaminetetraacetic acid
g: gramme
GC-MS: Gas chromatography mass spectrometry
Glu: Glutamic acid
h: hour
His : Histidine
HSPME: Head space solid phase microextraction
IGS: Intergenic spacer
ITS: Internal transcribed spacer
L: litre
Leu: Leucine
LSU:Large subunit gene
M: molar
MEA: Malt extract agar
MEB: Malt extract broth
min: minute
mL: milliliter
mm: millimiter
mM: millimolar
NaCl: sodium chloride
ng: nannogram
nm: nanometer
O_2: Oxygen
OMA: Oat meal agar
PCR: Polymerase chain recation
Phe: Phenylalanine
PI: Post inoculation
rpm: revolution per minute
s: second
SSU:Small subunit gene
TAE buffer: Tris- acetate-EDTA

Taq: Thermus aquaticus
TBZ: thiabendazole
Tyr : Tyrosine
ULO: Ultra low oxygen
UV: ultraviolet
V8A: Vegetable juice agar
Val: Valine
VOC: Volatile organic compounds
VS: Volatile substances

CHAPTER 1

1. INTRODUCTION

The consumption of fruits and vegetables is abundant in the Mediterranean diet, recognized worldwide as a health model to meet nutritional demands. Besides the strategic role in improving the diet of people around the world, these commodities have also monetary, social and cultural high value.

The world health organization has announced a global initiative to promote consumption of fruits and vegetables, while the European Community has allocated almost 90 million Euro for recent projects to increase fruit consumption overall among children in schools.

In fact, fruits and vegetables are rich in water, fibre, minerals vitamins and other phytochemicals; in particular they are good source of vitamin C (pepper, kiwifruit, papaya, orange, strawberry, cauliflower, broccoli), provitamins A (carrots, tomato, apricot, melon) and potassium (banana, kiwifruit, melon, apricot, artichoke, cauliflower, broccoli, tomato), essential to balance many important body functions and for correct growth, while flavonoids (blueberry, blackberry, cranberry, strawberry, redgrape, apple, plum, cherry, onion) provide most of the antioxidant activity involved in the prevention of cancer and cardiovascular diseases.

Apple is one of the most popular fruit favoured by health conscious, fitness freaks who believe in “health is wealth.” This wonderful fruit is packed with rich phyto-nutrients that in the true sense are indispensable for optimal health. However, growing apples profitably for today’s market is a challenge involving problems turnover on the long way from the orchard to the consumer’s table.

1.1. The importance of apple fruit production

Apples have been longer considered healthy, thus justifying the adage, *“an apple a day keeps the doctor away”.*

The name apple comes from the old English word “aeppel”, it is a pomaceous fruit of species *Malus domestica* Borkh. The wild ancestor of *M. domestica* is *M. sieversii* and is known as *Alma* from Alma Ata (Kszakistan) where it is native. The apple tree was probably the earliest tree to be cultivated; in fact, there are more than 7,500 known cultivars. Different cultivars are adapted to temperate, subtropical and tropical climates. Some of the commercially-popular apple cultivars are Golden Delicious, Red Delicious, Granny Smith, Jonagold, Fuji and Pink Lady ®.

Winter apples, are harvested in late autumn and stored at the recommended storage temperature of 0 to 2°C and 90 to 95% RH , they have been considered as an important food in Asia and Europe for millennia, as well as in Argentina and in the United States.

The recommended conditions for commercial storage of apples are -1 °C to 4 °C (30.2 to 39.2 °F) and 90 to 95% RH, depending upon variety. Typical storage periods for a number of varieties in cold rooms are shown in Table 1. The acceptable duration of cold storage has become shorter over the last several years as quality standards in the market have increased. Also, short-term controlled atmosphere (CA) storage is becoming more common as the period available for sale of cold-stored fruit has decreased (Watkins et al.,2005).

In 2009, 63 million tons (MT) of apples were grown worldwide (Figure n°1). In a recent, detailed study carried out by the State Department of Agriculture (USDA), world apple production for 2011/12 is estimated to be a record of 65.23 MT. This volume is 4% higher than the year before and 11% up compared to the average of the last five seasons (06/07-10/11).

China's participation in global supply of this species continues to grow at the expense of other producing countries. It is the world’s top producer with almost half of the total production (33,267 MT in 2010). The United States is the second leading producer, accounting for 6, 8% of world production with a production estimated at 4,212 million tons in 2010 (Faostat, 2010); In fact, more than 60% of the apples sold commercially are grown in Washington State.

Elsewhere in the Northern Hemisphere, crops increase also compared to last year, namely in China (+10%), in the USA (+2%), and most notably in Ukraine (+17%). (WAPA, 2011)

Table 1 - Potential months of storage at 0℃ of the main varieties of apple

Variety	Air	CA	Superficial scald susceptibility
Delicious	3	12	Moderate to very high
Empire	2-3	5-10	Slight
Fuji	4	12	Slight
Gala	2-3	5-6	Slight
Golden Delicious	3-4	8-10	Slight
Granny Smith	3-4	10-11	Very high
Jonagold	2	5-7	Moderate
Jonamac	2	3	Moderate
Law Rome	3-4	7-9	Very high
McIntosh	2-3	5-7	Moderate

Elsewhere in the Northern Hemisphere, crops increase also compared to last year, namely in China (+10%), in the USA (+2%), and most notably in Ukraine (+17%). (WAPA, 2011)

The European continent is one of the leading apple producer. The World Apple and Pear Association (WAPA) forecasts a European Union apple crop of 10,195 MT in 2011, which is still 5% lower than the average over the last three years. Poland, Germany, New Zealand, Italy, South Africa and Chili are among the leading apple country exporters.

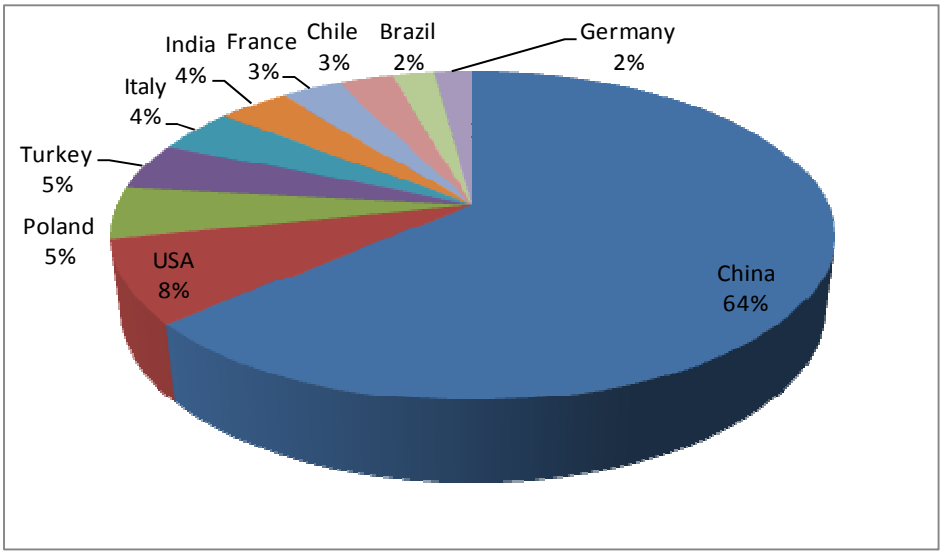


Figure 1 - Distribution of the world production of apple fruit (Faostat, 2010)

In 2011, the apple production in the EU increased by 5% compared to the low crop of last years. More specifically for apples, the 2011 European crop forecast is announced at 10,195 MT. This production remains however a 5% lower than the average for the last three years. In regard to varieties, Golden Delicious production is up by 5% to 2,533 MT. Gala increases by 7% to 1,059 MT. Jonagold is up by 14% at 594,000 tons, while Red Delicious decreases by 4% to 635,000 tons. Apple production in the European union of 15 member states (EU-15) was of 6,591 MT with a reduction of 7% comparing to 2009. This production is estimated to reach 6,940 MTin 2011. However, in the EU-12 area, the production reported in 2010 was of 3,205 MT (-18%). It remains more or less constant showing a slight increase in 2011 (3,255 MT) (WAPA, 2011) (Table. 2).

Italy is one of the major producing and trading countries; in 2011, its production reached 2,212 MT, over 33,000 tons above the previous season particularly of Golden Delicious

and Red Delicious more than the half of the Italian production of apple. The 46% of the Italian production comes from Alto Adige (1,066, MT) (Bridi, 2010) that with its 18,000 hectares of cultivations represent the cradle of the European production and distribution of apples.

Table 2- Apple production in the European Union (EU-27) by country (MT) (Faostat, 2011)

Country	2007	2008	2009	2010	2011	2011/2010 (VAR.%)
Austria	193	159	185	169	192	14
Belgium	358	336	344	288	298	3
Bulgaria	26	24	35	40	40	0
Czech Rep	113	157	145	103	66	-36
Denmark	32	26	24	21	20	-5
France	1676	1528	1651	1579	1655	5
Germany	1070	1047	1071	835	896	7
Greece	236	231	224	254	245	-4
Hungary	203	583	514	488	300	-39
Italy	2196	2164	2237	2179	2212	2
Latvia	31	34	13	12	8	-33
Lithuania	40	74	74	46	60	30
Netherlands	396	376	402	340	418	23
Poland	1100	3200	2600	1850	2300	24
Portugal	258	245	274	251	265	6
Romania	287	459	379	423	375	-11
Slovakia	10	42	48	32	33	3
Slovenia	80	68	64	66	73	11
Spain	599	528	470	486	502	3
Sweden	16	18	18	20	18	-10
UK	196	201	212	214	219	2
Total	**9117**	**11499**	**10984**	**9697**	**10195**	**5**

The latter is followed by Trentino (447,099), Piemonte (200,157), Veneto (194,290) and Emilia Romagna (146,895) (Table. 3).

Table 3 - Distribution of apple production in Italy (Tons) (Bridi, 2010)

Regions	2006	2007	2008	2009	2010	2010/2009 (VAR.%)
Alto Adige	921.314	978.815	1.058.701	1.157.524	1.066.047	-8
Trentino	362.536	458.792	433.827	420.658	447.099	6
Veneto	248.689	289.321	215.819	210.901	194.290	-8
Friuli	_	_	38.018	28.560	33.347	17
Lombardia	35.041	39.051	31.534	35.425	37.302	5
Piemonte	200.109	196.582	173.448	192.187	200.157	4
Emilia Romagna	162.325	189.082	168.334	157.023	146.895	-6
Others	60.000	45.000	45.000	35.000	40.000	14
Total	**1.990.014**	**2.196.643**	**2.164.681**	**2.237.279**	**2.165.137**	**-3**

1.2. Phytosanitary problems on stored apple fruits

Spoilage of fruits can occur before or after harvest during subsequent handling and it takes on great economic importance the closer it is to the sale of the fruit. Postharvest

losses in apple are mainly caused by fungal pathogens that limit the extension of storage life of fruit. Blue mould, caused by *Penicillium expansum* Link and grey mould, caused by *Botrytis cinerea* Pers.: Fr are common diseases of apple even in production areas where the most advanced storage technologies are available such European countries. Both of them are wound invading pathogens that cause decay on stored fruits damaged by insects, early splits and mechanical harvesting. Consequently, the control of apple fruit losses in the postharvest chain is decisive. It should take into consideration the numerous factors influencing decay development, and in particular fruit maturity at harvest and conditions applied during handling, storage and shelf life. The control of postharvest pathogens carried out with synthetic fungicides actually has undergone a strong reduction because of the concern about public health, environmental contaminations and the demand for fungicide-free fruit. Strange and Scott (2005) reported that pathogens which infect all major crops are a threat to global food security; they cause serious losses and also some of them may produce mycotoxins. Worldwide, these postharvest losses may reach as much as 50% during the shelf life of the fruit (Eckert and Ogawa, 1988; El-Ghaouth, 1997). Postharvest infections can occur either prior to harvest or during harvesting and subsequent handling and storage (Sommer, 1985). For example, every year in France, apple fruit loss due to fungal damage is estimated to be between 7 and 10% of production (Bernard et al., 1993).

According to Shewleft (1986), disease development during the postharvest phase depends upon the physiological status of the tissue and the constitutive and inducible resistance mechanisms of the harvested produce. In fact most harvested commodities are resistant to fungal infections during their early postharvest phase while during ripening and senescence they show more susceptibility to infection.

El Ghaouth (1997) reported that postharvest losses in developed countries may be lower comparing to the developing ones. This can be explained by the fact that these problems are often considered as serious from the producer's and consumer's standpoint.
The major postharvest pathogens were divided into two groups: those responsible of quiescent infection mainly via lenticels such as *Phlyctema vagabunda* Osterw (Syn *ex Gleosporium album*.), *G. perennans* Zeller and Childs (Syn. *Cryptoporiopsis curvispora*)

and *Nectria galligena* Bresad; and wound invading pathogens particularly *P. expansum* and *B. cinerea* followed by *Monilinia* spp., *Mucor* spp., *Rhizopus* spp., *Alternaria alternata* (Fr.) Keissl, *Stemphylium botryosum* Wallr, *Cladosporium herbarum* (Pers.) Link., *Colletotrichum gleosporioides* Penz (Syn. *G. fructigenum*), *Botryosphaeria* spp. and *Phytophtora* spp. that can become a serious problem during rainy seasons for fruits from orchards with heavy soils.

Finally, *Trichothecium roseum* Link, and species of *Phomopsis*, *Nigrospora*, *Fusarium*, *Epicoccum*, *Aspergillus* and *Trichoderma* are pathogens of minor importance, respecting to those listed previously (Eckert and Ogawa, 1988; Sanzani., 2007)

For italian apple production, the three most important postharvest pathogens are *P.expansum* the causal agent of blue mould, *B.cinerea* the causal agent of gray mould, and *P. vagabunda* the casual agent of lenticels rot.

1.2.1 *P. expansum*

P. expansum is the causal agent of blue mould also known as soft rot or wet rot, the most economically important postharvest disease of apples (Rosenberg, 1990).It is generally considered to be a wound parasite, and infection commonly follows rough handling and washing procedures after harvest. It may gain entry through previous *Gleosporium*, *Mucor* or *Phytophtora* infection sites or via lenticels (Snowdon, 1990).

1.2.2 *B. cinerea*

B. cinerea is the second most important postharvest fungal pathogen on apple next to *P.expansum*, hence considered to be also important postharvest pathogen of pears. It affects numerous commodities worldwide and causes significant losses in crops like strawberry, raspberry, kiwifruit, etc. Symptoms usually appear at the calyx or stem end of the fruit or at wound sites. Unlike blue mould, the rotted tissue tends to be firmer and rotted fruits have a relatively pleasant odour rather than a musty odour. Under humid conditions, a white or greyish-white mycelium forms on the surface of the rotted tissue, later spores colonize apple tissue wounded and infection can spread to healthy adjacent fruit by simple contact when fruits are mixed in dump tank or less frequently by exposure

from airborne conidia (Snowdon, 1990; Naqvi, 2004).

1.2.3 *Mucor piriformis* Fischer

M. piriformis is the causal agent of Mucor rot of apples, a disease of a worldwide importance in UK, South Africa and Europe. Infection usually occurs at wound sites alternatively at the stem-end, at the calyx-end, in the core region or anywhere on the fruit surface. Affected tissue appears light brown, soft and watery, with no odour. In humid atmosphere, a profuse production of sporangia bearing black spore-heads (sporangiospores) that are of prime importance in infection (Snowdon, 1990; Naqvi, 2004).

1.2.4 *Alternaria* spp.

Alternaria rot caused by *A. alternata* has been reported on apple and pears in Israel, Chile, Morocco, etc. It is responsible of various symptoms like skin blemishes, small black corky lesions or shallow-dark coloured rot on tissues that has suffered physical or physiological injury.

1.2.5 *Phlyctema vagabunda* Desm.

N. alba (E.J Gutrie) Verkley (anamorph *P. vagabunda*) is the causal agent of lenticel rot. It is one of the most frequent and damaging diseases occurring in stored apples in Italy and other European apple growing countries (Pratella, 2000; Mari et al, 2009). Fruit infection occurs in the orchard but the pathogen remains quiescent until the fruit reaches a certain stage of ripeness favorable for the invasion of fruit tissues. Disease symptoms are circular, pale brown spots with a darker outer ring, resembling those produced by the bull's eye rot pathogen on apple that occur in other parts of the world. They appear only several months after harvest (generally after 3-4 months of cold storage), when numerous lesions may develop on a single fruit. The microscopic examination of acervuli, developed in the rotted areas, revealed the presence of the fungus *N. alba* characterized by production of curved macroconidia and absence of microconidia (Henriquez, 2005).

1.2.6 *Monilinia* spp.

M. fructigena (Aderh. & Ruhl.) Honey is widespread in Europe, Asia and South America and causes serious losses in pome fruits contrarily to *M. laxa* (Aderh. & Ruhl.) that induces occasionally rot on apple and rarely on pear. Infected fruits show firstly small circular brown spots that turns on tufts of white mould break through the skin often forming concentric circles around the point infection under humid conditions. Copious production of grey-brown spores is observed in the presence of light and there may be no spore formation in the absence of light. In advance stage, the rot tends to remain firm and dry.

1.3. Blue mould rot

P. expansum is one of most important fungal pathogen responsible for postharvest losses of apple and pear (Mattheis and Roberts, 1992). It is widely known as a pathogen of stored pome fruits but it has also been isolated from other commodities such as sweet cherries, apricots, grapes, blueberries, peaches, strawberries and oilseed crops like walnuts, pecans, hazelnuts and acorns (Andersen et al., 2004; Murphy et al., 2006). Under favourable conditions (20°C; HR 85%), it can be responsible of over 50% of losses in all pome fruit growing areas (Vinas et al., 1993).

P. expansum is essentially a wound mediated pathogen. Wounds on the fruit peel such as punctures and bruises created at harvest and during postharvest handling or in packinghouses, where the spores number can be high (Sanderson and Spotts, 1995), are the major avenues for fungus infection (Rosenberger, 1990; Amiri and Bompeix, 2005; Morales et al., 2008b). *P. expansum* can germinate rapidly in the tissue. Highly contaminated facilities may induce an increase in the number of spores germinating in the same wound and influence the rot development.

Furthermore spores of *Penicillium* spp. are ubiquitous soil inhabitants (Domsch et al., 1980). They can thus contaminate apples preharvest from soil, crop debris, field bins and equipment. It is generally assumed that the initial inoculum of these decay-causing fungi is dispersed as dry or wet conidia that could land on fruit dry or wet with dew or rain.

Penicillium spp. are aggressive colonizers of both soil and plant litter (Domsch et al., 1980; Subramanian, 1983). Their battery of enzymes and production of antimicrobial compounds (Subramanian, 1983) give them a competitive edge over other organisms (including *B. cinerea*) in both substrates.

Giraud & Fauré, (2000) considered that risks of rot problems could be high when the airborne spore level exceeds 2.5 10^3 spores m^{-3}, however studies accomplished by Amiri and Bompeix (2005) showed that high levels of airborne *Penicillium* did not necessarily induce a high incidence of diseased apples. This can be explained by the fact that the waxy skin of a sound apple is a natural protection against mold infection which cannot readily be penetrated in the environment (Sydenham et al., 1995).

In addition, Lennox et al. (2003) found that population levels of *P. expansum* on fruit surfaces are significantly and positively correlated to decay in storage, but when fruits are wounded, also low inoculum concentration (<10 CFU cm^{-2}) is able to cause a significant levels of decay (Spotts and Cervantes, 2001).

Since 50% of *P. expansum* conidia died after approximately 6 days, the conidia landing on the fruit surface earlier than 2 weeks before harvest are unlikely to contribute to decay unless they form latent infections.

1.3.1 Taxonomy of *P. expansum*

P. expansum is one of the first species to be described in the genus *Penicillium*, a typical fasciculate specie producing terverticillate smooth-walled penicillin, synnemata. On rotted fruit, it produces conspicuous concentric zone of crust-like coremia. Conidiophores are predominantly mononematous, occasionally also synnematous, especially in marginal areas of fresh isolates; they are large regularly two-to three– stage-branched conidiophores with smooth walls and large ellipsoidal conidia. It is characterized by the rapid growth and the formation of the robust phialides. The colony showed a cream yellow to orange brown reverse colour after one week at 25℃ on yeast extract sucrose (YES) agar.The well developed coremia are occasionally present in marginal areas of fresh isolates.

P. expansum nomenclaturally typifies the genus *Penicillium* and it has been easily

recognized by later taxonomists based on the above characteristics. It's still regarded by most taxonomists as a unique species (Frisvad and Samson, 2004).

Frisvad (1981) was the first to suggest that extrolites, secondary metabolites often recognisable as diffusible colours, colony reverse colours and exudate colours, could be used directly in *Penicillium* taxonomy . They proved later to be particularly of high value in a taxonomic sense (Frisvad and Filtenborg, 1989, 1990; Frisvad et al., 1998).

Further studies confirmed the value of both non volatile and volatile extrolites in taxonomy (Lund and Frisvad, 1994; Larsen and Frisvad, 1995; Smedsgaard and Frisvad, 1996).However, the currently employed methods for identification of foodborne molds as *Penicillium* spp. require culture isolation and application of morphological and physiological tests (Gourama and Bullerman, 1995; Zhou et al., 2000), which are time-consuming, labor-intensive, and often require mycological expertise (Shapira et al., 1996). Consequently, genotypic characterization has proven to be useful.Recently, nucleic acid-based methods such as the polymerase chain reaction (PCR) has improved the situation (Hill, 1996), Although it is more suited for phylogenetic studies its application for classification and identification is relatively limited comparing to phenotypic data (Marek et al, 2003).

Skouboe et al.(1996, 1999, 2000) and Boysen et al.(1996) sequenced the ITS1 and the ITS2 region, including the 5.8 S region, of several terverticillate Penicilla and found rather few sequence differences among the species.

Furthermore, Peterson (2000) also found that the ribosomal DNA gene has too few informative differences to reveal the phylogeny of these Penicillia.

More recently, several DNA- based identification procedures has been used in order to detect intraspecific and interspecific variation in *Penicillium*. These methods include restriction fragment length polymorphism (RFLP), large sub-unit ribosomal (r) RNA sequence and the random amplified polymorphic DNA (RAPD) (Dupontet al., 1999; Laroche et al., 1995; Thompson and Latorre., 1999).

Several other *Penicillium* species were identified including, *P. commune*, *P. verrucosum*, *P. chrysogenum* and *P. regulosum*, *P. marinum* and *P. sclerotigenum, P. crustosum*, *P. aurantiumgriseum and P. solitum* The latter have been reported to cause decay on apples (Pitt, 1988; Pianzzola et al., 2004)

P. marinum and *P. sclerotigenum* are identified as synonyms of *P. expansum* based on the combination of micromorphological, macromorphological, physiological and extrolite characters.

1.3.2 Symptoms

Infection is first visible as a soft and sunken, yellow to pale-brown, circular lesion on the surface of the fruit which undergo rapid enlargement at temperatures between 20℃ and 25℃ and can quickly macerate the fruit under ideal conditions. It has been shown that the maceration is caused by a pectolytic enzyme produced by the fungus (Cole and Wood,1961). As the rot expands and tissue decays, the tissue becomes watery and the peel (skin) above the rot takes on a wrinkled appearance. A distinct margin is noticed between soft rotted flesh and firm healthy tissue.

In humid conditions masses of blue-green spores initially snow white in colour form on the surface of the lesion (Errampalli, 2004).The fungus can spread into neighbouring healthy fruits, forming nests of decay (Fig. 2). A diagnostic symptom of this rot consists on a strong earthy or musty odour and unpleased taste.

Figure 2- Typical symptoms of blue mould rot on apple and Kiwi fruits

1.3.3 Biology and epidemiology of *P. expansum*

P. expansum, is primarily a necrotroph and a wound parasite, most frequently gaining entrance through fresh mechanical micro-wounds, or after infections by other pathogens,

such as species of *Gleosporium*, *Phytophtora* and *Mucor* (Snowdon, 1990).

The fungus is a typical postharvest pathogen as it can establish infection even below 0°C (Koch, 1985; Baraldi et al., 2003). Although decay proceeds slowly at cold storage temperatures, rapid development ensues when the fruit is transferred to a warm environment. The optimal conditions of growth are temperatures ranging between 25 and 35°C and a pH wide range: from 2 to 10. The minimum temperature that has been reported for this species is –3°C. Water activity ranging from 0,82 to 0,95 is optimal for mycelium growth while the minimum water activity (a_w) for spore formation is 0,85 (Ostry et al., 2004). *P.expansum* grow well at low temperatures and poorly if at all at 37°C. It also grow well at low water activities and low pH values (Pitt and Hocking, 1998; Frisvad et al, 2000). It tolerates both quite acidic and alkaline conditions and it is psychrotolerant.

1.3.4 Disease cycle

The blue mould is not considered to be a serious problem in the field. Its incidence in the field is almost strictly confined to apples which have fallen to the ground. Blue mould is, however, a major disease in packinghouses and storage facilities, where airborne inoculum is almost always present. The primary source of inoculum in this environment comes from contaminated storage bins, picking boxes or decaying fruit lying on the floor or stuck to the walls of storage bins. Infection occurs when either air-or water-borne conidia enter fruit through the wound produced during harvesting or handling, through insect injuries, scab lesions, or open lenticels (Tepper and Yoder, 1982).

The water used for drenching and those in the water flumes harbour numerous spores as a result of successive washings of infected fruit and contaminated bins and can induce waterborne infections on fruits onto packing lines. Studies conducted in the Pacific North-Western United States, revealed that nearly 50% of samples of dump tank water contained between 10 to 100 conidia per millilitre (Spotts and Cervantes, 1993).

1.3.5 Production of secondary metabolites

Aside from direct economic considerations, some postharvest pathogens pose a potential health risk. A number of fungal genera such as *Penicillium*, *Alternaria* and *Fusarium* are

known to produce toxic metabolites (mycotoxins) under certain conditions. Generally speaking, the greatest risk of mycotoxin contamination occurs when diseased product is used in the production of processed food or animal feed (Morales et al., 2008a). Svendsen and Frisvad (1994) reported that the secondary metabolite profile in itself enables conclusive classification for many terverticillate Penicilla.

P.expansum, the most common foodborne fungi on apple fruit, is one of the most studied species in the genus since it is a consistent producer of toxic metabolites especially patulin. (Andersen et al., 2004). In pure culture, *P.expansum* is reported to produce no fewer than 50 different secondary metabolites such as citrinin, ochratoxin A (Bragulat et al., 2008), expansolides A et B (Massias et al., 1990), rubratoxin B (Paterson et al., 1987), roquefortine C, penitrem A (Bridge et al, 1989), PR-toxin (Paterson and Kemmelmeier, 1989), chaetoglobosins A and C (Frisvad, 1992), patulin (Brian et al., 1956; McKinley and Carlton, 1991; Andersen et al., 2004) and others like cyclopiazonic acid, brevianamide A, gentisyl alcohol, griseofulvin, mycophenolic acid (Bridge and Hawksworth., 1989), Raistrick phenols (Frisvad and Filtenborg., 1989), aflatrem (Paterson and Kemmelmeier., 1989). Citrinin, patulin, roquefortine C and communesin B are usually produced in higher amounts compared to other *P. expansum* metabolites (Gaucher et al., 1981). Andersen et al., (2004) have shown that the combination of metabolites produced by *P. expansum* is independant of geographic region and substratum and that this profile of metabolites is indicative for *P. expansum*.

However, recent studies suggest that the range of metabolite is much more limited (Frisvad and Thrane., 2002; Larsen et al., 1998). In fact, Andersen et al. (2004), has noticed that from 260 studied *P. expansum* isolates, only 4 of the 40 metabolites which are citrinin, gentisyl alcohol, patulin and roquefortine C were found. According to them, reports of metabolites produced by *P.expansum* other than those detected during their study were often based on misidentified fungal isolates.Gaucher et al (1981) reported that gentisyl alcohol and 6-methylsalicyclic are precursors for patulin since it was found in 50% of the *P. expansum* isolates tested.

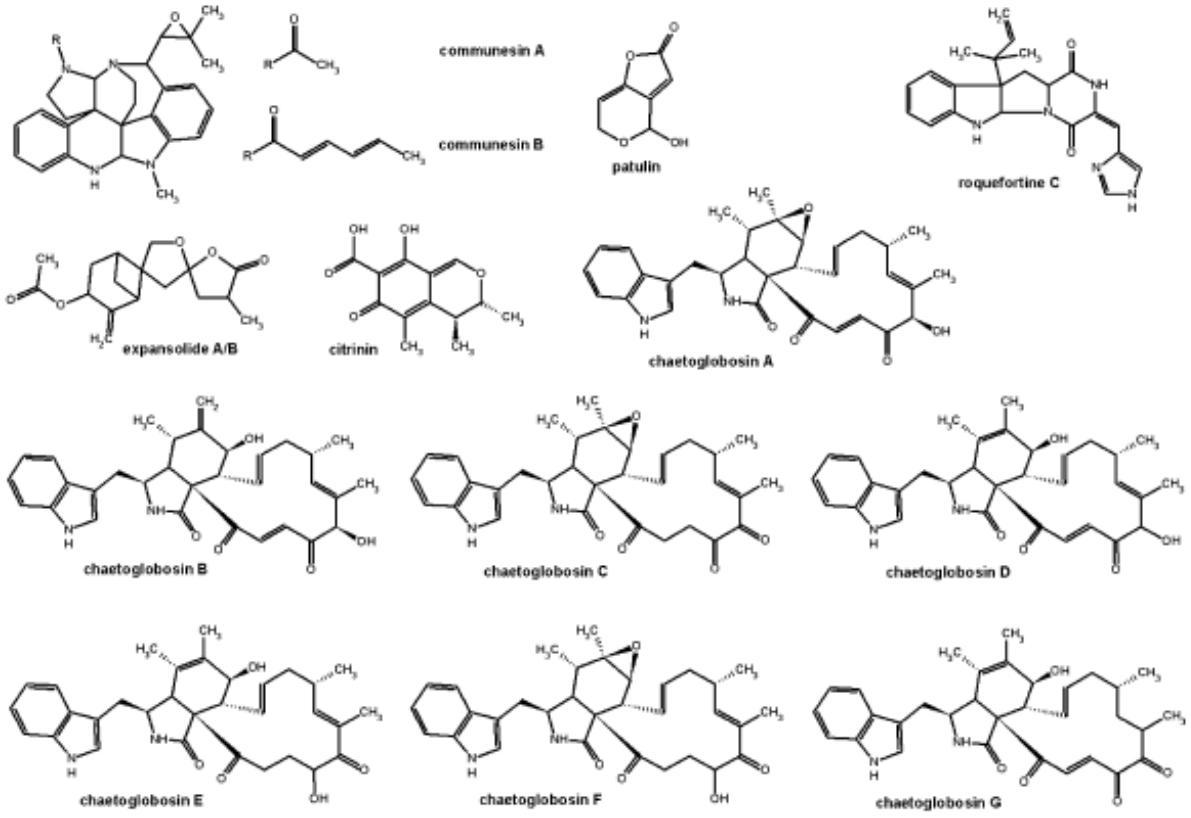


Figure 3- Structure of the metabolites produced by *P. expansum* (Andersen et al., 2004)

1.3.6 Patulin production

Patulin (4-hydroxy-4H-furo [3, 2-c]-pyran-2(6H)-one) is a mycotoxin produced mostly by species of *Penicillium* and *Aspergillus* (Engel and Teuber 1984; Harrison, 1989). It was first discovered as an antibiotic compound and given several names including claviformin, penicidin, clavatin, clavacin and expansin (Atkinson 1942; Chain et al. 1942; Bergel et al. 1943; Katzman et al., 1944; Oosterhuis, 1956), hence was banned because of its toxicity to human being (Boyd 1944). It has been reported to cause nausea, vomiting and gastrointestinal disorders (Lai et al., 2000; Moake et al., 2005), to be embryotoxic and at relatively high levels, immunosuppressive in animals (Escoula et al., 1988).

P. expansum is the most commonly patulin-producing fungus in apples and consequently apples and apple products are the major human dietary sources of patulin (Stott and

Bullerman 1975; Abramson, 1997; Bullerman, 2000; Karaoglanidis et al., 2011). It produces a higher patulin content in apples than in pear varieties (Menniti etal., 2010). Although the toxic effects of patulin in humans have not been proven conclusively, there is major concern about fruit processing industry since the use of moldy fruits contaminated with *P. expansum* greatly increases the risk of patulin contamination in fruit juices, especially apple juice, which are commonly consumed by infants and children. Surveys have revealed frequent occurrences of patulin in apple juice (Lindroth and Niskanen 1978; Brackett and Marth 1979; Wheeler et al., 1987; Burda 1992; Yurdun et al., 2001), leading to increased safety concerns regarding the implications of human exposure to high levels of patulin. For this reason, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a provisional maximum tolerable daily intake of 0,4 µg/kg body weight (WHO and JECFA, 1998) which was endorsed by the EU Scientific Committee for Food (SCF) in 2000. The maximum permitted levels of patulin in Europe are 10 µg/kg for fruit-based baby food, 50 µg/kg for fruit juice and 25 µg/kg for solid apple products (Moake et al., 2005). Patulin is a known toxicant due to its carcinogenic, mutagenic and immunotoxic characteristics (Pitt, 1997; Pianzzola et al., 2004). It has been reported to be toxic to many other organisms including protozoa, viruses, fungi, plants and mammals (Ciegler et al. 1971; Becci et al. 1981; Cole and Cox 1981; Engel and Teuber 1984; Smith et al. 1993; Llewellyn et al. 1998; Alves et al. 2000). Results of toxicological studies reported by Riley and Showker (2001), have shown that patulin is cytotoxic to continuous cell lines, and is believed to exert cytotoxic effects by forming covalent adducts with essential cellular sulfhydryl (SH, thiol) groups of proteins and glutathione. In addition, there is no clear evidence that patulin is carcinogenic, however, it has been shown to cause immunotoxic (Pacoud et al., 1990) and neurotoxic effects in animals (Deveraj et al., 1982). Patulin is also highly toxic to plants (Ciegler et al, 1971) and probably it may play a role in plant pathogenesis. Brian et al. (1956) stated that patulin is not always present in *P. expansum* rotted apples. Consequently, patulin is not involved in apple decay. However this hypothesis was not supported, since studies carried out by Wilson and Nuovo (1973) showed that all the isolates tested were able to produce at least 10 µg of patulin /ml of expressed juice from decayed apples. This amount may be sufficient to be cytotoxic to apple cells. Also, Andersen et al. (2004) reported that almost all *P. expansum*

strains are patulin producers and thus most decayed apples are likely to be contaminated with patulin if infection by this species occurs. Patulin content of rotted apple tissue is related to both population of mould spores and the length of time that *P. expansum* has been growing, consequently, patulin production does not provide *P. expansum* with a competitive advantage over other species. Moreover, its production may be also linked to nutritional stress (McCallum et al., 2002). Santos et al. (2002) suggested that patulin production is strain specific. Assays carried out by Morales et al. (2008c), have demonstrated that in more concentrated inocula, Isolate UdLTA-3.78 tended to increase patulin accumulation whearas isolate UdLTA-3.72 accumlutated less patulin.

In conclusion, prevention from mycotoxin contamination of apple fruits and apple by-products is one of the top properties in human and animal safety. Approaches to reduce the mycotoxin levels, particularly patulin in contaminated foods and feeds, include various physical, chemical and biological detoxification methods in stored and processed comodities. However, these techniques are still in developmental stages and have limited efficacy (Moake et al., 2005; Morales et al., 2010).

1.3.7 Production of volatile organic compounds

Fungal pathogens, included *P. expansum*, produce copious spores that may cause allergies and asthma (Zureik et al., 2002; Benndorf et al., 2008), and fungal toxins (mycotoxins), they are also producers of plenty of microbial volatile organic compounds (MVOCs) that are known to cause olfactory nuisance,.

Volatile substances (VS) are organic or inorganic compounds which are low in molecular weight (<300 Da) and low in polarity, but high in vapor pressure (Vespermann et al., 2007). Production of VS has been reported in bacteria (Fernando et al., 2005; Gu et al., 2007; Kai et al., 2007; Chen et al., 2008), *Streptomyces* spp. and other species of actinomycetes (Carpenter-Boggs et al., 1995; Sunesson et al., 1997; Schöler et al., 2002; Dickschat et al., 2005), fungi (Stinson et al., 2003, Koitabashi et al., 2004; Strobel et al., 2007) and higher plants (AL-Taweel et al., 2004; Kulakiotu et al., 2004). Fungistatic and fungicidal effects of VS occur naturally (Archbold et al., 1997) such as those produced by the endophytic fungus *Muscodor albus* isolate CZ-620 effective in the control of fruit post-harvest diseases caused by *B. cinerea*, *C. acutatum*, *Geotrichum* spp., *M. fructicola*,

Penicillium spp., and *Rhizopus* spp. (Mercier and Jiménez, 2004; Mercier and Smilanick, 2005).

Introduced in the early nineties by Arthur and Pawliszyn (1990), solid phase microextraction (SPME) has been known to be a reliable and rapid analytical tool to determine very low quantities of analytes. This principle has been advantageously applied to low dose MVOC analysis (Fiedler et al., 2001; Wady et al., 2003; Wady and Larsson, 2005); SPME in combination with GC-MS was used to explore the volatile secondary metabolites released by different fungal species grown on different substrata.

In addition, Magan and Evans, (2000) and Paolesse et al. (2006) reported that the detection of volatile organic compounds (VOCs) produced by fungi could be a non-invasive method for the monitoring of fungal growth in food. For example, trans-1,10-dimethyl-trans-9-decalol (geosmin) is a potential growth indicator for *Penicillium* strains. Studies carried out by Larsen & Frisvad, (1995) have demonstrated that 47 taxa of *Penicillium* have shown different volatile metabolites production profiles. They often include several sesquiterpenes, indicating the potential of profiles of volatile fungal metabolites for use in chemosystematics of *Penicillium* taxa. *P. expansum* emitted a large number of VOCs, geosmin has been reported to be the main odour compound produced by *P. expansum* (Mattheis and Roberts, 1992), and also by an isolate of *A. versicolor* (Sönnichsen and Keller, 1997). It is the primary component of the odour associated with *P. expansum.* It has been identified as a component of the off-flavor of dry beans (Battery et al., 1976) and contributes to the natural flavor of red table beets (Acree et al., 1976). Geosmin was reported by Mattheis and Roberts (1992) as a possible specific chemical marker for *P. expansum*. The presence and also the concentration of the MVOCs strongly depended on the age of the mould culture. In particular aliphatic and aromatic ethers (except 3-methylanisole) and sulphur compounds (dimethylsulfide, dimethyldisulfide) were found in cultures of higher age, while geosmin was found to be characteristic of the initial growth stage of *P. expansum* whearas 3-methyl-1-butanol was detected in the log phase (Fiedler et al., 2001). During the growth phase, 2-phenylethanol (PEA) was produced as well, however, at high air exchange rates (2 l/24 h), this alcohol was not produced (Fiedler and Schütz, 2000). The formation of methylketones, for

example 2-butanone, 2-pentanone, 2-hexanene, 2-heptanone and 2-octanone was also very typical of *P.expansum* (Fiedler et al., 2001).

1.3.8 Management of *P. expansum*

More than 90 fungal species have been described as causal agents of apple decay during storage (Jones and Aldwinckle, 1991; Morales et al., 2008a). The relative importance of each pathogen depends on climatic and storage conditions.

P. expansum seems to be the more aggressive comparing to other species. It is recovered most frequently from pear and apple, however other species can be find in green mold rot as in order of decreasing frequency by *P. solitum, P. commune* and *P. aurantiogriseum* (Sanderson and Spotts, 1995). The benzimidazole fungicides were introduced in the early 1960's as antithelmintic drugs (Brown et al., 1961) and were quickly shown to be active against a wide spectrum of organisms. Therefore benzimidazoles found application in various fields, including human and veterinary medicine and agriculture (Cargo and Dewey, 1970; De Brabander et al., 1976). Within benzimidazoles, thiabendazole (TBZ) was widely used because of its effectiveness in controlling blue mould on fruit and as the only chemical compounds allowed in Italy for the control of postharvest blue mould during the last decades. It belongs to the class of benzimidazoles and is included in Annex I of EC Council Directive 91/414 (European Commission 2001). The fungicide was commonly applied as either a drench treatment prior to the cold storage and/or spray treatment in packing lines (Rosenberger and Meyer, 1979; Koffmann and Penrose, 1987).

Hence, the concomitant development of fungicide-resistant fungal strains, the public awareness of pesticide residues and high development costs of new chemicals have resulted in a significant interest in the development of alternative non chemical methods for disease control. According to Eckert (1988) and Delp (1988), rotation with non benzimidazoles fungicides alone or in a combination with products that have different modes of action are two possible fungicide-resistance management strategies to control benzimidazole-resistant populations.

In an urgent attempt to find alternate fungicides to TBZ, two fungicides, cyprodinil and

anilinopyramidine were evaluated for their efficacy against blue mould rot caused by thiabendazole-sensitive and resistant isolates of *P expansum (*Errampali, 2004; Sholberg et al., 2005a; Smilanick et al., 2006; Xiao and Boal, 2009). Cyprodinil, and fludioxonil fungicides were rated as "reduced risk" toxicology fungicide by the United States Environmental Protection Agency (USEPA, 1998). Cyprodinil, belonging to the class anilinopyrimidine, does not affect spore germination but inhibits germ-tube elongation and initial mycelial growth of *B. cinerea*. It was found to be effective against blue mould of apple under laboratory conditions (Zhou et al., 2002) but also against TBZ-sensitive and resistant *P. expansum* strains on Empire apples (Errampalli and Crnko, 2004). Fludioxonil, belonging to the chemical class of henylpyrroles, is a nonsystemic fungicide acting through the inhibition of spore germination, germ tube elongation, and mycelium growth of *P. expansum* and able to reduce decay caused by fungal isolates on apple (Errampalli and Crnko, 2003). They have modes of action different from each other and from that of the TBZ (Errampalli and Crnko, 2004). Fludioxonil affects cell-wall synthesis and induces accumulation of glycerol in mycelia cells contrarily to cyprodinil that blocks the excretion of hydrolylitic enzymes and inhibits methionine biosynthesis in fungal cells (Leroux, 1996). Rosenberger et al, (2002), noticed that a higher concentration of fludioxonil (300 µg/mL) was required to control blue mould existing prior to the fungicide treatment in apples stored for 4 months or longer. The absence of cross-resistance between TBZ and fludioxonil or cyprodinil provides options for the use of these two fungicides and their mixture for disease management of *P. expansum* in packinghouses (Errampalli and Crnko, 2004**)**.

Since *P. expansum* is a classical high risk pathogen from the view of resistance development and the site specific fungicides (Frac, 2010), recently, a resistance risk was demonstrated for cyprodinyl and fudioxonil (Karaoglanidis et al ., 2011) as was reported in the recent past for the benzimidazoles and DMIs (Rosenberg et al., 1991; Vinas et al., 1991, 1993; Baraldi et al., 2003; Morales et al.,2008a; Sholberg et al., 2005b; Morales et al., 2008a). In recent *in vitro* fungitoxicity bioassays carried out in Greece, Karaoglanidis et al. (2011) have found that from a total of 236 *P. expansum* isolates tested for their sensitivity, 43% showed reduced sensitivity to cyprodinil with EC_{50} values significantly higher (up to 100-fold) than those of the most sensitive ones while only 7.5 % of the

isolates showed decreased sensitivity to fludioxonil. In addition, a study regarding the influence of fungicide resistance on the mycotoxin production shown that most cyprodinil-resistant isolates produced patulin at concentrations higher than the sensitive ones while a significant reduction in the patulin production was found in most isolates with reduced sensitivity to fludioxonil; these results contradicted those reported previously by Doukas et al. (2008), Makoglou et al. (2008; 2009) which demonstrated that *P. expansum* resistant to the phenylpyrrole fludioxonil produced significantly higher levels of mycotoxins (ochratoxins, patulin and fumonisins) compared to the parental sensitive strains.

In conclusion, the extensively and inappropriate use of fungicides in crop protection may lead to reduced sensitivity and certain mycotoxin-producing fungi, and thus to an increased contamination of commodities with mycotoxins. Consequently, careful implementation of the commercial use of these compounds is needed to maintain their effectiveness and to preserve the quality and the safety of pome fruit and their by-products.

The continuous trend to restrict or ban the use of current synthetic fungicides has intensified exploratory and developmental research for biocontrol agents, unlikely to leave harmful residues on the fruit surface as substitutes that should be the one of choice.

In recent years, biological control using microbial antagonists has emerged as one of the most promising alternative control measures and has become an important field for research. It has made great advances, especially during the past decade, the usefulness of this approach has been proven under commercial conditions (Wilson and Wisniewski, 1994; El Ghaouth et al., 1997; Janisiewicz, 1998; Janisiewicz and Korsten, 2002). Several antagonistic microorganisms, naturally- occuring bioactive compounds, have been isolated from various sources including fermented food products and the surfaces of leaves, fruit and vegetables. In most reported cases, pathogen inhibition is greater when the antagonist is applied prior to infection taking place. For this reason, control of quiescent field infections (e.g. *Colletotrichum* spp.) using postharvest applications of antagonists is often more difficult to achieve than control of infections occurring after harvest (e.g. *Penicillium* spp.).

Antagonistic bacteria and yeasts have been shown to control decay of a variety of harvested commodities including citrus, pome and stone fruits. Effective control of decay

of citrus fruit caused by *P. digitatum* (Pers.: Fr) Sacc., and *P. italicum* Wehmer was reported with bacterial antagonists such as *Bacillus subtilis* (Ehrenberg) Cohn, *Burkolderia* (*Pseudomonas*) *cepacia* Palleroni & Holmes and *Pseudomonas syringae* Van Hall (Smilanick and Dennis-Arrue, 1992; Huang et al., 1995; Bull et al., 1997)
Two biofungicides, Aspire (Ecogen, Inc., Langhore, PA) based on the yeast *Candida oleophila* (Droby et al., 1993, 1998) and BioSave 100 and 110 (JET Harvest Solutions, Longwood, FL) containing saprophytic strains of *P. syringae* (Janisiewicz and Marchi, 1992; Janisiewicz and Jeffers, 1997) have been registered for postharvest use on pome fruit in the USA (Fravel and Larkin, 1996; Janisiewicz and Jeffers, 1997), but not in Canada. In fact, Buckner (2005), Holmes and Edmunds (2005) and Stockwell and Stack (2007), underligned the fact that the use of BioSave has been continually increasing and the original registration for application to apples, pears and citrus fruit has been extended to cherries, potatoes and sweet potatoes. In a preliminary study it was shown that $5 \cdot 10^{10}$ CFU /ml of *P. syringae* (*P. syringae;* BioSave) reduced 89% of blue mould in Empire apples in cold storage (Errampalli, 2003).

Although the biocontrol agents were found effective, the control level could not reach the 95–98% that was often approached by the chemical fungicides. Also lack of consistency was reported as one of the drawbacks for the biocontrol (Chalutz and Droby, 1997).
Integrating biocontrol agents with fungicides or exogenous chemicals have been proposed to enhance the efficacy of biocontrol agents (Chand-Goyal and Spotts,1996, 1997; Sugar and Spotts, 1999; Zhou et al., 2002, 2005), and indirectly to reduce fungicide concentrations resulting in reduced residue levels on the fruit (Papadopoulou- Mourkidou, 1991).

1.4. Resistance to Thiabendazole: mutation of the β-tubulin gene

Benzimidazole resistance represented the beginning of serious fungicide resistance problems. This happened in the 1970's because benzimidazoles were used widely, alone and intensively for crop protection, but also they are specific-site inhibitors (Horst, 1987).

Their eradicative properties can be attributed to penetration through the waxy cuticle of the host and arrival at the infection site (Ben-Arie, 1975). This characteristic was responsible of the persistence of the fungicide over long storage periods, but at the same time it became the cause of its continuous selection pressure on populations of *Penicillium* spp. (Prusky et al., 1980; Lee, 1985).

In fact the intensive use of TBZ, along with antiscalding agent, diphenylamine (DPA), has resulted in the development of benzimidazole resistant pathogens in packinghouses in the United States (Rosenberger and Meyer, 1985); British Columbia (Sholberg and Haag, 1996); and Ontario (Errampalli, 2004) in Canada.

In most cases, resistance has been associated with point mutations in the β-tubulin gene. The latter is a highly conserved gene that is ubiquitous in all eukaryotic cells. In fact, the benzimidazoles bind to β-tubulin and inhibit microtubule polymerisation. These mutations altered the amino acid sequences at the benzimidazole-binding site (Davidse and Ishii, 1995; Michailides 2005). Cabanas et al. (2009b), reported that changes at different codons in the β-tubulin gene were responsible for benzimidazole resistance of different mutants fungi. Mutations conferring resistance to benzimidazoles were detected at codons 6, 50, 134, 165, 198, 200 and 257 in *A. nidulans* (Jung & Oakley, 1990). In *Saccharomyces cerevisiae*, mutations were identified at codons 167 and 241 (Li et al., 1996) while in *Neurospora crassa* mutations, were located at codons 167 and 198 (Koenraadt et al., 1992).

In the genus *Penicillium*, the β-tubulin mutations conferring resistance to TBZ seem to be restricted to several positions. Mutations at codons 198 or 200 confer resistance to benzimidazoles benomyl and TBZ in *P. italicum*, *P. aurantiogriseum* and *P. digitatum* (Scholberg et al., 2005; Schmidt et al., 2006) whearas mutations in *P. solitum*, *P. puberulum* and *P. viridicatum* were found only at position 198.

Baraldi et al. (2003) reported that mutations associated with natural resistance and laboratory induced TBZ resistance in *P. expansum* involve codon 198 (Glu to Ala, Glu to Val or Glu to Lys) and codon 167 (Phe to Tyr).

CHAPTER 2

2. OBJECTIVES

This work aimed to study the resistance to TBZ among *P. expansum* strains. Preliminary screening on mycelium growth and spore germination assays will be carried out into MEA plates amended with commercial or pure TBZ. They will be confirmed by a microtiter assay.

Moreover, a comparison between pure and commercial TBZ and their fungicidal effect on the growth of *P.expansum* strains will be performed. The eventual presence of mutation on the β-tubulin gene related to the emergence of resistance will be also studied. For this aim, some *P. expansum* strains with different levels of resistance will be cloned and mutations associated with TBZ resistance will be identified.

Possible alternative means of control will be tested *in vitro* such as the storage of *P. expansum* strains under controlled atmosphere with ultra low oxygen levels (ULO). Assays of biological control will be effectuated using a *P. expansum* strain LB8-99 as a biocontrol agent both *in vitro* and *in vivo* against other fungal pathogens including *P. expansum* strains.

Furthermore, we will focus on the study of the identification of VOCs produced by a *P. expansum* strain LB8-99 and particularly the antifungal effect of the phenylethyl alcohol.

More quantitative tests will be performed to determine the lethal concentrations of phenylethyl alcohol to fungal spores and mycelium and to study the sensitivity of the target postharvest pathogens and specific fungal structures including mycelium and spores which can be differentially affected by the volatile substances.

CHAPTER 3

3. MATERIAL AND METHODS

3.1. Isolates

Isolates were taken from common (apple) and non common host species including kiwi, lemon, peach and plum fruits, showing symptoms of blue mould, collected from the fields or packinghouses located in Emilia Romagna region and some of *P. expansum* strains were taken from CRIOF-UNIBO collection. For the isolation, the following protocol was used: sporulating areas of lesions of infected fruits were rubbed and conidia were suspended in sterile distilled water added with Tween 80 (0.05%). Each suspension of conidia was streaked onto Malt Extract Agar (MEA) plates. MEA was prepared by dissolving 50 g of MEA powder (Oxoid, Hampshire, UK) in 1 L of distilled water. Plates were kept at 25°C for 48 h, then a single hyphal tip was excised with a sterile scalpel and cultured on fresh MEA plate. At the end of the selection forty-eight fungal isolates were obtained.

All isolates were maintained as single spore cultures on MEA Petri dishes at 4°C with periodic transfers through apples. The pathogen conidial suspension was prepared from 3 days old MEA cultures. The inoculum was harvested by adding a small amount of sterile distilled water with 0.05% Tween 80 to each plate and gently rubbing the surface of the culture with a sterile inoculation loop (10 µL). The resulting conidial suspension was filtered through a sterile hydrophilic compress 10x10 cm (Farmatexa-Farmac-Zabban Italy). The spore concentration of the pathogen was determined with a haemocytometer (Fein-Optik ,Bad, Blakenburg, DDR).

3.2. Identification of the isolates

3.2.1 Morphological identification

The isolates were preliminary identified under microscope (Carl Zeiss, Germany) basing on their morphological features. For this aim, a drop (about 20 µL) of conidial suspension of each isolate prepared as reported previously was placed in the center of a glass microscope slide; similarly, some of the young sporulating structures were transferred to microscope slide containing a small drop of sterile water using a fine needle. All the slides were coverd with a clean cover slip and observed under microscope (Nikon). The measure of the conidia diameter, the length of stipes metulae and phialids were obtained using universal computer image analysis (LUCIA image software, version 4. 61).

3.2.2 Molecular identification by Polymerase Chain Reaction (PCR)

3.2.2.1 Extraction of genomic DNA from isolates

All reagents used were purchased from Sigma Aldrich Co. (St. Louis, MO) unless indicated otherwise. A standard DNA extraction protocol was followed as described by Chen and Ronald (1999), with slight modifications. To obtain fungal mycelia, isolates were grown on plates of MEA that were overlaid with sterile cellophane (autoclaved at 121°C for 20 min). A spore suspension (0.1 mL containing 10^3 spores/ mL) of each isolate was spread over the cellophane and the cultures were incubated at 20°C for 72 h. The mycelia growing on the cellophane, was lifted off, collected and transferred into sterile 1.5-mL Eppendorf tubes.

The tubes were submerged in liquid nitrogen for 30 s to freeze the mycelia. Immediately following freezing, the mycelia were crushed using a sterile micro pestle. Tubes of 2 mL filled of 0.8 mL of DNA extraction buffer containing 0.1M Tris, 5 mM EDTA, 1.4 M NaCl, pH 8.0. were frozen on the ice then added with crushed mycelium.

The tubes were vortexed then thawed in a 67℃ water bath for 20 min. After, 0.4 mL of phenol (pH ca. 7-8) and 0.4 mL of chloroform:isoamyl alcohol (49:1) were added and tubes were vortexed, then centrifuged at 14,000 rpm for 10 min at 4℃. The upper phase (aqueous phase) was added to 0.8 mL of isopropanol kept on the ice and gently mixed by inversion of the tubes several times. The tubes were centrifuged at 14,000 rpm for 25 min at 4℃ and the supernatant was poured off. The pellet was washed in 0.5 mL of 75% ethanol and centrifuged at 14,000 for 5 min at 4℃ and the supernatant was removed. Pellets were dried for at least 15 min in air,dissolved in 0.1 mL of 10 mM Tris (pH 8.0) and resuspended in 5 µL RNase for 30 min at 37℃. Finally, the DNA was quantified spectrophotometrically using the nanodrop and stored at –20℃.

3.2.2.2 Polymerase Chain Reaction

DNA extracted from isolates following the protocol of Chen and Ronald (1999) reported previously was amplified by polymerase chain reaction (PCR) using universal primers ITS1F and ITS4 designed to amplify the ITS1-5.8S-ITS2 rDNA region of filamentous ascomycetes (Table 4). The forward primer ITS1F anneals at the 3' end of small subunit (SSU) gene, whereas the reverse primer ITS 4 anneals at the 5' end of large subunit (LSU) gene. Biometra (Gottingen, Germany) thermal cycler was programmed for optimum PCR conditions. Initially, the reaction mixtures were heated at 92 ℃ for 5 min (denaturation), followed by 30 cycles at a melting temperature of 94 ℃ for 1 min, annealing at 55 ℃ for 45 s, and extension at 72 ℃ for 10 min, and a final extension for 10 min at 72 ℃. PCR reactions were performed in a total volume of reaction mixtures of 50 µl containing 2 µL of genomic DNA (200 ng), 5 µL 10XLA PCR Buffer, 4 µL dNTP (2.5 mM), 4 µL $MgCl_2$ (2mM), 0.3 µL of Taq polymerase (5 units/µL) (TaKaRa, Japan), 2.5 µL (10 picomol) of each primer and 27.7 µL of nuclease free water. Amplicons were analysed by electrophoresis in 1% agarose gels in TAE buffer (Sambrook *et al*., 1989), visualized by staining with ethidium bromide (2 µg/mL) and detected by UV fluorescence. One-kb DNA ladder (Promega) was used as a molecular size marker.

Table 4- Primers pairs used to amplify the IGS regions

Primers	Sequence (5' - 3')
ITS-1-F (Forward)	CTTGGTCATTTAGAGGAAGTAA
ITS-4-R (Reverse)	TCCTCCGCTTATTGATATGC

PCR products were purified using Nucleospin plant II kit (Machery- NAGEL) following the supplier's protocol and sequenced using ITS-1-F primer by BMR Genomics (Padua, Italy). Sequence homologies were checked by Basic Local Alignment Search Tool (BLAST) analyses to explore all of the available DNA sequence data in international databases.

3.3. *In vitro* study of thiabendazole resistance of P*. expansum* strains

3.3.1 Preliminary assays

Thirteen *P. expansum* strains randomly chosen within the isolates previously selected, were assessed for their susceptibility to commercial fungicide TBZ [2-(thiazol-4yl) benzimidazole] as Tecto, Syngenta, (France).The commercial dose of 400 µg/mL was selected as discriminatory dose for resistance or sensitivity to fungicide. The assays on TBZ resistance were carried out determining its effects on some growth parameters as dry weight mycelium, mycelium growth and conidial germination.

3.3.1.1 Dry weight mycelium

The dry weight mycelium (DWM) of strains was evaluated in a liquid medium: malt extract broth, (MEB), MEA without agar. The medium was prepared dissolving 20 g of MEB powder (Oxoid) in 1L of distilled water and adding commercial TBZ at a final concentration of 400 µg/mL. After sterilization, 40 mL of lactic acid solution (10% w/w) was put into the medium to avoid the bacteria contamination. The inoculum suspensions were prepared as previously described and adjusted to 10^3 conidia/mL. Sterile Erlenmeyer flasks (50 mL) containing each 10 mL of MEB amended with fungicide were inoculated with 100 µL conidial suspension of *P. expansum* strains, then incubated at 20°C. After 10 days post inoculation (DPI), the mycelium was harvested through filtration using Whatman filter n. 1 (Whatman GmbH, Dassel, Germany), previously desiccated and preweighed. After filtration, filters containing the deposited fungal material were dried in an oven at 80°C until reaching a constant weight with a precision balance.The difference between the filter weight and the combined weight of the filter with fungal mycelium represented the dry mass of the pathogen expressed in mg of DWM.

3.3.1.2 Mycelium growth

The sensitivity of *P. expansum* strains to TBZ was evaluated on MEA plates supplemented with 400 µg/mL of commercial TBZ. Control media did not contain the fungicide. Both substrates were amended with lactic acid (10%) as previously described. The strains were first grown on Czapek medium to limit the conidia production. Czapek is a poor media prepared by dissolving 45.4 g of Czapek Dox agar (Oxoid) on 1 L of sterile distilled water. The growth rate (colony diameter) was estimated by placing a plug (6 mm diameter) removed from a growing colony on Czapek, onto TBZ-amended and non amended MEA and colony diameter measurements were performed after 3 days growth at 20°C.The results were expressed in term of average colony diameter (mm) and the inhibition of mycelium growth respect to control was calculated.

3.3.1.3 Conidial germination

For this purpose, aliquots (0.1 mL) of a conidial suspension (10^3 conidia/mL) of each *P. expansum* strain were spread on TBZ-amended medium (400 µg/mL) in Petri dishes, and plates were incubated at 20°C. After 3 days the number of germinated conidia was counted and expressed as colony forming units (CFU) per plate and the inhibition of conidial germination respect to control was calculated.

In all three experiments, non amended TBZ medium was used as the control and the assays were replicated 3 times (3 dishes) per each strain and repeated twice.

3.4. Resistance of *Penicillium* spp. strains to pure and commercial thiabendazole

The resistance to TBZ was investigated on all *Penicillium* spp. collection containing 48 strains. MEA was amended with commercial or pure TBZ at the concentration of 400 µg/mL. Control was represented by non amended MEA. Assays were performed on conidial germination and mycelium growth. In the first case, aliquot (0.1 mL) of a conidial suspension (10^3 conidia/mL) was spread on MEA Petri dishes. After incubation for 3 days at 20 °C, CFU/plate were counted and the inhibition of conidial germination respect to control was calculated.

The same assay was performed for mycelium growth, by putting a plug of 6 mm diameter of each strain in the center of MEA plates in order to assess the colony diameter (mm). The experiment was replicated 5 times (dishes) per each strain and repeated three times.

3.5. Dose-response curves of *Penicillum* spp. strains to thiabendazole: microtiter assay

The dose-response curves to TBZ was tested on ten TBZ-resistant *P. expansum* strains and they were generated to identify concentrations of TBZ that inhibited growth of pathogen by 50 and 95 % (ED_{50} and ED_{95} respectively). For this purpose a microtiter assay was adopted as described by Spiegel and Stammler (2006) and Stammler and Speakman (2006) with some modifications. Pure TBZ was solved in dimethylsulfoxide to reach a concentration of 200.000 µg/mL and the dilutions were prepared in MEB diluted with water (1:2). The assay was performed in 96-well, flat-bottom, polystyrene microtiter plates (IWAKI, Japan). Each well contained 50 µL of fungicide solution and 50 µL of *P. expansum* spore suspension adjusted to 2.10^4 conidia/mL. The following final fungicide concentrations were used in the microtiter assays: 0, 3.125, 6.25, 12.5, 25 and 50 µg/mL. For each isolate and fungicide concentration, four replicate wells were performed. All microtiter plates included internal standards of diluted MEB without spores of *P. expansum*, at each concentration of TBZ. Wells containing MEB non amended with TBZ represented the control.

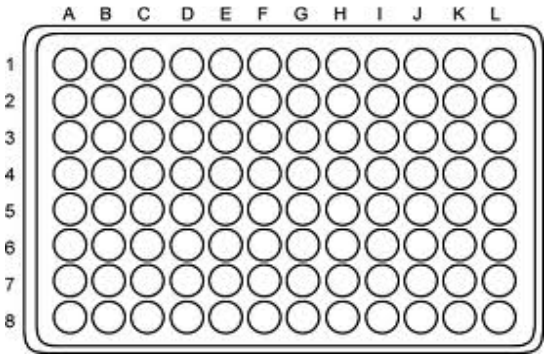


Figure 4 - A 96-well, flat-bottom, polystyrene microtiter plates

The microtiter plates were incubated at 20℃ in darkness and two days after inoculation, the absorbance measurements per each *P. expansum* strain was performed using a spectrophotometer at 620 nm. The values were corrected by comparison with the blanks. The ED-values were determined for each isolate by probit-analysis. A linear regression of the probit of percent inhibition of conidial germination versus fungicide concentration was calculated for each isolate, and the effective fungicide concentration (ED) estimated to produce 50% (ED_{50}) and 95% (ED_{95}) inhibition were determined by interpolation from the regression line. ED-values were assessed for each strain in two independent tests and the mean value were expressed in µg/mL.

3.6. Mutation of β-tubulin gene in P. *expansum* strains

Ten *P. expansum* strains with different levels of susceptibility to pure and commercial TBZ were tested. Fungal genomic DNA was extracted as described previously. PCR amplification of the β-tubulin gene was achieved using specific primers “Pen-for”, 5’-GCNAARGGNCAYTAYACNGARGGN-3’ and Pen-rev, 5’GCRAANCCNACCATRAARAARTG-3’ (Baraldi et al., 2003).

PCR reactions were performed in 50 µL reaction mixture containing 2 µL genomic DNA (200 ng/µL), 5 µL buffer 10x, 27.7 µL ultra pure sterile water, 4 µL dNTP (2.5 mM), 2.5 µL of each primer (50 pmol/µL), 2 µL BSA and 0.3 µL Taq TAKARA (5 U/µL DNA) (TAKARA). Amplification was performed on a Biometra (Gottingen, Germany) thermal cycler programmed for 45 cycles of 1min denaturation at 95℃, followed by primer annealing for 45 s at 63℃, and extension for 1 min 20s at 72℃, followed by a final 10 min elongation step at 72℃.The PCR products were run on a 1% agarose gel, then purified using exosap-it, PCR DNA Purification kit, (Affymetrix, USA), following the supplier’s protocol and cloned in TOP10 chemically competent *E.coli* following the TOPO-TA Cloning kit protocol (Invitrogen). Plasmid DNA preparations were done using overnight cultures containing 50 µg/ml ampicillin. Cloning was verified through a PCR colony carried out in a final volume of 50 µL composed of 5 µL DNA template, 8 µl of dNTP (2.5 mM), 5 µL Buffer 10x, 29.5 ultra pure sterilewater, 1 µL of each primer M13 For and M13 Rev (provided in

TOPO-TA Cloning kit, Invitrogen) and 0.5 µL Taq polymerase (5u/µL) (Fermentas). The PCR program consisted of an initial denaturation for 2 min 30 s at 94°C followed by 40 cycles of 94°C for 30 s, 52°C for 1min and 72°C for 1 min 30s followed by a final extension step of 5min 30 s at 72°C. The control consists on 45 µL master mix added with 4 µL of ultrapure sterile water and 1 µL of control provided by TOPO-TA Cloning kit; it is useful to detect the presence of false positive samples.Cloned fragments of the β -tubulin gene were purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen) sequenced (two clones from each isolate) using both M13 Forward and M13 Reverse primers, The predicted amino acid sequences were aligned using clustal x software (Thompson et al., 1997; Jeanmougin et al ., 1998).

3.7. Influence of ultra low oxygen conditions on P*. expansum* strains

In order to evaluate the influence of controlled atmosphere on the growth of *P. expansum,* three different modified atmosphere with low oxygen levels (0.5% O_2; 1% O_2; 2% O_2) were assayed at 0°C. The level of CO_2 in all atmospheres was of 0.3%.

For this purpose, an inoculum suspension of six *P. expansum* strains were prepared in sterile distilled water amended with Tween 80 (0.05%), adjusted to 10^3 conidia/mL and spread on MEA plates. The plates were introduced in the jars (0.015 m^3 volume) at the oxygen levels previously cited, The CFUs were counted every week for 1 months.

Mycelium growth was also studied by placing a plug (6mm diameter) from actively growing cultures on the center of a MEA Petri dish and colony diameter (mm) was measured every 4 days for 1 month. Both experiments were replicated 3 times (3 dishes) per each strain. Normal atmosphere (21% O_2-0.04.CO_2) was considered as control. After each inspection the jars were closed as soon as possible, and the previous atmosphere were restored within 30 min.

3.8. Study of volatile organic compounds production by P. *expansum*

3.8.1 Antifungal effect of the LB8/99 *P. expansum* strain *in vitro* and *in vivo*

The LB8/99 *P. expansum* strain, from Criof collection is TBZ-sensitive strain and in previous trials showed an activity against certain fungal pathogens (data not shown). In order to study its antifungal effect on mycelia growth and conidial germination of some pathogens, a fungal filtrate was prepared. The LB8/99 strain was cultured on MEA for 3 days at 20°C in order to prepare a conidial suspension. One litre bottle of MEB was inoculated with LB8/99 conidial suspension, at a final concentration of 10^6 conidia/mL and incubated at 20°C. Ten days later, the mycelium was separated from medium by centrifugation (4000 rpm/min, 20 min) and the supernatant was filtered through sterile filter paper (0.45µm) (Whatman GmbH, Germany).

3.8.1.1 Target pathogens

A. alternata (Fr.) Keissl, *Aspergillus* spp., *B. cinerea* (De Bary) Whetzel, *Cladosporium* spp., *C. acutatum* J.H. Simmonds, *Fusarium culmorum* (W. G. Smith) Sacc., *F. graminearum Schwabe* , *F. poae* (Peck) Wollenw. in Lewis, *M. laxa (Aderh.& Ruhl.),* six *P. expansum* strains and *Phialophora* spp. used in the experiments were singularly isolated from infected tissue of host fruit and kept at 4°C on MEA as monoconidial culture until use. In order to obtain a conidial suspension, each pathogen grown in the following conditions: *B. cinerea* grown on Oat Meal Agar (OMA: 60g of oatmeal, 10 g sodium nitrate, 30 g of saccharose and 12 g of agar per 1000 mL of distilled water), the cultures were incubated at 25°C under UV (350-420 nm) light for 12 h daily. *C. acutatum* was cultured on Potato Dextrose Agar (PDA) at 20°C for 10 days while *A. alternata*, *Aspergillus* spp., *Cladosporium* spp., *F. culmurum*, *F. graminearum*, *F.poae* and *Phialophora* spp. were cultured on PDA at 25°C for 3 days. *M. laxa* grown on V8Agar (V8A: 250 mL of V8 vegetable juice and 40 g of agar per 1000 mL of distilled water) Petri dishes, incubated at 25°C with 12 h dark:12 h light cycles for 10 days. *P. expansum* were cultured on MEA for 3 days at 25°C.

3.8.2 *In vitro* fungitoxicity assays

3.8.2.1 Dry weight mycelium

An aliquot of 20 mL from culture filtrate of LB 8/99 was added into sterile 50ml-flasks then inoculated with 100 µL of conidial suspension (10^3 conidia/mL) of *B. cinerea, C. acutatum, M. laxa* and six *P. expansum* strains and incubated at 20°C for 7 days. Later, the content of the flasks was filtered using Whatman filter n.1 (preconditioned overnight at 80°C), dried in an oven at 80°C until constant weight as previously described. Flasks containing 20 mL of MEB and inoculated with target pathogens were considered as the control. The sample unit was represented by 3 replications (3 flasks) for each pathogen. Trial was repeated twice.

3.8.2.2 Conidial germination

The fungitoxic effect of LB8/99 *P. expansum* strain was assayed *in vitro* on 6 *P. expansum* strains. The experiment was carried out using the cavity slides. A 0.1 mL of LB8/99 filtrate was placed inside the depression of sterilized cavity slide. Conidial suspension of each *P. expansum* strain was individually added into LB8/99 filtrate to a final concentration of 10^3 conidia/mL. The cavity slides were put inside Petri dishes that were fitted with moist filter paper. Each assay was performed three times. Conidia inoculated in sterile MEB represented the control. After 12 hours incubation at 20°C, microscopic observations were made and the measurement of the length of germ tube for each strain (mm) was recorded by Lucia Image software.

3.8.3 Double Petri dish assay

The antifungal activity of VOCs produced by LB8/99 was tested for inhibition of mycelial growth and conidial germination of *B. cinerea*, *C. acutatum, M. laxa* and *P. expansum* on MEA . The bioassay was done in closed Petri dishes (90 mm in diameter) in the presence of pathogens and LB8/99. In the test for inhibition of mycelial growth, a mycelial plug removed from actively colony margin of pathogens, cited above, was inoculated in a Petri dish containg 20 ml of MEA. Subsequently the cover of MEA plates containing LB8/99, inoculated and incubated at 20°C for 2 days before, was removed and MEA dish inoculated with mycelial agar plug of target pathogens was put above. The set of the

double dishes (DD) was sealed immediately using a double layers of parafilm (Parafilm M, Chicago) to make a closed DD chamber of almost 180 cm^3 in volume (Huang et al., 2011). For inhibiton of conidial germination, aliquots, 100 µl/dish, of a conidial suspension (10^3 conidia/ mL) of target pathogens were pipetted onto MEA in petri dishes (90 mm in diameter). The conidial suspension in each dish was evenly spread on the surface of MEA. Cover of MEA plates containing LB8/99 as described for mycelial growth was removed and covered with dish inoculated with conidia of tested pathogens and the DD set was sealed with parafilm. For both assays, the control treatment was represented by dishes inoculated only with target pathogens and sealed with parafilm (Fig. 5). The DD sets and control dishes were incubated at 20℃ for 3 days. In order to investigate the spectrum of activity of VOCs produced by LB8/99, preliminary assays were also performed on *A. alternata*, *Aspergillus* spp., *Cladosporium* spp., *F. culmurum*, *F. graminearum*, *F. poae* and *Phyalophora* spp. following the same protocol. Growth inhibition by volatiles produced by LB8/99 was assessed based on the percentage of inhibition of diameter growth and conidial germination with respect to control. Both values were calculated as follows (Trivedi *et al*., 2008): (T1-T2) / T1)x 100, where: T1 = diameter growth or cfu of target pathogen not exposed to LB8/99 (control); T2 = diameter growth or cfu of a target pathogen exposed to LB8/99. The assay was conducted in five replicates (dishes) and repeated twice.

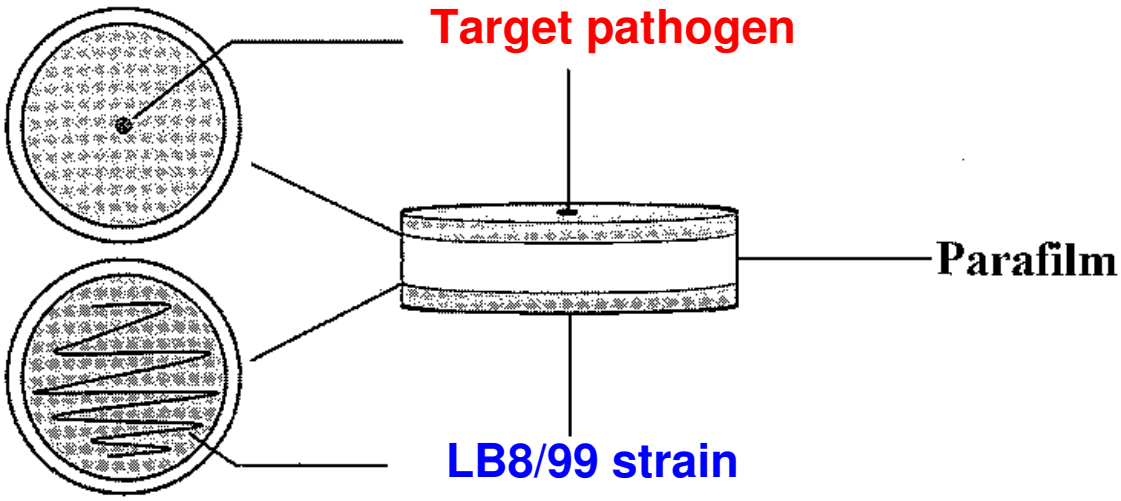


Figure 5- A schematic diagram showing the method of double Petri dish for testing antifungal activity of the volatile organic compounds of *P. expansum* strain LB8/99 on mycelium growth and conidial germination of target pathogens.

3.8.4 *In vivo* interaction between LB8/99 and P13 *P. expansum* strains

The *in vivo* assays were conducted on apple 'Golden Delicious' obtained from local packinghouse, selected for uniformity of size, maturity and free from wound and decays. Fruit were washed with 1% NaCl amended water, rinsed with sterile water, left to dry, and wounded (3x3x3 mm) with a sterile needle at equatorial region.

Apple were divided in three sets of 20 fruits each. The fruits of the first set were inoculated with 20 µL of LB8/99 conidial suspension (10^3 conidia/mL), while fruits of the second set were inoculated with P13 strain (TBZ-resistant) at the same concentration. Fruits of the third set were inoculated with 20 µL mixture of LB8/99 and P13, both of them at the concentration of 10^3 conidia/mL. After inoculation, fruits were kept at 20°C and the disease severity (lesion diameter of infected fruit) was recorded after 3 days. In order to identify the isolate responsible of the rot, MEA plates amended or not with TBZ (400 µg/mL) were inoculated with conidial suspension prepared from blue mould present on lesions of fruit inoculated with the mixture of LB8/99 + P13. Twenty Petri (replicates)

TBZ-amended and twenty non amended were singly inoculated with conidial suspension derived from blue mold present in a single fruit. The experiment was repeated twice.

3.8.5 Preliminary extraction and identification of volatile substances

Head space volatiles from LB8/99 were qualitatively analysed and first identified using solid-phase micro-extraction (SPME) (Strobel et al., 2001) coupled with Gas chromatography-Mass spectrometry (GC-MS) technique (Agilent 7890A Series GC System, USA). Fresh cultures of LB8/99 strain were prepared following the protocol of the double Petri dish assay.

The needle of the SPME device, containing the extraction fiber, coated with 85 microM film (supelco, Bellefonte, PA, USA) was inserted into each plate through a small hole and the fiber was exposed to the gas phase for 20 min at 22℃. The needle of the SPME was then removed from the Petri dish and inserted into the gas chromatograph injector port. Thermal desorbtion of extracted compounds was performed at 250℃ for 2 min and subsequent compound separation was achieved through a 30 x 0.25 mm varian capillary column HP-MS5 (film thickness 0.25 µm) at a flow rate of 1 mL/min with helium as carrier gas. The column temperature was set at 45℃ for 3 min and then programmed from 40° to 300℃ at 20℃/min. The temperature of the injection port and ion source were set at 250℃ and 280℃ respectively; splitless injection mode and electron impact ionization (70 eV) were established. Sampling was performed for 100 hr from the inoculation at different times (48, 52,65,72, 76,89,96 and 100 hr) and VOCs GC peak areas were recorded and considered for their kinetic production profile valuation.

The VOCs were identified considering their mass spectra, their retention time as comparing to reference substances on NIST (National Institute of Standards and Technology) PBM library. Similarly, the VOCs emitted by CADRP28, a resistant *P.expansum* strain were identified.

3.8.6 Effect of pure phenethyl alcohol on mycelium growth and conidial germination of fungal pathogens

The antifungal activity of pure PEA (Sigma Aldrich, St. Louis, MO, USA), recognized by SPME-GC-MS analysis as one of the VOCs produced by LB8/99, was assayed on mycelium growth and conidia germination of target pathogens following the method described above with some modifications. A plug (6 mm diameter) from an actively growing pathogen culture or 100 µL of a conidial suspension were respectively placed or spread in the centre of MEA plates. In each case different aliquots of pure PEA corresponding to 77; 148; 308; 615 and 1230 mg/mL were placed, using a microsyringe, on a paper filter (Whatman No.1, 90 mm diameter), positioned inside the cover. The dishes were quickly closed and sealed with parafilm and incubated at 20°C. After 7 days or 2 days, the dishes were opened and mycelium growth and conidia germination were respectively evaluated. Petri dishes inoculated with pathogens but treated with distilled water in place of PEA were considered as the control. Five replicates per each dose were prepared and the experiment was repeated twice. Mycelium growth (mm) was gauged with ruler. Conidia germination was determined by counting the CFUs developed on MEA. The ED_{50} values of inhibition of fungal colony growth and conidial germination were calculated as reported earlier (see microtiter assay).

3.8.7 Kinetic of production and quantification of phenethyl alcohol

3.8.7.1 Kinetic of production

A method was designed to quantitatively establish the kinetic of production of PEA released by LB8/99 and CADRP28 (a *P. expasnum* strain, chosen randomly from strains of CRIOF-UNIBO collection). One hundred µL drop of a conidial suspension of LB8/99 adjusted to 10^5 conidia/mL was spread on MEA plates that were incubated at 20°C. The fungal PEA production was followed by headspace SPME coupled with GC-FID analysis. and sampling was performed for 100hr at different intervals of time: 48, 52,65,72, 76,89,96 and 100 hr from inoculation.

The SPME sampling system was performed in identical way as described above. An Agilent 7820A gas chromatograph equipped with a flame ionization detector (FID) was used for the chromatographic analysis. Instrument settings were as follows: the injector and detector temperature were set to 250℃ and 300℃ respectively; the oven program started at 40℃ for 3 min and raised to 300 with a rate of 20℃/min; the flow rate of the carrier gas (He) was 1 mL/min and the splitless injection mode was established. Solution of an individual standard (synthetic PEA) purchased from Sigma-Aldrich (Milwaukee, WI) was prepared in the laboratory to correctly identify natural PEA by GC-FID.
The kinetic of production of the naturally released PEA was established by determining the mean value of 5 area measured at each sampling time. Three replications were performed for each quantification assay.

3.8.7.2 Quantification

A calibration curve for the natural produced PEA quantification was established by SPME-GC-FID analysis using synthetic PEA standards. For this aim, five microliters of the synthetic PEA water solutions at different concentrations were injected into closed MEA Petri dishes through a hole made just before injection with a gastight syringe. The final PEA head space concentrations used in the trial were 207, 502 and 1041 µg/mL. Plates were then incubated for 10 min at 20℃ before head space-SPME sampling. Five replications were performed per each concentration in order to reduce variability. The chromatographic data were collected, stored, and processed with excel and a calibration curve was defined by plotting GC-FID peak area versus PEA concentration.

3.8.8 Effect of pure phenethyl alcohol at the concentration naturally produced by LB8/99 strain on conidial germination and mycelium growth

In vitro trials were performed to study the effect of PEA at the real concentration naturally produced by LB8/99 previously calculated from the calibration curve (596 µg/mL of headspace) and at 2 times the natural concentration (1192 µg/mL of headspace) on *B.cinerea, C. acutatum, M.laxa* and *P. expansum*. PEA solutions were obtained diluting

95 µL or 190 µL of pure PEA on 10 mL of sterile distilled water and vortexing for 3 min to homogenize the solution. Two compartment Petri dishes were used; in one compartment a drop of 5 µL PEA solution was put in the center, while the second compartment hosted the target pathogen allowing its exposure to the volatile PEA.

The assessment of mycelium growth and the conidial germination of the target pathogens was carried out as reported previously. Control was represented by target pathogen not exposed to PEA. All Petri dishes were wrapped with one layer of parafilm. The growth of *C. acutatum, M. laxa*, and *P.expansum* was determined after 3 days and for *B. cinerea* after 24 hours of exposure by measuring the diameter of the colony from two orthogonal diameter measurements or by counting the number of CFUs. Five replicate plates were used for each pathogen and the experiment was repeated twice.

3.9. Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) using the statistical package Statistica for Windows (Statsoft Inc.). Separation of means was performed using LSD test at $P<0.05$. All experiments were carried out in a completely randomized block design.

CHAPTER 4

4. RESULTS

During this work, 48 isolates supposedly belonging to the *Penicillium* genus, were obtained from rotted fruit, with typical blue mould symptoms, collected from the orchards and some packinghouses located in the Emilia Romagna (Italy) region. Out of 48, 16 were isolated from apple, 10 from kiwi, 4 from peach, 8 from plum, 2 from apricot and 8 from lemon fruits.

4.1. Identification of the isolates

4.1.1 Morphological identification

Preliminary conventional identification was based on morphological characteristics of the isolates grown on MEA, useful for phenotypic identification of isolates through asexual structures, although species classified in sub-genus *Penicillium* are morphologically similar. Isolates were identified with the help of keys developed by Pitt (1991) and Frisvad and Samson (2004). Colonies of *P. expansum* cultured on MEA plates and incubated at 25℃ for 4 days showed a reverse colour, cream yellow to orange brown to the naked eye. Microscopic observation showed the presence of conidiophores single or in fascicles, appressed with stipes usually smoothwalled, and terverticillate; metulae are more or less cylindrical measuring 10~17 × 3~4 µm; phialids are ampulliform (9~12 × 2~4 µm) and conidia are ellipsoidal to subglobose, smooth-walled with a diameter of 3~3.5 × 2.5~3.0 µm. Colony characteristics and micromorphology of the fungus reported above agreed well with the description of *P. expansum* reported by Frisvad and Samson, (2004)

However, morphological identification remains a time consuming procedure, labor-intensive that often requires mycological expertise and PCR could be a rapid tool for screening the presence of *P. expansum* on fruits.

4.1.2 Molecular identification: polymerase chain reaction

Amplification of genomic DNA with primers ITS1F and ITS4 yields fragments of approximately 500 bp (Fig. 6). Analyses of sequence homologies by Basic Local Alignment Search Tool (BLAST) of the partial sequence obtained after purification and sequencing showed that all the 48 terverticillated *Penicillium* strains recovered from apple, pear, kiwi and apricot were identified as *P. expansum* with sequence similarity ranging from 95 to 100%.

Seven out of eight of the isolates recovered from plum fruits were *P. expansum*, only one was identified as *P. commune.* While all 4 isolates taken from peach fruits were *P.vinaceum* (1 isolate) or *P. commune* (3 isolates) with sequence similarity ranging from 95 to 100%. The isolates recovered from lemon fruits were *P. italicum* and *P.digitatum* as reported in bibliography.

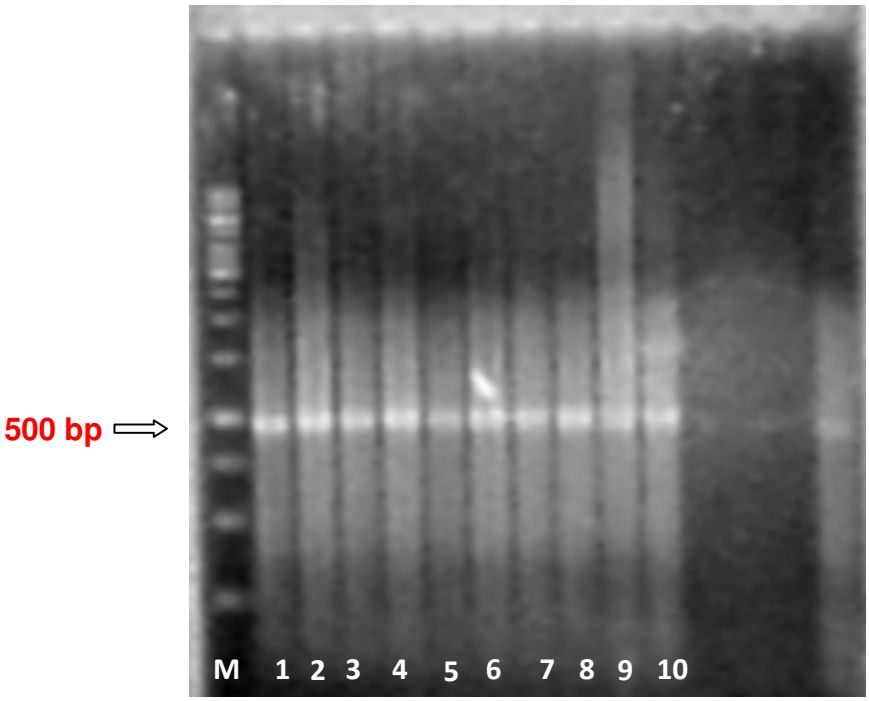


Figure 6 - Agarose gel of ten *Penicillium* spp. strains amplified with primers ITS4 and ITS1F. line M: marker (1kb, fermentas); lanes 1-10 differents *Penicillium* spp isolates loaded as PCR product.

4.2. *In vitro* study of thiabendazole resistance of P. *expansum* strains

4.2.1 Preliminary assays

A screening of fungicide resistance of thirtheen *P. expansum* strains (derived from infected fruit) was performed *in vitro*, following common traditional methods: inhibition of DWM, mycelium growth (colony diameter) and spore germination (CFU) on medium amended with TBZ (400 µg/mL) with respect to control. *P. expansum* strains studied split into two discrete distributions, one sensitive and the other resistant. The strains classified as sensitive (S) did not grow on TBZ amended medium or grew with a significantly lower (P<0.05) rate with respect to the control. Among the resistant strains, those that showed, on TBZ amended medium, a conidial germination or a mycelium growth similar to that observed on non TBZ-amended medium (control) are considered resistant (R); whearas,

strains for which TBZ induces a significantly higher (P<0,05) percentage of conidial germination or mycelium growth are classified as highly resistant (RR).

4.2.1.1 Dry weight mycelium

The DWM of ten *P. expansum* strains was reduced significantly comparing to the control (P<0.05). The percentage of inhibition ranged from 49 % (P10) to 91.9% (P4) and they were consequently classified as sensitive. However, there was no significant difference observed between the DWM produced by strains P6, P12 and P13 in fungicide amended medium and the control. All three strains showed a DWM production of 24 mg, 22 mg and 24 mg respectively similar to that produced by controls (26 mg, 25 mg and 26 mg respectively) (Fig. 7) thus they were considered resistant.

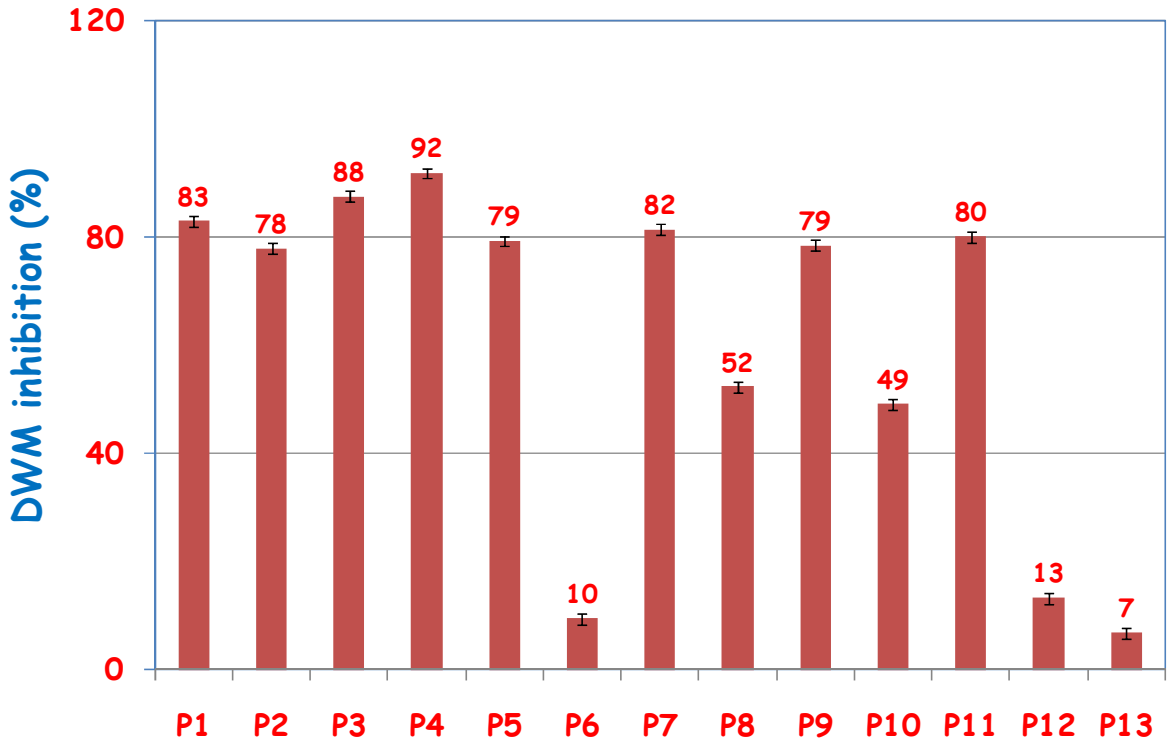


Figure 7- Effect of commercial thiabendazole on dry weight mycelium (DWM) production of thirteen *P. expansum* strains. The inhibiton is expressed as the percentage of reduction of DWM with respect to control. Data on the histogram represent the mean of 3 replicates ± standard errors.

4.2.1.2 Mycelium growth and conidial germination

Commercial TBZ reduced the mycelial growth and conidial germination of 10 (77%) out of 13 *P. expansum* strains. In the absence of fungicide, the colony diameter for *P. expansum* strains on MEA after 3 days at 20°C showed an average of 50 mm and the rate of conidial germination was 99%. In the presence of TBZ (400 µg/mL), the mycelial growth as well as the conidial germination were almost completely inhibited for 9 strains, these strains were classified as sensitive (S). Four strains (P3, P6, P12 and P13), were considered TBZ-resistant (R) since they showed a low mycelial growth inhibiton ranging from 5% (P6) to 31% (P12) with respect to the control (Fig.8). Basing on conidia germination results, two strains P6 and P11 were classified as resistant since they were able to germinate on TBZ-amended medium, while the 2 strains P12 and P13, for which TBZ showed a stimulatory effect on conidial germination were classified as highly resistant (RR) (Fig. 9).

In conclusion, an agreement was noticed between the results of the effect of TBZ on conidial germination and mycelium growth for all 11 *P. expansum* strains screened for sensitivity to TBZ except 2 strains P3 and P11 which behaved differently in confront of TBZ. A little effect on spore germination of P11 strain was observed, while mycelium growth was completely inhibited. In the contrary, P3 showed a significant reduction of conidial germination (99% inhibition) and DWM (88% inhibition) and was considered as a sensitive strain based on these 2 parameters, but a low mycelium growth inhibition of 23% was reported and thus the strain was classified as resistant (R)

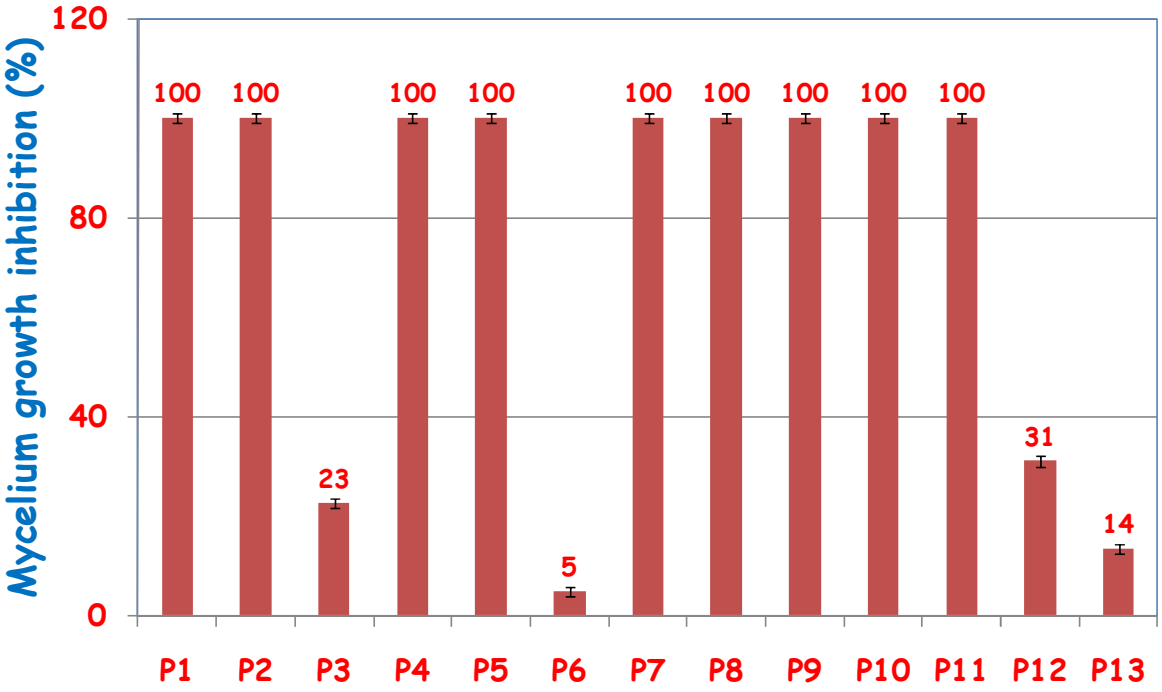


Figure 8 - Effect of commercial thiabendazole on the mycelium growth of thirteen *Penicillium expansum* strains, expressed by the percentage of inhibition of mycelium growth. Data on the histogram represent the mean of 3 replicates ± standard errors.

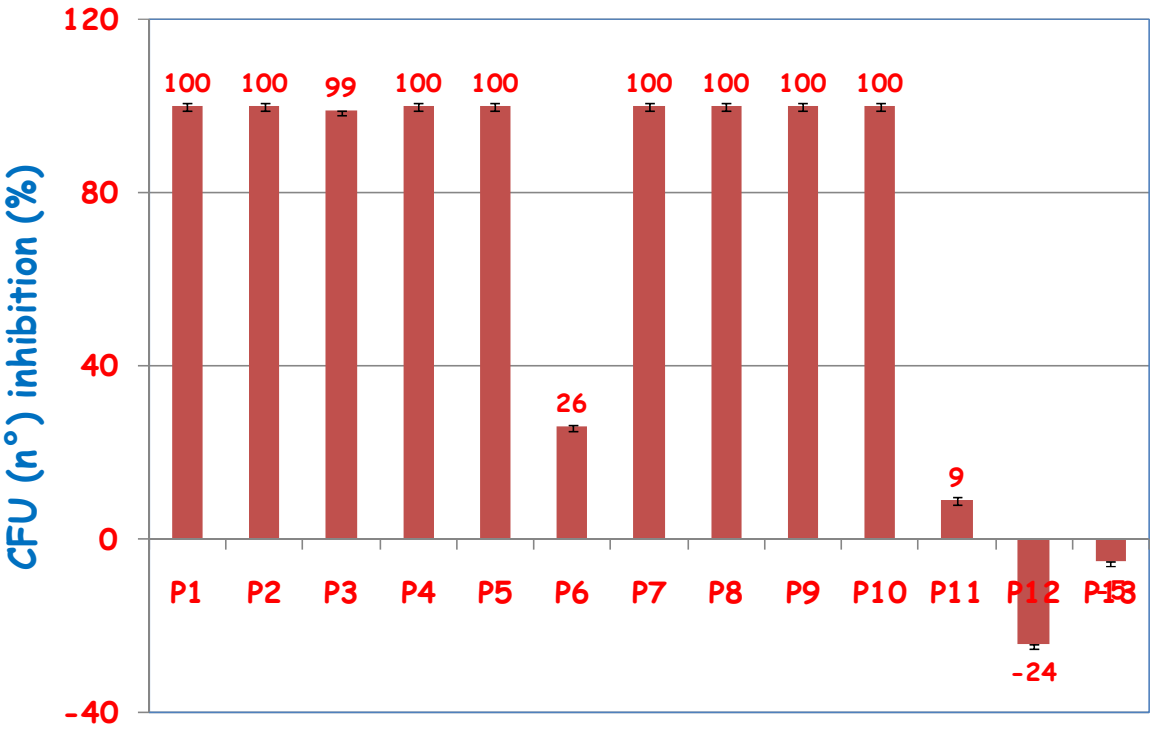


Figure 9 - Effect of commercial thiabendazole on the conidial germination of thirteen *P. expansum* strains expressed by the percentage of inhibition of conidial germination. Data on the histogram represent the mean of 3 replicates ± standard errors.

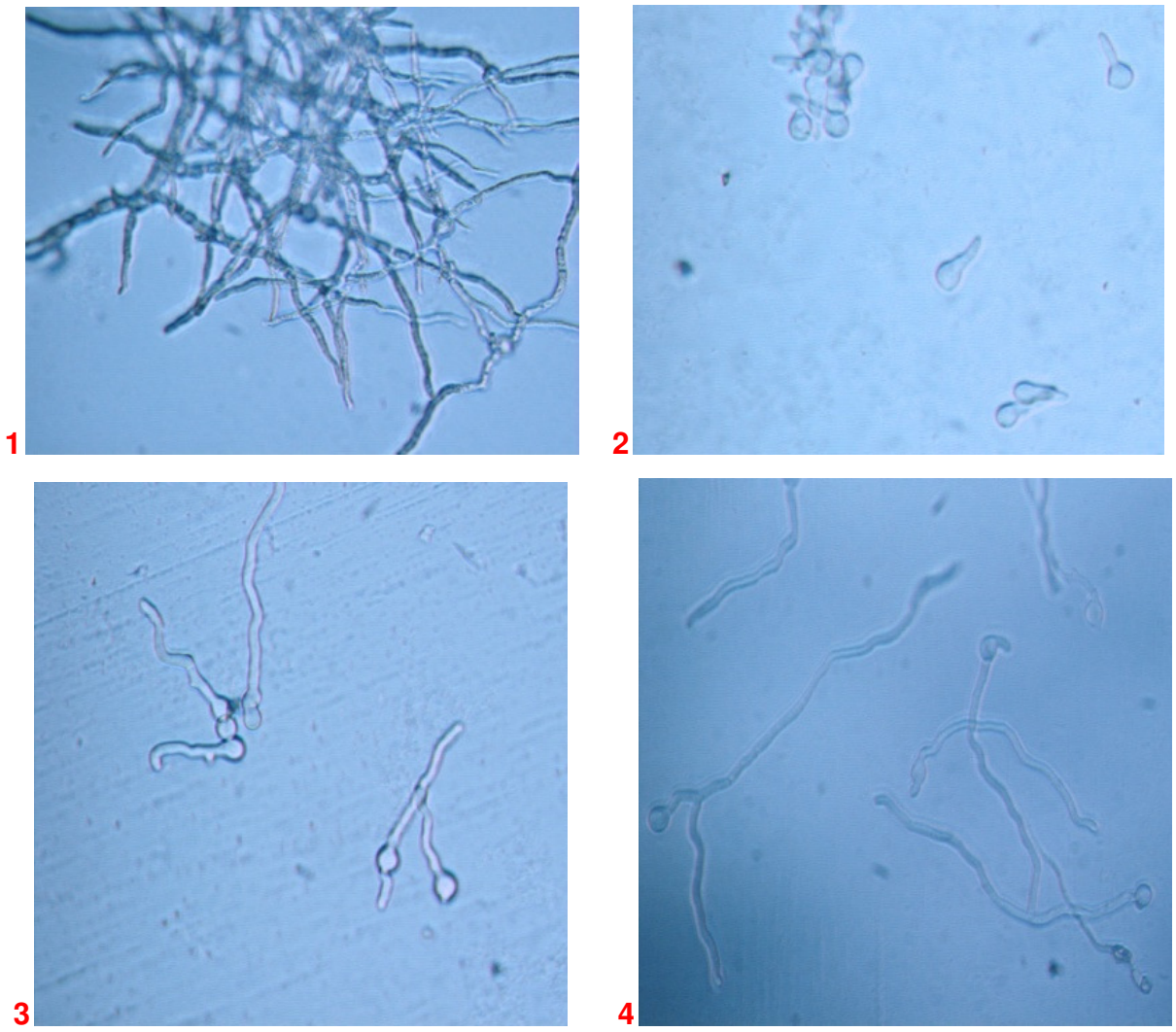


Figure 10 - Influence of commercial thiabendazole (400 µg/mL) on the growth of *P. expansum*. Spores of resistant strain (P13) are able to germinate (3-4) while there was no development of the germination tube for the sensitive one (P3) (2). Picture (1) shows the spore germination of strain P3 on TBZ-non amended medium (Control).

4.2.2 Resistance of *Penicillium* spp. strains to pure and commercial thiabendazole *in vitro*

The resistance to commercial and pure TBZ, was assayed for all 48 *Penicillium* spp. strains previously collected (Table 5). Thirteen strains were found resistant on MEA amended with both commercial or pure TBZ (400 µg/mL) confirming their resistance to commercial product and active ingredient alone (pure). A difference was found in

TBZ-resistant strains: PEN 1, K11 and plum from Argentina strain: on medium amended with commercial TBZ they appeared high resistant (RR) while on medium amended with pure TBZ they were only resistant (R). The contrary happened for strain P14, that showed only resistance (R) when grown on medium amended with commercial TBZ and high resistance (RR) when grown on medium amended with pure TBZ.

Table 5 - Classification of *Penicillium* spp. strains based on their level of susceptibility to commercial and pure thiabendazole

	Strains	Source	Commercial TBZ	Pure TBZ
1	P1	Apple	S	S
2	P2	Apple	S	S
3	P3	Apple	S	S
4	P4	Apple	S	S
5	P5	Apple	S	S
6	P6	Apple	R	R
7	P7	Apple	S	S
8	P8	Apple	S	S
9	P9	Apple	S	S
10	P10	Apple	S	S
11	P11	Apple	S	S
12	P12	Apple	R	R
13	P13	Apple	R	R
14	P14	Apple	R	RR
15	Mela Stark	Apple	S	S
16	PEN1	Apple	RR	R
17	Pear1	Pear	S	S
18	K1	Kiwi	S	S
19	K2	Kiwi	S	S
20	K3	Kiwi	S	S
21	K4	Kiwi	S	S
22	K5	Kiwi	S	S
23	K6	Kiwi	S	S
24	K7	Kiwi	R	R
25	K10	Kiwi	R	R
26	K11	Kiwi	RR	R
27	K12	Kiwi	RR	RR
28	S1	Plum	S	S
29	S2	Plum	S	S
30	S3	Plum	S	S
31	S4	Plum	S	S
32	S5	Plum	S	S
33	S6	Plum	S	S
34	S7	Plum	S	S
35	Plum argentina	Plum	RR	R
36	Agrobiochimica	Plum	S	S
37	1-Albicocca	Apricot	S	S
38	2-Albicocca	Apricot	S	S
39	1-Pesca	Peach	S	S
40	2-Pesca	Peach	S	S
41	3-Pesca	Peach	S	S
42	4-Pesca	Peach	R	R
43	L1	Lemon	S	S
44	L2	Lemon	S	S
45	L3 (B)	Lemon	S	S
46	L4 (B)	Lemon	RR	RR
47	L4 (V)	Lemon	RR	RR
48	L6	Lemon	S	S

4.2.3 Dose-response curves of *P.* expansum strains to thiabendazole: microtiter assay

Ten resistant strains of *P. expansum* were tested in microtiter assays in order to determine the ED_{50} and ED_{95} values. The photometric measurement (620 nm) of the growth demonstrated that all resistant strains grew in MEB diluted at 50% and were able to germinate at high concentrations of pure TBZ.

The regression rates elaborated between the logarithm of TBZ concentration and the percentage of inhibition of conidial germination (efficacy index) transformed in probit, used for the determination of ED_{50} and ED_{95}, are significant ($P \leq 0.05$), the coefficients of correlation ranged from 0,90 (K7) to 0,98 (Plum Argentina) (Table. 6).

First inhibiting effects of TBZ were observed with 6.25–12.5 µg/mL. The minimum inhibitory concentrations (MIC: concentration with the 100% inhibition) of TBZ in *P. expansum* were superior to 1000 µg/mL; they ranged between 1200 and 20000 µg/mL. Microscopical observations of conidial germination showed that the spores were able to germinate even at the highest concentration tested (50 µg/mL) but the germ tube elongation was completely stopped . For all samples, the ED_{50}-values ranged from 54 (PEN1) to 320 µg/mL (P6) and the ED_{95}-values from 410 (P12) to 733 µg/mL (PEN1). There were no significant differences on the ED_{50} values found between strains derived from different host as presented in Fig.11, showing the distribution of the ED_{50}- and ED_{95}-values. The averages of ED_{50} values for apple-strains were similar to those from kiwi and plum; However, a significant difference was reported for ED_{95} values since those of the strains deriving from apple-showed the highest value comparing to strains isolated from the other commodities. No false positive strains were identified.

We have noticed that the ED_{95} values of *Penicillium vinaceum* strains from Peach (766 µg/mL) were significantly higher ($P<0.05$) with respect to those of *P. expansum* strains from apple, Kiwi and Plum (Data not shown).

Table 6 - ED_{50}-values [µg/mL] and ED_{95}-values [µg/mL] of *P. expansum* strains from different commodities to pure thiabendazole.

Strains	Level of resistance	ED_{50}	ED_{95}	MIC	Regression curve	Coefficient of correlation
P6	R	320	710	7200	Y=9,03+2,07X	0,96
P12	R	170	410	1200	Y=12,7+4,36X	0,90
P13	R	134	690	5600	Y=9,3+2,29X	0,96
P14	RR	132	670	5300	Y=9,4+2,32X	0,97
K7	R	174	418	1300	Y=12,6+4,34X	0,90
K10	R	177	422	1300	Y=12,7+4,38X	0,92
K11	R	177	442	1400	Y=12,3+4,16X	0,91
K12	RR	112	467	2800	Y=10,2+2,64X	0,94
PEN1	R	54	733	20000	Y=8,3+1,44X	0,95
Plum Argentina	R	204	537	1800	Y=11,6+3,89X	0,98

X: Dose of thiabendazole (µg/mL)

Y: Probit of the percentage of inhibition of conidial germination

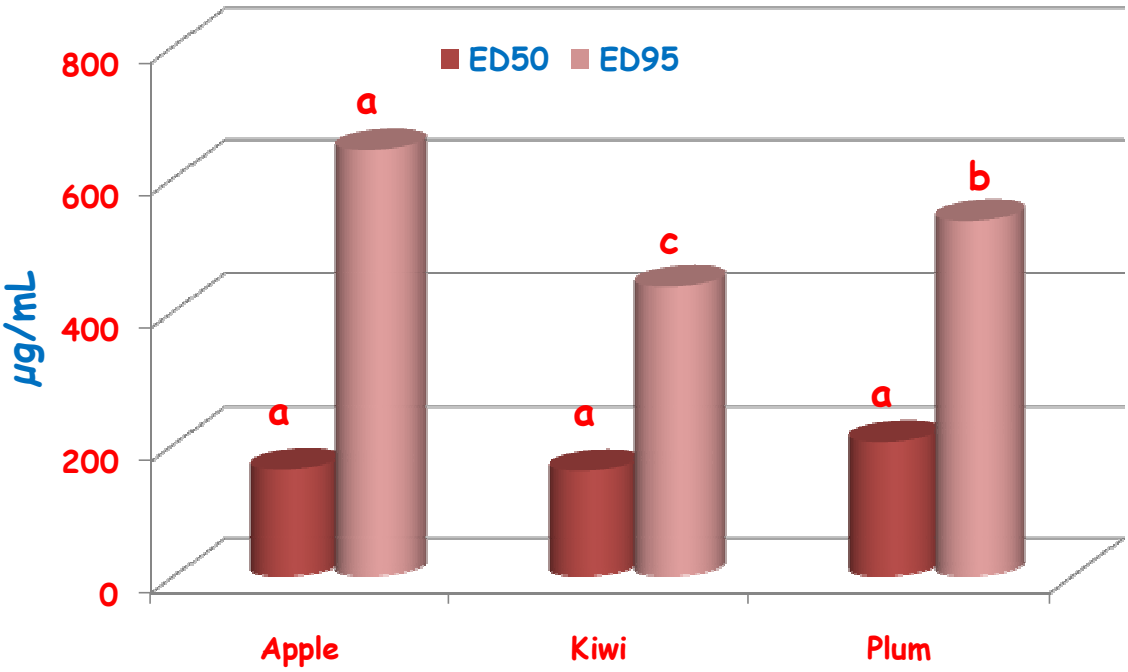


Figure 11 - Sensitivity of *P. expansum* to thiabendazole. The diagram shows the distribution of the ED_{50} and ED_{95}-values (µg/mL) of ten strains of *P. expansum* for each comodity with the described microtiter assay

4.2.4 Mutation of β-tubulin gene in *P. expansum* strains

PCR amplification of the genomic DNA of 10 *P. expansum* strains using specific primers corresponding to a β-tubulin gene sequence of 940 base pairs (bp), generated a unique fragment of 500 bp (Fig.12). The β-tubulin gene sequences from all the representative sensitive and resistant strains (3 and 7, respectively) shared 99 to 100% sequence similarities, with respec to *P.expansum* type strain FJ012871.1.

Based on homologous protein sequences of *P. expansum* β-tubulin proteins, the predicted β-tubulin amino acid sequences consisted of 191 aminoacids, from residue 167 to residue 357. The comparison of the deduced amino acid sequences of all 10 *P. expansum* strains with *P. expansum* type strain (Fig.13) revealed that one (P3) out of 3 ensitive *P.expansum* strains had a single point mutation at codon 167, with Phe changed

to leu. Four strains (P6-P12-K11-K12) were mutated at codons 198 (Glu to val) and 240 (Leu to Phe) while P14 strain had mutation only at codon 240 (Leu to Phe). A unique exception is the resistant strain P13 in which the mutation at residue Leu 250 to Phe was detected. Of the seven strains classified as resistant to TBZ, only one strain Plum Argentina had no mutations in the sequenced region.

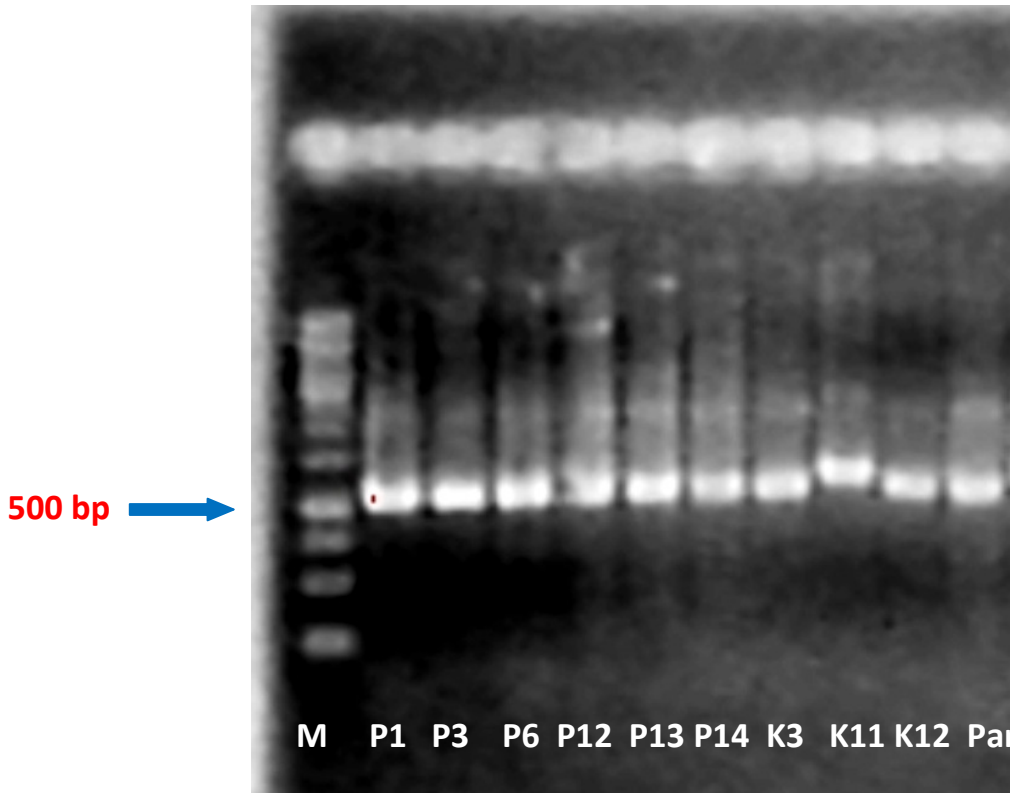
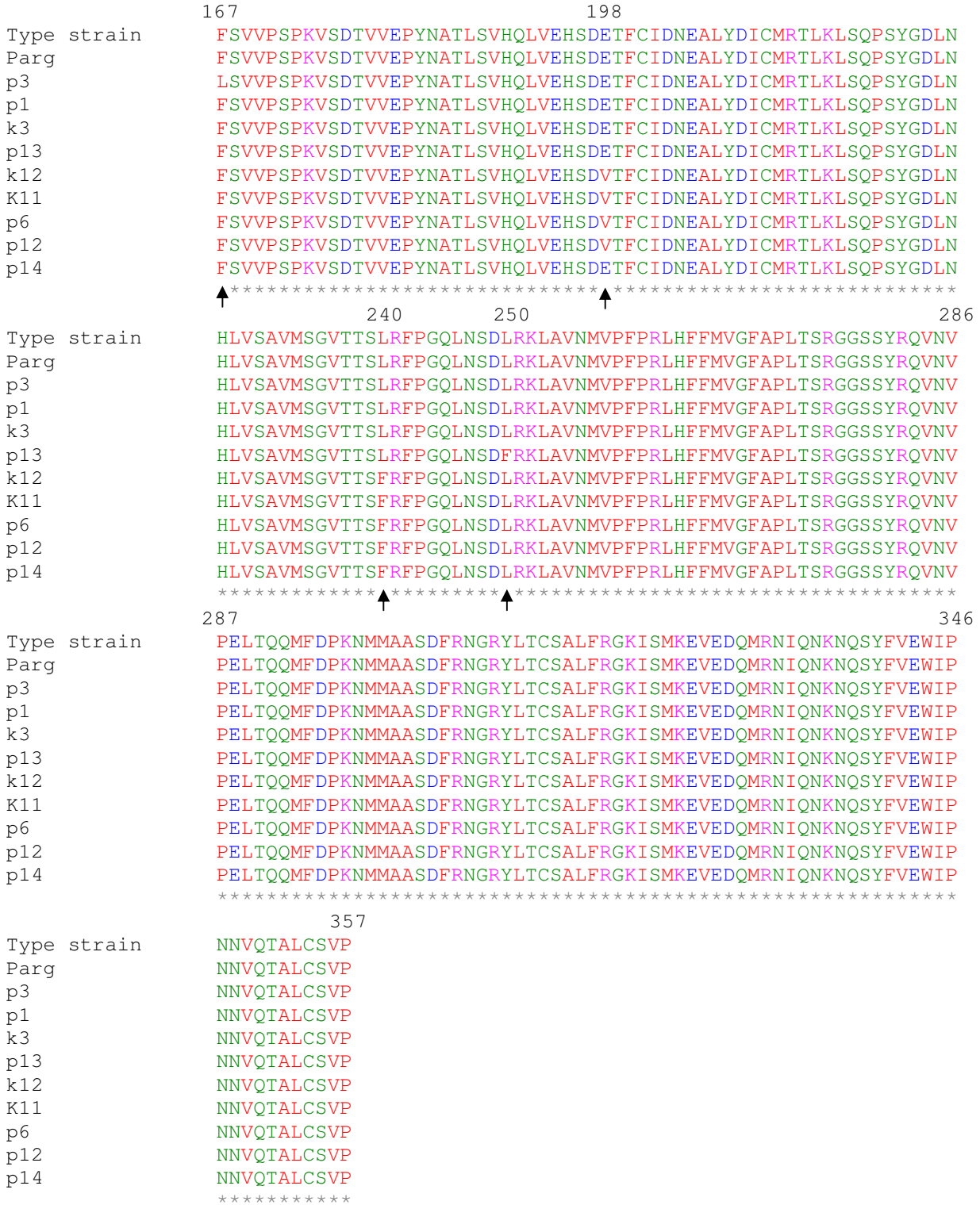


Figure 12 - PCR colony corresponding to the cloned β-tubulin gene of 10 *P. expansum* strains. Lane1: marker (1kb, fermentas); Lanes 2 -11: *P. expansum* strains. P1, P3 and K3 are TBZ-sensitive strains (S) while P6, P12,P13, K11, K12 and Plum Argentina (Par) are TBZ-resistant strains.


167 198
Type strain FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
Parg FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
p3 LSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
p1 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
k3 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
p13 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
k12 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDVTFCIDNEALYDICMRTLKLSQPSYGDLN
K11 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDVTFCIDNEALYDICMRTLKLSQPSYGDLN
p6 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDVTFCIDNEALYDICMRTLKLSQPSYGDLN
p12 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDVTFCIDNEALYDICMRTLKLSQPSYGDLN
p14 FSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETFCIDNEALYDICMRTLKLSQPSYGDLN
240 250 286
Type strain HLVSAVMSGVTTSLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
Parg HLVSAVMSGVTTSLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
p3 HLVSAVMSGVTTSLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
p1 HLVSAVMSGVTTSLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
k3 HLVSAVMSGVTTSLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
p13 HLVSAVMSGVTTSLRFPGQLNSDFRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
k12 HLVSAVMSGVTTSFRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
K11 HLVSAVMSGVTTSFRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
p6 HLVSAVMSGVTTSFRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
p12 HLVSAVMSGVTTSFRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGGSSYRQVNV
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287 346
Type strain PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
Parg PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p3 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p1 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
k3 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p13 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
k12 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
K11 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p6 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p12 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
p14 PELTQQMFDPKNMMAASDFRNGRYLTCSALFRGKISMKEVEDQMRNIQNKNQSYFVEWIP
357
Type strain NNVQTALCSVP
Parg NNVQTALCSVP
p3 NNVQTALCSVP
p1 NNVQTALCSVP
k3 NNVQTALCSVP
p13 NNVQTALCSVP
k12 NNVQTALCSVP
K11 NNVQTALCSVP
p6 NNVQTALCSVP
p12 NNVQTALCSVP
p14 NNVQTALCSVP

Figure 13 - multiple sequence alignment of the β-tubulin protein (residue 167-357) from 10 *P. expansum* resistant and sensible strains and *Penicillium expansum* type strain (FJ012871.1). Mutated residues are indicated with an arrow.

4.3. Influence of ultra low oxygen conditions on *P. expansum* strains

The results of the effect of ULO conditions, generally used for fruit storage, showed that the storage in atmosphere with a reducted oxygen levels can not be considered an effective alternative means for blue mould control.

Data of the assessment of the mycelium growth on MEA plates inoculated with *P. expansum* strains and stored at low oxygen level for 1 month showed that there was no significant difference between the colony diameter developed under low oxygen levels (2%, 1%, 0.5%) (22; 23; 20 mm respectively) and the control (21%) (21 mm) as illustrated on table 7(A), except for strain ISCI12 and EMFP6 which mycelium growth at 1% O_2 (23 mm and 28 mm respectively) was significantly (P<0.05) higher than that observed at 21% O_2 level (20 mm). The same behaviour was observed on conidia germination after 30 days of storage at ULO conditions. Rating of conidia germination (CFU/plate) of *P. expansum* strains was 73%, 73% and 84% under the 3 different atmospheres composition: 0,5%, 1% and 2% O_2 respectively, similarly to control 21% O_2 (73%) (Table. 7B). Only ISCI12 strain showed a percentage of spore germination of 96% at 2% O_2 level, significantly greater than those observed at 21%, 1% and 0.5% of O_2 respectively, 75%; 83% and 70%.

We have noticed also that both conidial germination and mycelium growth of *P.expansum* strains were completely inhibited during the two first weeks of storage., which can be due probably to the effect of the low temperature (0°C).

Table 7A - Effect of ultra low oxygen conditions on the mycelium growth of *P. expansum* strains at 0°C expressed as the colony diameter (mm). Within each strain, different letters represent a significant difference between the different O_2 levels according to LSD test ($P<0.05$)

Strains	Oxygen levels (%)			
	21	2	1	0.5
P6	20a	23a	19a	23a
P4	18a	19a	22a	17a
ISCI12	20b	20b	23a	17c
P32	23a	23a	24a	19a
CADRP28	21a	23a	20a	24a
EMFP6	20c	23b	28a	21bc

Table 7B - Effect of ultra low oxygen conditions on the conidial germination of *P. expansum* strains after 30 days at 0°C expressed by the number of colony forming units (cfu/plate). Within each strain, different letters represent a significant difference between the different O_2 levels according to LSD test ($P<0.05$)

Strains	Oxygen levels (%)			
	21	2	1	0.5
P6	62a	59a	60a	56a
P4	33a	35a	34a	35a
ISCI12	75b	96a	83ab	70b
P32	91a	129a	100a	89a
CADRP28	91a	86a	78a	77a
EMFP6	89a	102a	84a	108a

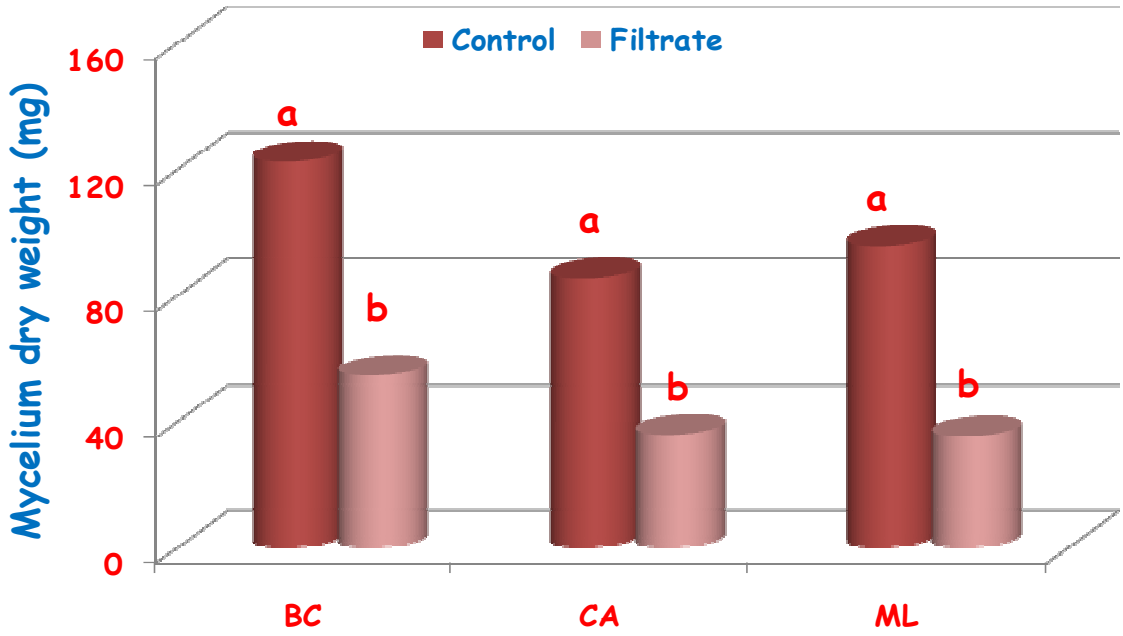
4.4. The antifungal effect of the LB8/99 P. *expansum* strain *in vitro* and *in vivo*

4.4.1 In vitro fungitoxicity assays

4.4.1.1 Mycelium dry weight

The fungicidal activity of secondary metabolites produces by LB8/99 strain in culture filtrate was assayed on DWM of some fungal pathogens. All tested pathogens showed a significant decrease of DWM when grown in sterile cultural filtrate of LB8/99 with respect to the control. The highest growth inhibiton was observed in *P. expansum* strains (-75,5%) (Fig.14B) followed by *M. laxa* (-63%), *C. acutatum* (-58%) and *B. cinerea* (-56%) (Fig. 14A).

A)



B)

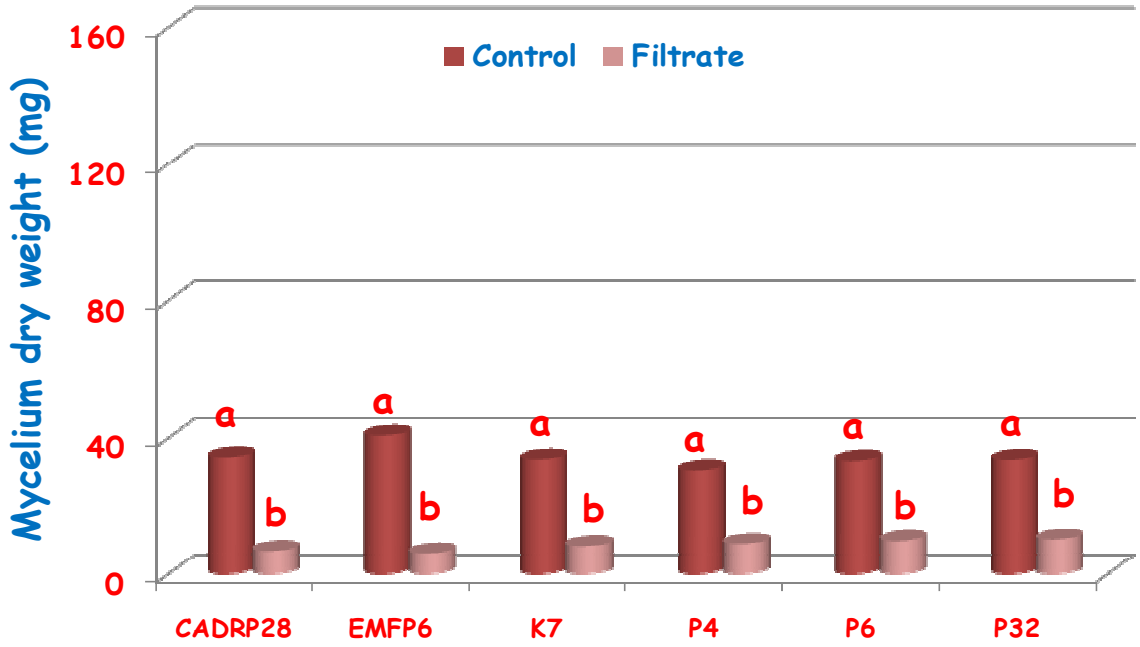


Figure 14 - Effect of LB8/99 strain filtrate on the growth of an isolate of *B. cinerea* (BC), *C. acutatum* (CA), *M. laxa* (ML) (Fig.13A) and six *P. expansum* strains (PE) (Fig.13B). Data in columns represent the mean of 3 replicates, the experiment was repeated twice. Within each strains, different letters represent a significant difference according to DMS test (P<0.05).

4.4.1.2 Conidial germination

The conidia germination of the six *P.expansum* strains treated with sterile culture filtrate was not inhibited, rather in some cases (K7, P4, P6 and P32) was stimulated (Fig.15). Microscopic observations of germinated conidia revealed a consistent increase of the length of the germ tube in all tested strains comparing to the control (Fig. 16 A-B). The germ tubes of the germinated conidia were 4 times longer than those of the control for P4; 2 times for P6, K7 and P32 and 1 time for CADRP28 and EMFP6 (data not shown). However, the treated germ tube appeared to be abnormal comparing to the one grown on MEB (control). It was more branched and showed the absence of some fragments (more septate) which can explain the reduction of the mycelium dry weight reported previously (Fig. 16B).

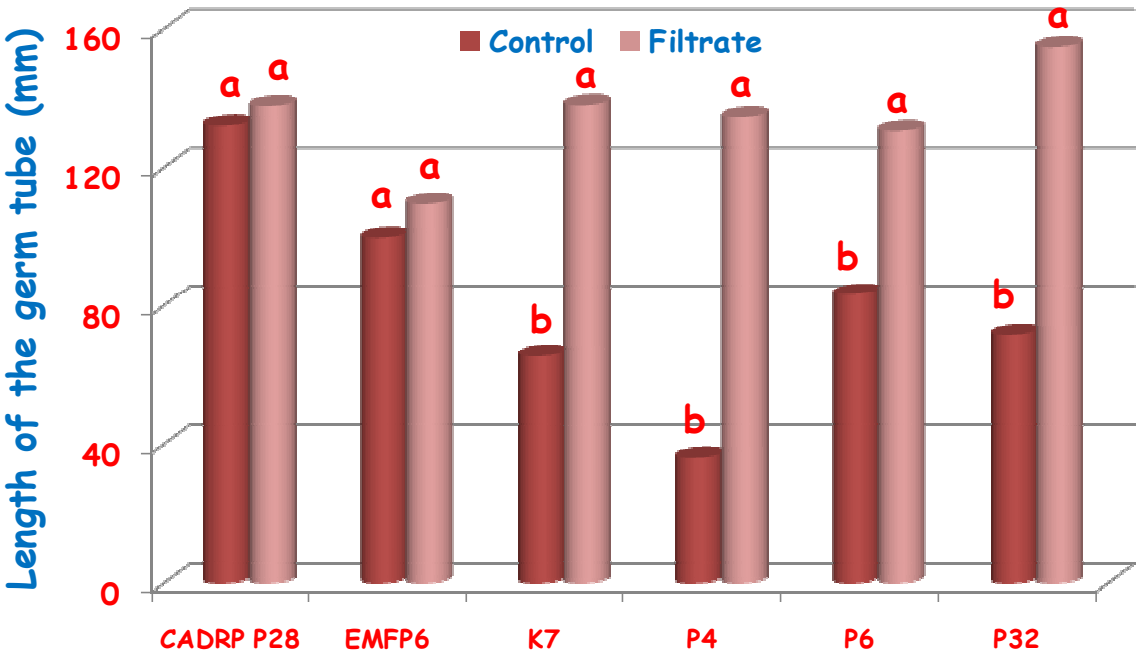


Figure 15 - Effect of LB8/99 strain filtrate on the conidial germination of six *Penicillium expansum* strains. Data in columns represent the mean of 3 replicates, the experiment was repeated twice. Within each strain, different letters represent a significant difference according to DMS test (P<0.05)

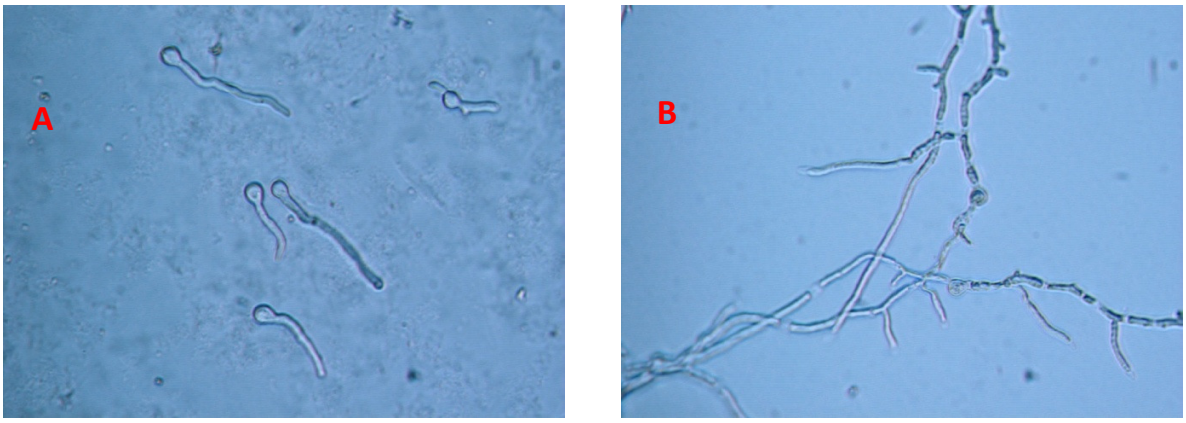
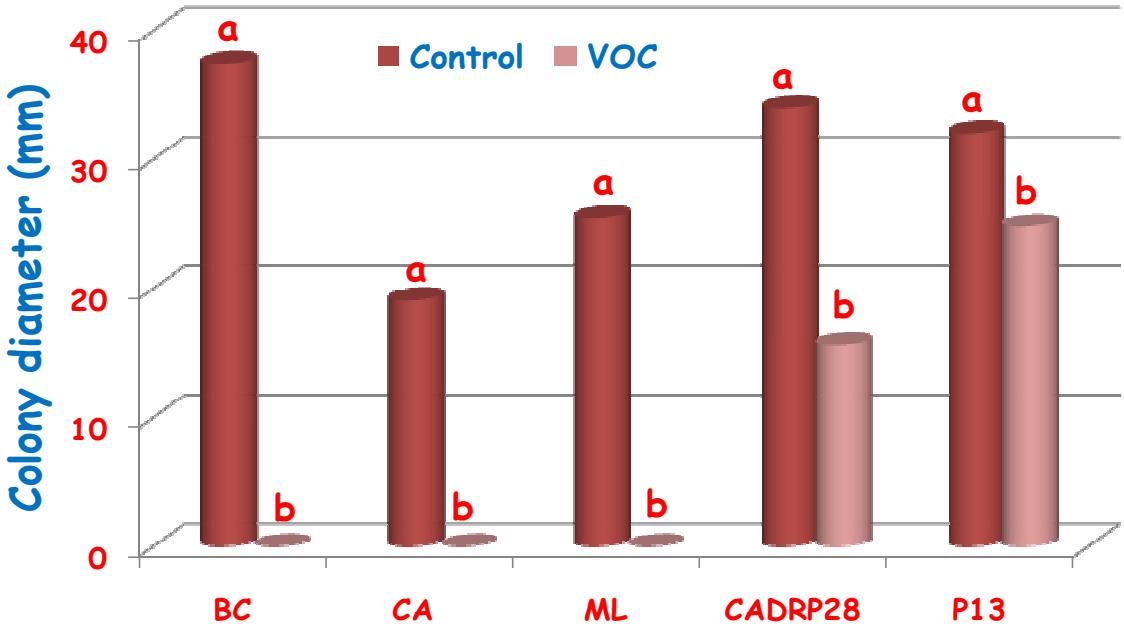


Figure 16- Effect of LB8/99 strain filtrate on *P. expansum* conidial germination after 12h incubation. (A) normal germ tubes from a culture on MEB; (B) abnormal germ tube from a culture on LB8/99 filtrate showing branched mycelium and the formation of empty segments.

4.4.1.3 Double Petri dish assay

In the double Petri dish assay, where there was not either physical contact between LB8/99 strain and pathogens or fungal diffusion through the culture medium, the antifungal effects observed on mycelium growth and conidia germination, could be attributed to the production of VOCs generated by LB8/99. The VOCs produced by LB8/99 strain inhibited completely the mycelium growth of *B. cinerea*, *C. acutatum*, and *M. laxa*, while in the case of *P. expansum* strains (P13 and CADRP28), the inhibition was of 69,7% and 46% respectively (Fig.17A). These data were also confirmed by results obtained from inhibition of conidial germination; in this case no germination was observed in *B. cinerea*, *C. acutatum* and *M. laxa* conidia exposed to VOCs of LB8/99 strain, after 3 days of incubation (Fig.17B). While the conidia germination of *P. expansum* strains was inhibited only by 18,1% and 32 %, however the reduction was significant with respect to the control.

A)



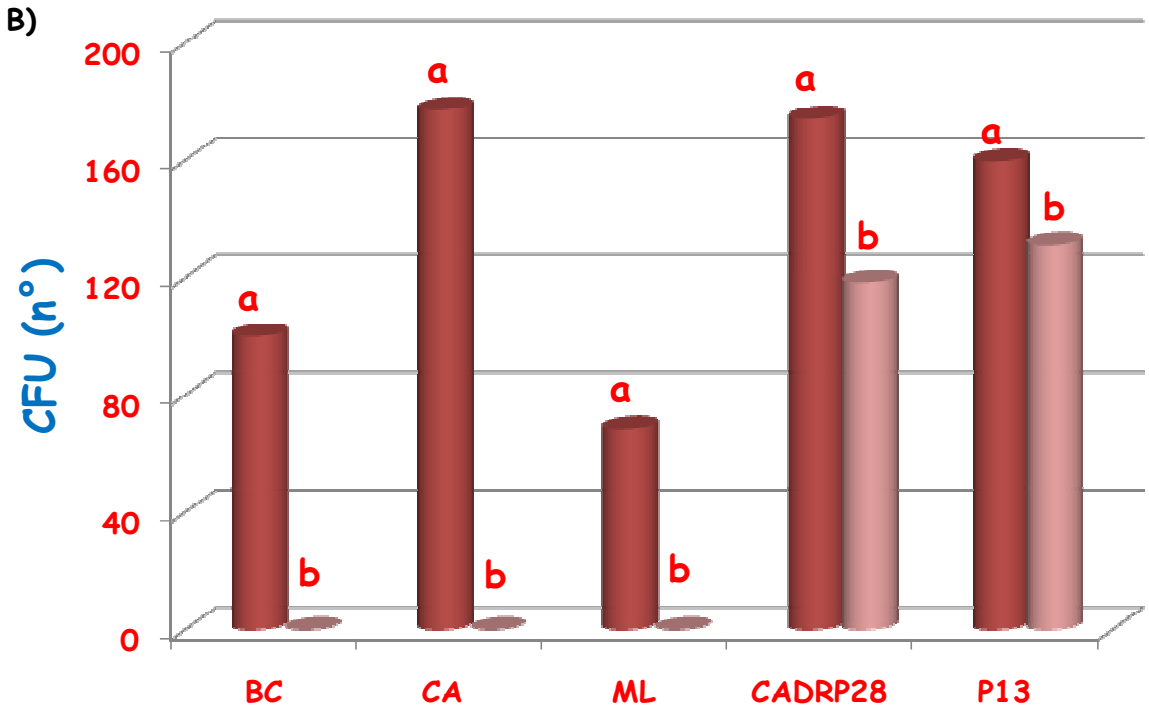


Figure 17- Effect of volatile organic compounds (VOCs) produced by LB8/99 strain on the growth of *B. cinerea* (BC), *C. acutatum* (CA), *M. laxa* (ML) and two *P. expansum* strains (CADRP28 and P13). A) Colony diameter was determined after 4 days of incubation at 20°C. B) colony forming unit was determined after 3 days at 20°C. Data in columns represent the mean of 5 replicates, the experiment was repeated twice. Within each pathogen different letters represent a significant difference according to LSD test (P<0.05).

Following the detection of the antifungal effect of the VOCs produced by LB8/99, further preliminary assays were performed against other fungal pathogens in order to determine the spectrum of activity of the volatile compounds.

After two days of incubation at 25°C, mycelium growth of *F. culmurum, F.graminearum* and *F. poae* cultured on MEA plates and exposed to VOCs emitted by LB8/99 strain was significantly reduced compared to the control (P≤0.05) with a percentage reduction of 54.6%, 32.7% and 56,2% respectively. Hence, conidial germination of *Aspergillus* spp. and mycelium growth of *A. alternata, Cladosporium* spp. and *Phialophora* spp. were completely inhibited (Table. 8).

Table 8 - Effect of volatile organic compounds (VOCs) produced by LB8/99 strain on the mycelium growth of *A. alternata*, *F. culmurum*, *F. graminearum*, *F. poae* and *Phialophora* spp. and conidial germination of *Aspergillus* spp. and *Cladosporium* spp.. Data represent the mean of 5 replicates, the experiment was repeated once. Within each pathogen different letters represent a significant difference according to LSD test (P<0.05).

Pathogens	Colony diameter (mm)		Conidial germination	
	Control	VOCs	Control	VOCs
A. alternata	24a	0b	nd	nd
Aspergillus spp.	nd	nd	151a	0b
Cladosporium spp.	nd	nd	126a	0b
F. culmurum	44a	20b	nd	nd
F.graminearum	42a	28b	nd	nd
F. poae	43a	19b	nd	nd
Phialophora spp.	14a	0b	nd	nd

4.4.2 *In vivo* interaction between LB8/99 and P13 *P. expansum* strains

After three days of incubation at 20°C, no significant differences were found between the lesion diameter (disease severity) of fruit inoculated with a mixture of LB8/99+P13, or with LB8/99, and P13 alone (Fig.18A)

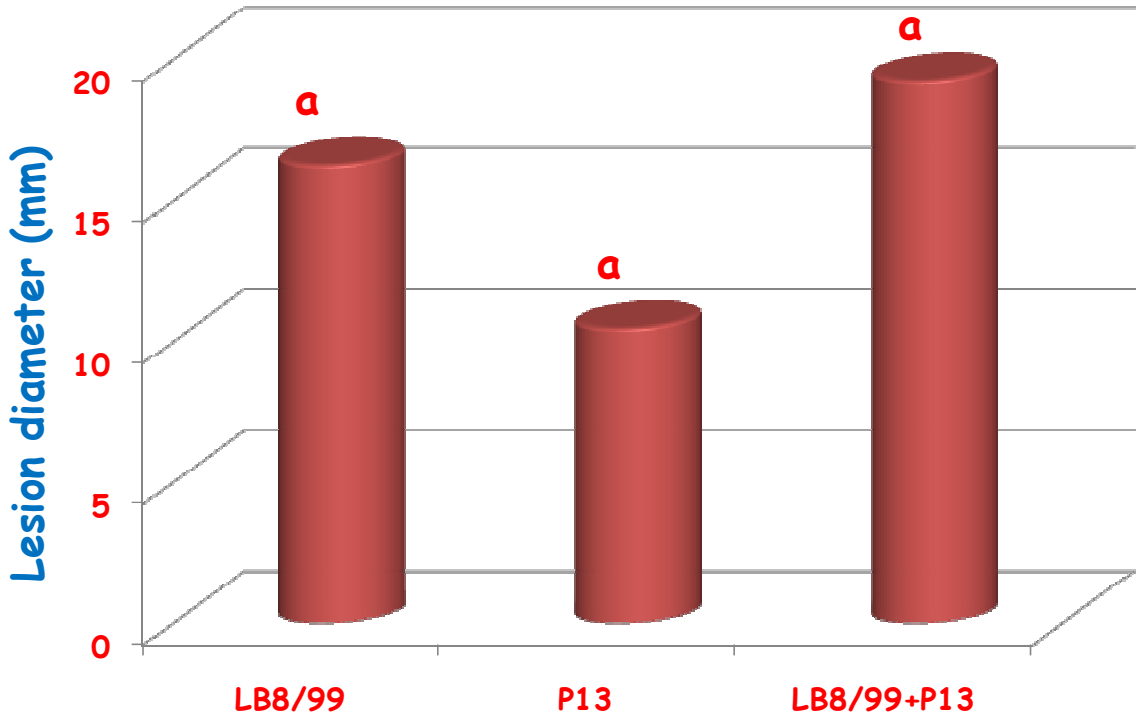


Figure 18A- Interaction between two *P. expansum* strains, LB8/99 and P13, on apple. The histogram shows the lesion diameter (mm) after 3 days of incubation at 20°C on apple inoculated with conidial suspension of LB8/99, P13 or LB8/99+P13. LB8/99 is TBZ-sensitive and P13 is TBZ-resistant. Different letters represent a significant difference according to LSD test (P<0.05).

However in the *in vitro* screening assay carried out on MEA plates amended with TBZ and inoculated with small pieces of tissue taken from blue mould present on lesions of fruit inoculated with the mixture of LB8/99 + P13 strains, revealed that the LB8/99 (TBZ-sensitive) was the strain responsible of the decay, indeed, no fungal growth was observed on TBZ amended medium (Fig.18C). Similarly, no colonies grew on MEA amended with fungicide and inoculated with conidial suspension prepared from blue mould present on lesions of fruit inoculated with LB8/99 strain. While those prepared from lesion of apple inoculated with strain P13 (TBZ-resistant) developed on MEA amended with fungicide (Fig. 18B).

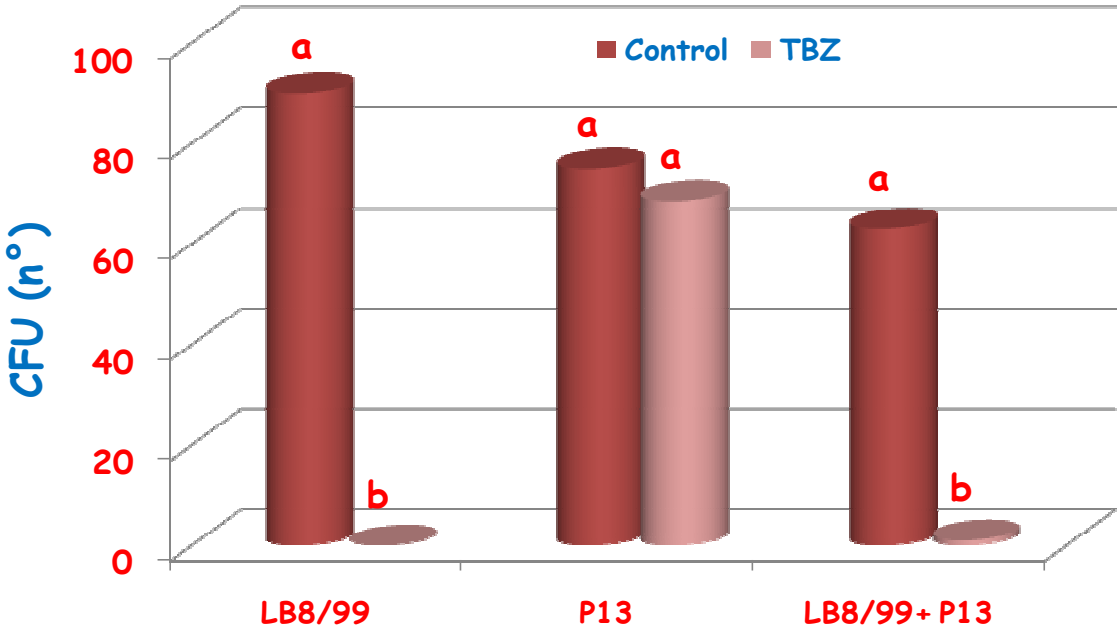


Figure 18B - Interaction between two *P. expansum* strains, LB8/99 + P13, on apple. The histogram shows the colony forming unit developed on malt extract agar amended with TBZ (400 µg/mL) or not (Control) and inoculated with conidial suspension prepared from blue mould present on lesions of apple fruit previously inoculated with LB8/99, P13 or LB8/99+P13. LB8/99 is TBZ-sensitive and P13 TBZ-resistant. Different letters represent a significant difference according to LSD test (P<0.05).

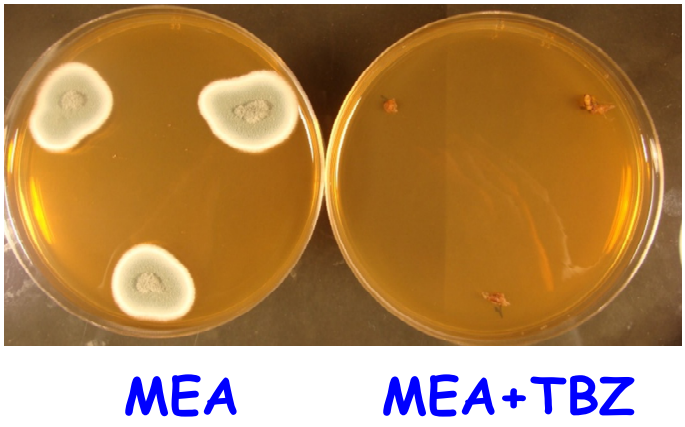


Figure 18C - Malt extract agar plates amended with TBZ (400 µg/mL) and inoculated with small pieces of tissue taken from lesions of fruit inoculated with the mixture of LB8/99 + P13 strains

4.4.3 Preliminary extraction and identification of volatile substances

A qualitative analysis was performed in order to identify the VOCs with antifungal activity produced by LB8/99. SPME coupled with GC-MS analysis permitted the preliminary investigation of the gas phase in the double Petri dish test previously performed. Results showed the presence of numerous compounds probably derived from LB8/99 culture grown on MEA plates (Fig.19). During four days of incubation at 20°C, the most commonly produced VOCs were alkanes, aldehydes, ethers, terpenes and terpene derivatives, alcohols, esters, ketones, and sulfur compounds. The three most representative VOCs were: geosmin (1, 10-dimethyl-trans-9-decalol) and phenethyl alcohol (PEA) as the major terpenoid and alcohol respectively, and a third unknown substance recognized as 1, 6, 10-Dodecatriene, 7, 11, dimethyl-3-methylene with a 74% match factor (Table. 9).

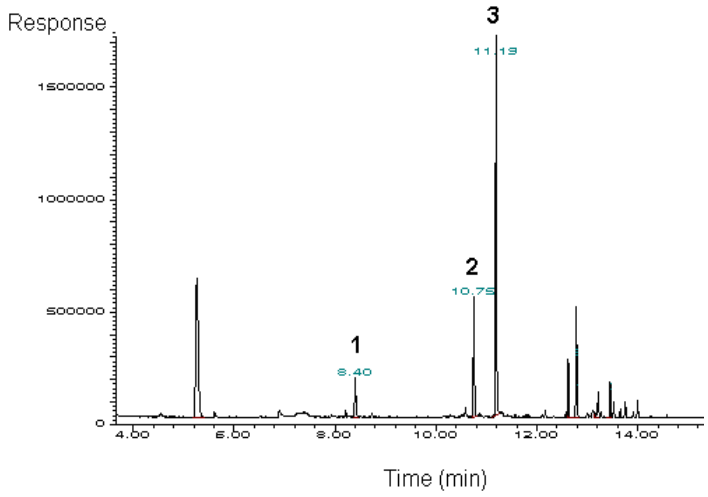


Figure 19- Chromatogram corresponding to volatile organic compounds produced by *P. expansum* strain LB8/99 grown on MEA. Labeled peaks are the most representative compounds found in the headspace of fungal culture samples by HS-SPME-GC-MS. (1) Phenethyl alcohol; (2) Geosmin; (3) unknown substance

Table 9- Identification of volatile organic coumpounds released by *P. expansum* strain LB8/99 grown on MEA at 20°C using HS-SPME-GC-MS.

VOCs	Retention time (min)
PEA	8,4
Geosmin	10,75
Unknown substance	11,19

The dynamics of production of these compounds was followed for 4 days incubation at 20°C of LB8/99 strain cultured on MEA (Fig. 20). The production of both geosmin and PEA was detected after 2 DPI. The emission of geosmin was rapid initially, starting at 44 hr PI, remained relatively stable over the following 48 hr –period, then declined rapidly. The production of the latter was favored by the conidial germination of the fungal pathogen. The emission of PEA seemed to be also favored by fungal sporulation, peaking at 72 hr (3 DPI) and declining fast thereafter to an undetectable level. The release of the unidentified substance appeared later, after 67 hr PI, when mycelium starts to be covered with typical blue conidia of *P. expansum,* and kept increasing slowly and constantly until 96 hours before completely disappear at 100 hr.

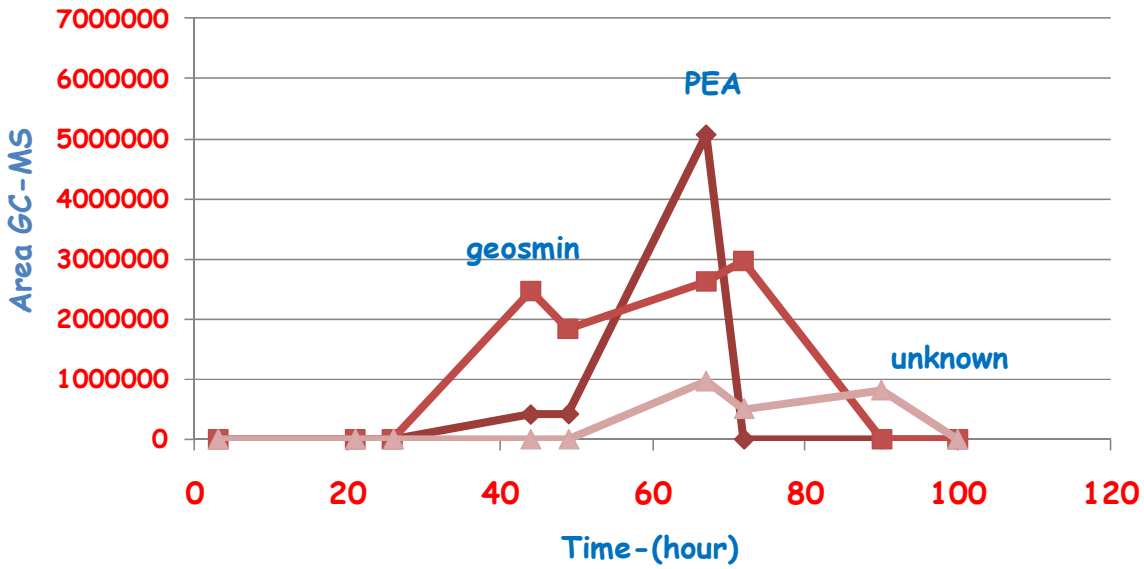


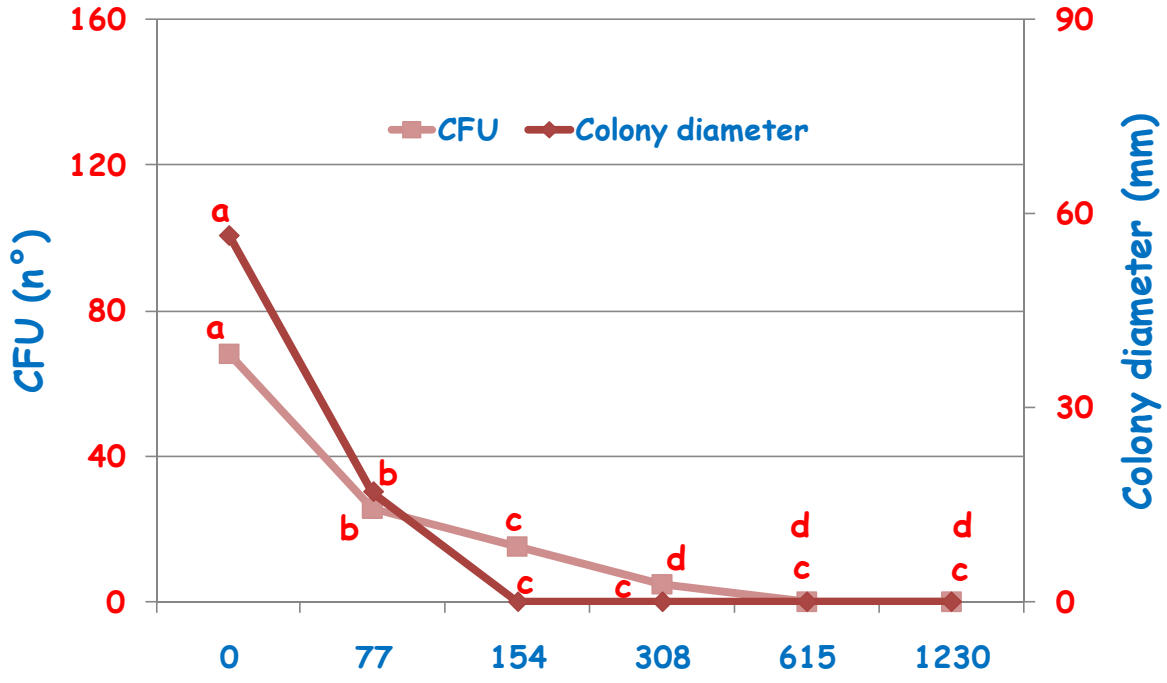
Figure 20 - Kinetic of production of the most representative volatile organic compounds produced by *P. expansum* strain LB8/99 grown on MEA. Labelled curves corresponds to the average hourly area of each compound measured using HS-SPME-GC-MS.

CADRP28, a *P. expansum* strain has also been found to produce the same VOCs as LB8/99, however in the case of CADRP28, VOCs emission was slower in time. The reason for that probably is found in the different behavior of the two fungi: LB8/99 proved to have a rapid and abundant growth and sporulation compared to CADRP28 and consequently, much VOCs were reported after the same time of incubation (Data not shown).

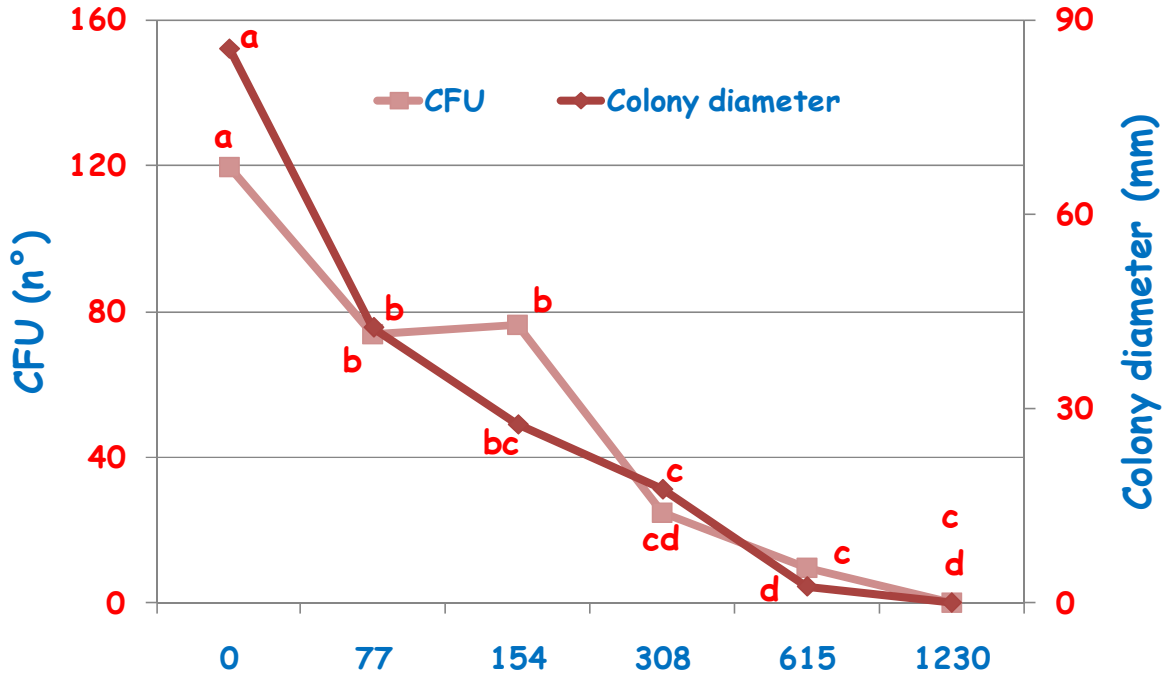
4.4.4 Effect of pure phenethyl alcohol on mycelium growth and conidial germination of target pathogens

The effects of pure PEA were tested on conidia germination and mycelium growth of target pathogens by biofumigation assay. The highest concentration of PEA (1230 mg/mL of headspace) inhibited completely both conidia germination and mycelium growth of all pathogens, except for conidial germination of *P. expansum* strain, that was reduced by 90% with respect to untreated control (Fig. 21 A,B,C,D). Mycelium was more sensitive to PEA than conidia; at the lowest tested concentration (77 mg/mL of headspace), mycelium of *M. laxa* was completely inhibited, while mycelium growth of *C. acutatum* and *B. cinerea* was reduced more than 60% with respect to control, only the mycelium of *P. expansum* showed a low reduction (33%).

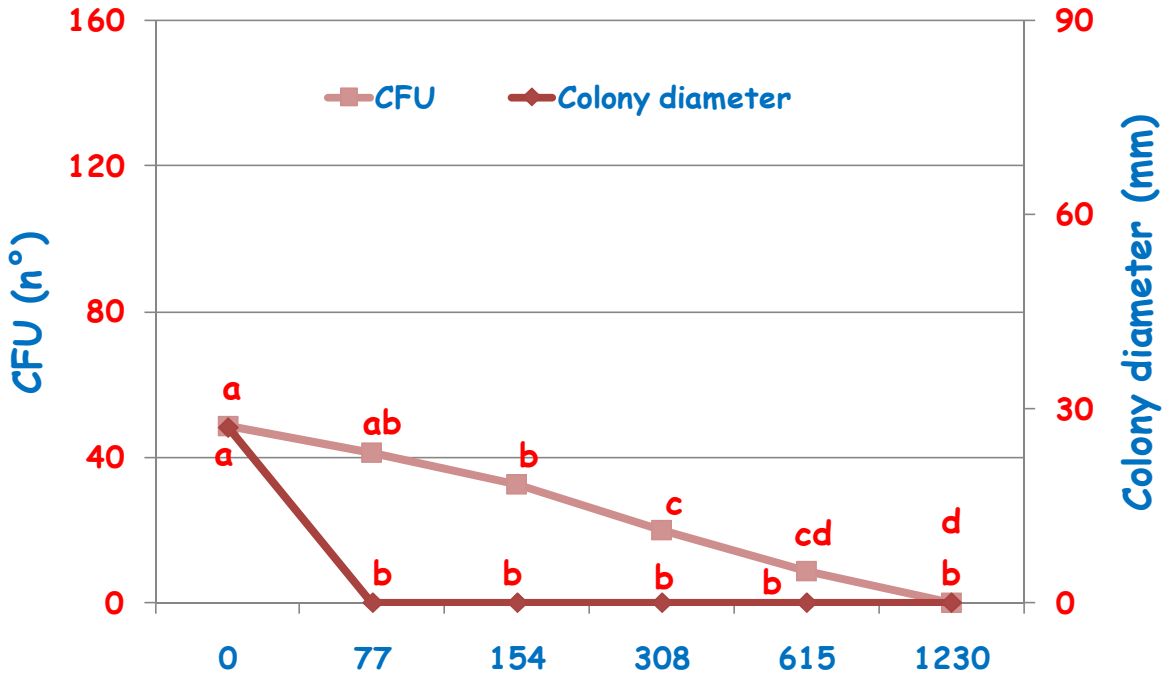
A)



B)


160
120
80
40
0
90
60
30
0
CFU (n°)
Colony diameter (mm)
CFU
Colony diameter
a
a
b
b
b
bc
c
cd
c
d
c
d
0
77
154
308
615
1230

C)


160
120
80
40
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0
CFU (n°)
Colony diameter (mm)
CFU
Colony diameter
a
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d
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b
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0
77
154
308
615
1230

D)

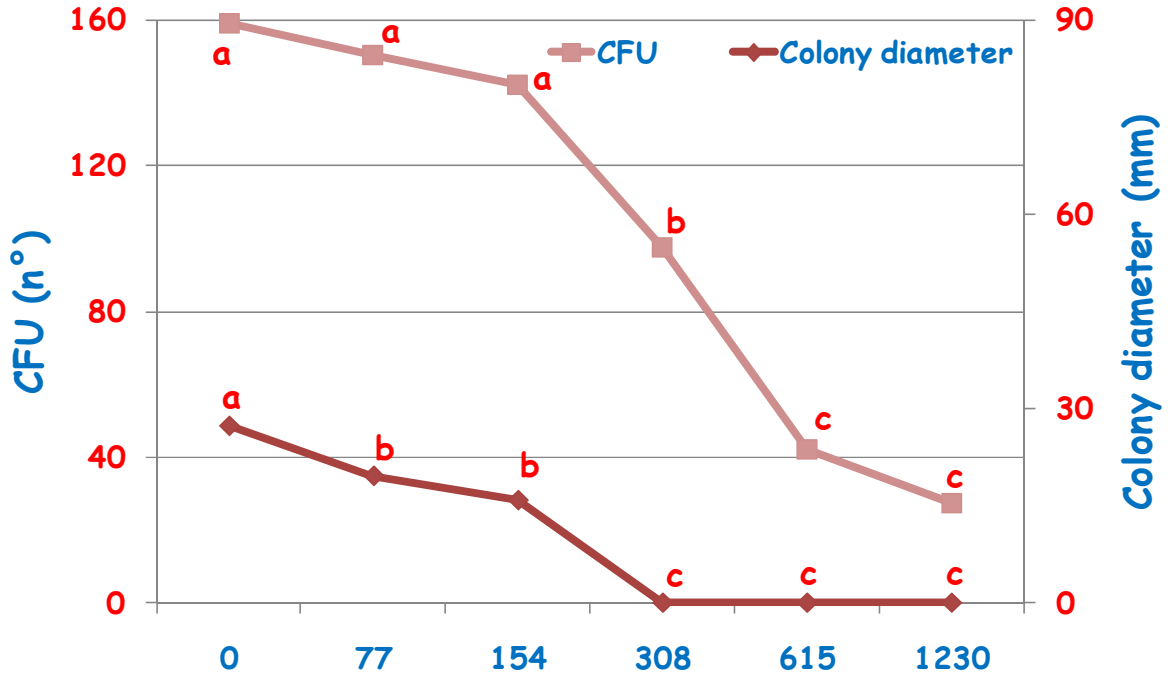

Figure 21 - Effect of pure phenethyl alcohol on the growth of *B. cinerea* (A), *C. acutatum* (B), *M. laxa* (C) and *P. expansum* (D). Colony diameter was determined after 7 days and CFU was determined after 3 days of incubation at 20°C. Each data represents the mean of 5 replicates. The experiment was repeated twice. Within each pathogen and curve different letters represent a significant difference according to LDS test (P<0.05).

The determination of ED_{50} value of PEA had confirmed the previous results. PEA proved to be a growth inhibitor as it had the lowest ED_{50} value of 832 mg/mL and 1023 mg/mL against mycelia growth of *C. acutatum* and *P. expansum* isolates respectively but also the lowest MIC (77 mg/mL) against mycelium growth of *B.cinerea* and *M. laxa*. Contrarily , the EC_{50} value for conidial germination were higher except for *B. cinerea* with only 676 mg/mL (Table.10).

Table 10 - ED_{50} values of phenethyl alcohol on the inhibition of mycelial growth (colony diameter) and conidial germination (CFU) of *B. cinerea*, *C. acutatum*, *M. laxa* and *P. expansum.*

	ED_{50} (mg/ml)			
	B. cinerea	*C. acutatum*	*M. laxa*	*P. expansum*
Colony diameter (mm)	**< 77**	**832**	**< 77**	**1023**
CFU (n°)	**676**	**1445**	**2100**	**3700**

4.4.5 Kinetic of production and quantification of phenethyl alcohol

4.4.5.1 Kinetic of production

The dynamic of production of PEA was followed by SPME and GC-FID analysis and appeared considerably variable among the two *P. expansum* strains LB8/99 and CADRP28 (Fig. 22). The measurement of hourly PEA production by LB8/99 grown on MEA Petri dishes, showed that PEA alcohol emission profile was variable during incubation period (100 hours). The area of PEA released by both LB8/99 and CADRP28 reached the peak after 72 hours PI, however, the area of PEA emitted by CADRP28 was 2 times lesser than that emitted by LB8/99 and remained almost stable for 24 hours.

It was interesting to notice that the variation of PEA emission profile was probably influenced also by the growth rate of LB8/99 and CADRP28 *P. expansum* strains, since, CADRP28 has shown a slow growth and retarded sporulation comparing to LB8/99.

These results confirm evidence found in preliminary investigation by GC-MS analysis.

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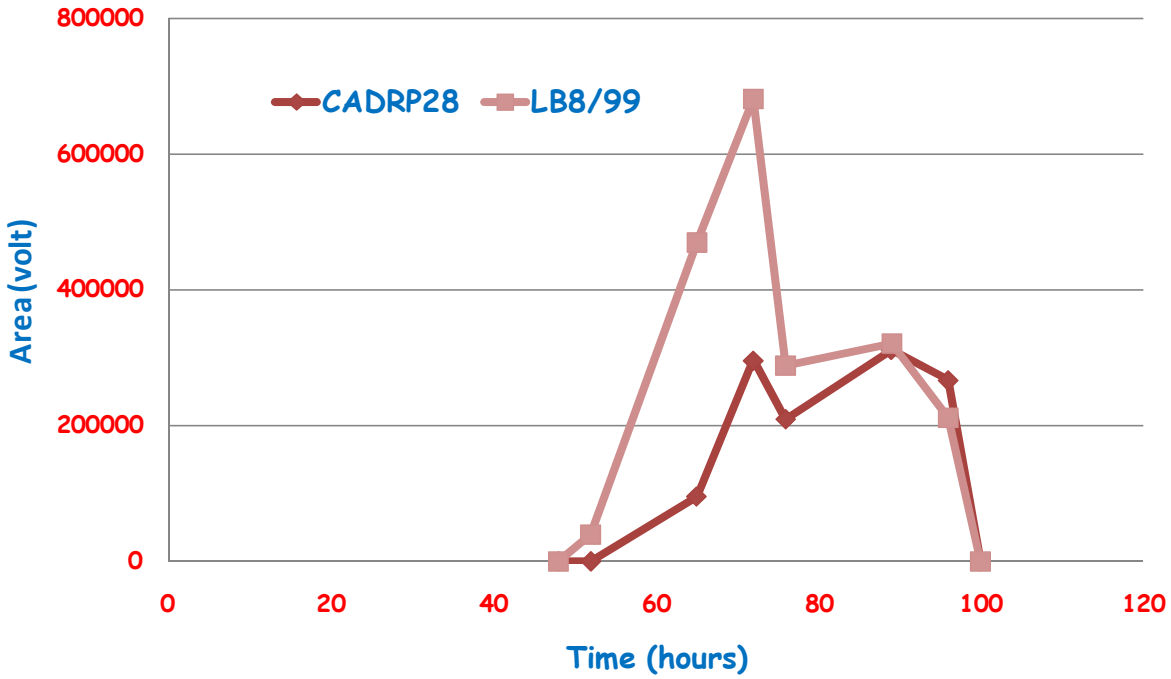


Figure 22 - The dynamic of production of phenethyl alcohol produced by both two *P. expansum* strains LB8/99 and CADRP28. Curves corresponds to the average hourly area of phenethyl alcohol measured using HS-SPME-GC-FID.

4.4.5.2 Quantification

The calibration curve designed using pure PEA solutions at the same conditions of kinetic production assays showed excellent linearity (Fig. 23) and permitted the quantification of the naturally PEA produced by LB8/99. The maximum concentration of PEA found in the Petri dish headspace during kinetic assays was therefore calculated from the maximum GC-FID area recorded and corresponded to 596 µg/mL. This concentration was too low comparing to the effective concentration of pure PEA that showed a high antifungal effect *in vitro* against some fungal postharvest pathogens as reported previously. *In vitro* assays were repeated at the same conditions used for kinetic study, in order to verify the PEA antifungal effect at the real concentration produced by LB8/99.

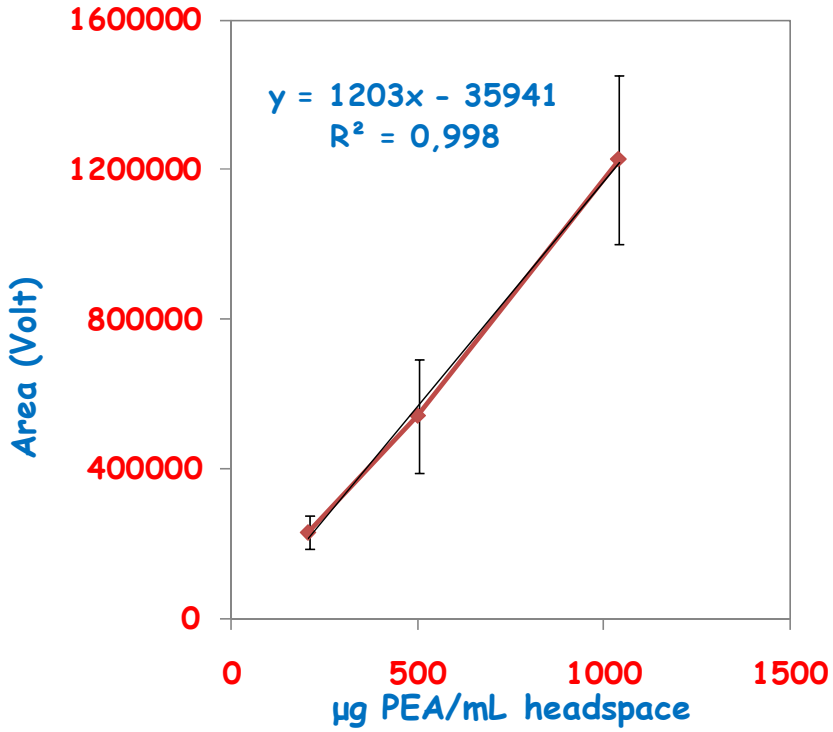


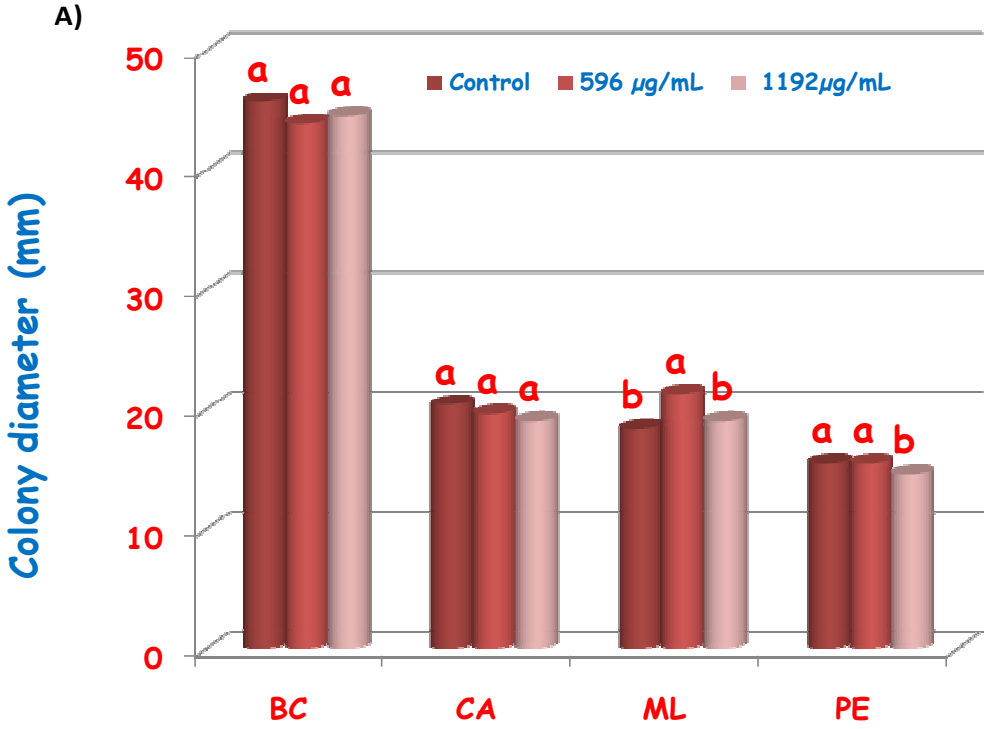
Figure 23 - Calibration curve for phenethyl alcohol (PEA) designed with three different concentrations of pure PEA solutions (207; 502 and 1041 µg/mL).

4.4.6 Effect of pure phenethyl alcohol at the concentration naturally produced by LB8/99 strain on conidial germination and mycelium growth

Exposure of fungal pathogens to pure PEA at the real concentration emitted naturally by LB8/99 at the maximum of production (596 µg/ mL) showed no inhibitory effect against mycelium growth and conidial germination of *B. cinerea*, *M. laxa* and *P. expansum* except for *C. acutatum* where a very slight significant reduction of conidial germination was reported (23%) (Fig. 24B). While, a stimulatory effect was noticed on the mycelium growth of *M. laxa* as illustrated in the Figure 24A.

Increasing the concentration of PEA at 1192 µg/mL (2 times the real concentration) permitted also the growth of all the fungal pathogens reported previously with the exception for *P.expansum*, which colony diameter was reduced significantly by 6% with respect to the control.

A)


Colony diameter (mm)
50
40
30
20
10
0
Control
596 µg/mL
1192µg/mL
a a a
a a a
b a b
a a b
BC
CA
ML
PE

B)

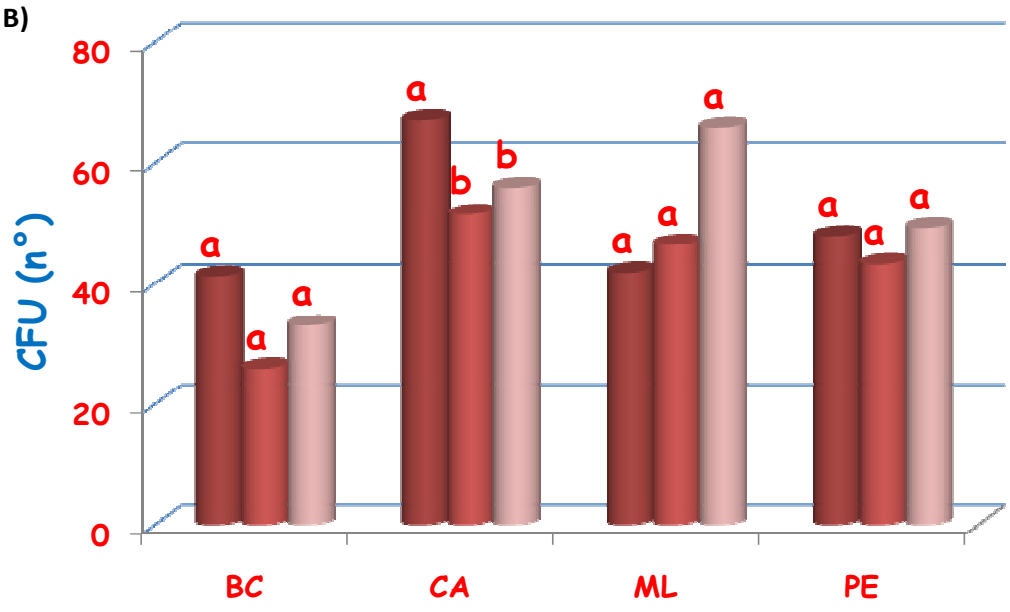

CFU (n°)
80
60
40
20
0
a a a
a b b
a a a
a a a
BC
CA
ML
PE

Figure 24 - The effect of pure phenethyl alcohol at the real concentration emitted at the peak of production by LB8/99 strain (596 µg/mL) and at 2 times the real concentration (1192 µg/ mL) on the colony diameter (A) and conidial germination (B) of the phytopathogenic fungi *B. cinerea (BC)*, *C. acutatum (CA)*, *M. laxa* (ML) and *P. expansum (PE)*. Different letters represent a significant difference according to LSD test (P<0.05).

CHAPTER 5

5. DISCUSSION

Blue mould rot is caused by various *Penicillium* spp species. During the present study, almost all the fungi isolated from the rotted tissue of host (apple, pear) and non host (kiwi, apricot and plum) commodities were identified as *P. expansum*. The latter is the most aggressive species and the most frequently associated with blue mould during apples storage in Italy and worldwide (Romano et al.,1983; Pitt and Hocking 1997; Piazzola et al., 2004; Sholberg et al., 2005a-b). Moreover, in this work two *Penicillium* species, *P. commune* and *P. vinaceum,* were isolated from rotted peach fruits with symptoms of blue mould rot.The former has been yet described as the causative agent of blue mould rot but with decreasing frequency, as it was reported by Sanderson and Spotts (1995), while no previous reports on *P. vinaceum* were found in the bibliography. *P. vinaceum* was defined as an endophytic fungus that has proven to be rich sources of biologically active secondary metabolites such as quinazoline alkaloid and therefore has attracted increasing attention in recent years. Consequently more investigations are needed to determine if it can be also considered a pathogenic fungus in some commodities under certain conditions as senescence. .

In addition to *P. expansum*, *P. solitum* has been recognized recently as an agent of blue mould on apple in Uruguay (Pianzolla et al., 2004). Both were recovered most frequently from pear and apple dump tank water, however the majority of fruit were infected with *P. expansum*, followed in order of decreasing frequency by *P. solitum, P. commune* and *P. aurantiogriseum* (Sanderson and Spotts, 1995).

The management of blue mould rot relies mainly on the use of synthetic fungicides, TBZ, belonging to benzimidazole class, has been widely used in the past and its repeated application has led to the emergence of resistance among the *P. expansum* strains, considered to be the cause of ineffective disease control . The limited success of benzimidazoles in the control of postharvest fungal pathogens was reported by Bolay et

al. (1974) and Bryk (1997) on *B.cinerea* and *P. alba,* on *M. fructicola (*Cox et al.,2009*)* and *P. expansum* (Baraldi et al., 2003*;* Sanchez- Torres and Tuset, 2011).Therefore resistance to this class fungicide has been studied and documented by many authors (Rosenberger and Meyer, 1985; Smith, 1988)

In the present study, the preliminary *in vitro* screening has been useful to discriminate TBZ-resistant and -sensitive strains. The results of monitoring of the occurrence and the distribution of the resistant strains consisting on direct-planting of pathogen strains on MEA plates amended with commercial or pure TBZ (400 µg/mL), revealed that the percentage of TBZ-resistant strains isolated from apples is two-times lesser (31%) than those reported by other authors (Pianzzola et al. 2004; Errampalli et al. 2006). The low frequency of the occurrence of resistant strains can be explained by the fact that the use of TBZ was abondonned many years ago (10 years) and that the integrated management program adopted probably has reduced the risk of the fungicide resistance emergence.

In addition, our resistant strains of *P. expansum* could be less fit, implying that the population might tend to return to its original state of balanced adaptation in absence of selection pressure by the fungicide as reported by Prusky et al., (1985),

Discriminatory concentration is often used for determining whether or not isolates are resistant to a fungicide (Chapeland et al., 1999; Baroffio et al., 2003; Moyano et al., 2004; Russell, 2004).In this work, all the isolates labeled as resistant (R) were those that could grow at the commercial dose of 400 µg /mL of TBZ and sensitive (S) if they could not (Baraldi et al., 2003). In fact, the discriminatory concentration of TBZ for sensitivy screening tests is variable: some authors considered isolates resistant to TBZ those that grown on amended media with 5 µg/mL TBZ (Errampalli et al. 2006; Li and Xiao 2008), or in a range of 4 and 16 µg/mL (Cabanas et al., 2009a). Sholberg et al. (2005a) grouped *Penicillum* spp. isolates as sensitive if they did not grow at 1 µg/mL, moderately resistant if they grew at 50 µg/ mL, but not at 100 µg /mL and highly resistant if they grew at 100, 500 and 1000 µg/mL.

Study of the effects of TBZ at 400 µg/mL on *P. expansum* resistant strains has revealed a weak effect on conidial germination, germ-tube elongation and initial mycelial growth. Among the TBZ- resistant strains scored in this work, seven strains showed higher percentage of conidial germination on TBZ- amended medium (RR) than control.

According to Baraldi et al, (2003), TBZ may have a stimulatory effect on conidial germination. Such effect has been studied earlier for metalaxyl (aphenylamidefungicide) on the vegetative growth of some isolates of *Phytophtora infestans* (Zhang et al., 1997).
The behavior of two strains P11 and P3 in presence of TBZ observed in this work (Fig. 6-7-8) is not well documented. Trials on TBZ resistance of P11 strain showed a greater inhibition of mycelium growth than conidial germination, similar results were discussed also by Cabanas et al. (2009a). TBZ seems to inhibit spore germination, but it's more effective when germination has begun. Allen and Gottlieb (1970) added that TBZ is fungicidal and causes stunting and malformation of the germ tubes once they have begun to emerge from spores. More investigations are needed in order to elucidate the effect of TBZ on P3 strain.
Our results suggest that the germination assay based on the counting of CFUs on fungicide amended medium is suitable for phenotyping strains for resistance to TBZ. However, spore germination assays, consisting on the assessment of the percentage of germination, are not appropriate (Cabanas et al., 2009a), in fact, a great number of spores are considered germinated, as the germ tube are still longer than the diameter of the spores although the germ tubes were shortened and twisted as the concentration of fungicide increased.
Since the traditonal techniques are time-consuming and labour intensive, in this study we described a microtiter assay called also 'broth microdilution method' (Cabanas et al., 2009a) developed to test the susceptibility of pathogens causing invasive infections such as *Verticillium dahliae* (Rampersad, 2011), *M. fructicola* (Cox et al., 2009), *B. cinerea* (Stammler and Speakman, 2006) and last but not least *Staphylococcus epidermidis* (Pettit et al., 2005). It was performed according to the guidelines of the CLSI document M38-A (Clinical and laboratory Standards Institute (CLSI), 2002) with some adaptations.
The proposed method is scalable for large sample sets; three *P. expansum* strains were tested on each microplate, saving time and culture media. Spore density and incubation time, 2 important growth parameters, were set up after numerous preliminary trials. Based upon microtiter assays conducted at different range of spore concentrations, we determined that the optimal spectrophotometric measurements occurred with *P. expansum* at 2.10^4 conidia/mL within 48 hours incubation at 20°C. In contrast, Cabanas et

al. (2009a) used spores density in excess of 10^5 conidia/mL; however such concentration produced inconsistent and unreliable results with our *P. expansum* strains and also with *M. fructicola* tested for sensitivity to fenbuconazole (Cox et al., 2009). In both *B.cinerea* (Pelloux-prayer et al., 1998) and *M fructicola* (Cox et al., 2009) colorimetric microtiter assay (AB assays), the optimal colorimetric signal occurred at 10^5 conidia/mL within 24h. We hypothesize that microtiter assay is species dependent and optimal spore density would have to be empirically determined to apply this technique on different fungal pathogens.

The artificial medium is an important component that must provide enough nutrients for optimal and homogenous germination of spores at a level quantifiable by spectrophotometric means but also must prevent the fungus from accessing alternative media (Kuhajek etal., 2003). Spiegel and Stammler (2006), have noticed that rich media induce incomplete inhibition of growth or germination of spores *in vitro* even at high concentrations and would result in high EC values. This explained why 2 folds- serially-diluted MEB was used in our assay and not full-strength MEB; and 20% V8 juice broth for *M. fructicola* (Cox et al.,2009).

Decreasing concentrations of TBZ (50; 25; 12.5; 6.25 and 3.125 µg/mL) tested have shown to be suitable to evaluate the 10 resistant *P.expansum* strains. Higher concentrations were not tested to avoid solubility problems (Pijls et al, 1994).

At the highest TBZ-dose tested (50 µg/mL), growth of all resistant isolates was not inhibited, so no definitive MIC value could be stated. The MICs values determined using the regression curve ranged between 1200 and 20000 µg/mL. In agreement with our results, Koffmann et al. (1978); Sholberg et al. (2005a) reported MICs >1000 µg/mL for resistant isolates of *P. expansum*.

For all the samples, mean ED_{50} values were > 50 µg/mL, ranging between 54 and 320 µg/mL. while Baraldi et al. (2003), Li and Xiao (2008) and Cabanas et al, (2009a) found ED_{50} values higher than 200 µg/mL. The discrepancy in ED_{50} may result from different media and inoculum used in these three studies. In fact the former, has used conidial suspension prepared from an aged PDA-culture of 7-day-old that are less sensitive and could give rise to false positive results (Birchmore and Forster, 1996).

Although the excellent concordance of the results obtained by the broth microdilution method and the agar dilution methods reported by many authors, the first one is recommended because it potentially allow for a high throughput screening of multiple isolates and multiple fungicide concentrations on few time.

Resistance to benzimidazoles has been associated with mutations on the β-tubulin gene; sequencing analysis of the β-tubulin gene revealed that all the TBZ-resistant strains had a similar sequence with only one or two different base pairs located in coding regions. In this study, the β-tubulin aminoacid sequence from codon 167 to 357 was analysed since it included most of the codons found mutated in *P. expansum* and other fungi resistant to benzimidazole. The deduced aminoacid sequences of all *P. expansum* strains were identical to the one found in strains belonging to genetic type 1 reported by Cabanas et al. (2009b).

The mutations of β-tubulin that confer resistance to benzimidazoles seems to be restricted to several positions including codons 165, 167,198, 200, 240 and 258 (Thomas et al., 1985; Orbach etal., 1986; Jung & Oakley, 1990; Fujimara et al., 1992;Jung et al., 1992; Li et al., 1996; Albertini et al., 1999; Cabanas et al., 2009b).

In our study, mutations at codon 198 (Glu to Val) and codon 240 (Leu to Phe) conferred resistance to thiabendazole in *P. expansum* strains. These results were similar to those reported by Cabanas et al. (2009). Contrarily to *P, expansum*, mutations found accociated with resistance to benzimidazole (benomyl and TBZ) in *P. italicum*, *P. aurantiogriseum* and *P. digitatum* involved codons 198 or 200 (Koenraadt et al., 1992; Sholberg et al., 2005b; Schmidt et al., 2006); whearas, in *P. solitum*, *P. puberulum* and *P. viridicatum* mutations were detected only at residue 198 (Koenraadt et al., 1992; Sholberg et al., 2005b).

In four resistant *P.expansum* strains with mutations at codon 198 (Glu to Val), the substitution of glutamic acid by lysine or by alanine was not observed. In accordance with our results, Koenraadt et al. (1992), Baraldi et al. (2003), Sholberg et al. (2005b) and Cabanas et al. (2009b) reported that a change of glutamic acid to valine at this position confers resistance to TBZ in *P. expansum*. According to Hollomon et al. (1998) replacement of a polar aminoacid at codon 198 with a small neutral one clearly alters the protein sufficiently to reduce the binding of thiabendazole, which also confers resistance.

Mutations at codon 240 (Leu to Phe) has been found on the same 4 resistant strains (P6; P12; K11; K12). Analogous mutations (Leu 240 Phe) were identified in *Tapesia acuformis* and *T. yallundae* (Albertini et al., 1999), and in natural (Cabanas et al., 2009b) and laboratory-induced thiabendazole-resistant isolates of *P.expansum* (Baraldi et al., 2003). The mutation leucine to phenylalanine was also identified in one resistant strain (P13) at codon 250 rather than at codon 240. Also Baraldi et al. (2003), identified the same mutation at residue Phe 200 to Leu. However, this aminoacid substitution (Phe to Leu) may be phenotypically silent with respect to thiabendazole resistance since both phenylalanine and leucine are bulky hydrophobic amino acids and their substitution may not cause major changes in the protein structure and function of the β-tubulin gene (Baraldi et al., 2003). This hypothesis gives a logic explanation for the identification of such mutation not only in TBZ-resistant strains but also in the sensitive ones and it may be applied to the mutation Phe 167 to Leu identified in one sensitive *P. expansum* strain (P3). In the present work, no mutation was found in Plum Argentine (Parg) resistant strain, suggesting that TBZ resistance can be determined by factors other than a single point mutation at codon 198, but it may be associated with mutations in a different region of the β-tubulin gene or in different genes including α-tubulin or genes encoding for microtubules-regulating proteins. Analysis of DNA sequences of the β -tubulin gene showed a point mutation at codon His 6 to Tyr in benomyl resistant strains of *M. fructicola* (Ma et al., 2003). While in *Sclerotinia cerevisiae*, Richards et al. (2000) reported some mutations in an α-tubulin gene (TUB1) increasing benomyl sensitivity.

Other molecular mechanisms may be involved such as ATP-binding cassette (ABC) transporters known to be responsible for multidrug resistance in fungi (de Waard, 1997). In addition to ABC transporter genes, PMR1 and PMR5 , associated with resistance to demethylation inhibitors (DMI) and benzimidazoles (Nakaune et al., 1998, 2002), recently the CYPR51 gene was shown to be responsible for resistance to (DMI) in *P. digitatum*, it exhibited a five tandem repeat sequence in resistant isolates and only one tandem repeat in the sensitive ones (Sanchez-Torres and Tuset, 2011).

Like other physical means, ULO is considered as a promising management strategy (Mari et al., 2003). The influence of the ultra low oxygen (ULO) was assayed on

the growth of *P. expansum in vitro*. No significant differences were observed on mycelium growth and conidial germination within the different O_2 concentrations (2%; 1% and 0.5%) after 30 days. In accordance with our results, Baert et al. (2007) found no influence on pathogen development at 20%; 3% and 1% O_2 levels and more recently Mari et al. (2010) working under low O_2 levels (6%;3%; 1.5% and 0.75 %) found the same conclusions. This finding on the tolerance of *P.expansum* to low O_2 levels was pointed by Sommer et al. (1981) who demonstrated that the pathogen's growth *in vitro* is lower in carbon monoxide enriched atmosphere than in low O_2. The ULO storage inhibitory effect (Karabulat and Baykal., 2004; Qin et al., 2004) and its contribution to extend fruit storage life (Taniwaki et al., 2001;Kader., 2002) was demonstrated on postharvest pathogens *in vivo* . Although few data report the effect of low O_2 levels on the disease incidence, Sitton and Patterson (1992), showed a significant reduction of the lesion diameter in Golden Delicious, Red Delicious and McIntosh apples inoculated with conidia of *P. expansum,* probably due to a delay in apple maturity than a direct effect on pathogen. Careful management of O_2 and CO_2 levels during fruit storage and limitation of maximum storage duration could have a significant impact on fruit maturity and consequently in decreasing fruit susceptibility to decay.

In the present study, some compounds produced by the *P. expasnum* LB8/99 strain revealed an antifungal activity against the mycelium growth of important postharvest pathogens. Fungi of the genus *Penicillium* are more known as antibacterial than antifungal substances producers although in the last decades numerous authors reported the activity of secondary metabolites from *Penicillium* spp. against fungal plant pathogens. The protein PAF secreted by *P. chrysogenum* strain Q176 was found active against *Aspergillus* spp., *B. cinerea*, *Fusarium* spp. etc. (Kaiserer et al., 2003), while the substance produced by *P. oxalicum* strain PY-1 was effective against numerous plant pathogenic fungi (Yang et al., 2008). *Phytophthora* root rot was controlled by *P. striatisporum* strain Pst10 and the suppression of disease may be due, at least partially, to the production of toxic metabolites that had specific activity against several *Phytophthora* spp. (Ma et al., 2008). Very few are the works on antifungal metabolites extracted from *P. expansum* (He et al., 2004) and to our knowledge, this is the first work on the potential of *Pencillium* against either other *P. expansum* strains or postharvest pathogens. The

presence of antifungal substances in liquid culture of LB8/99 strain was evaluated on the dry mycelium production of six *P. expansum* strains and of single isolates of *B. cinerea*, *C. acutatum* and *M. laxa* and a significant reduction of growth of all pathogens tested was noticed. While on conidial germination and germ tube elongation of *P. expansum* strains the control was limited. The mycelium was abnormal, unusually thinner in diameter and more heavily branched showing separated empty segments that can explain the previous reduction of the DWM. Similar malformations were induced by *B. subtilis* volatiles on *B .cinerea* mycelium (Chen et al., 2008). Thin-layer chromatography tests revealed that the extracts from the liquid filtrates of LB8/99 with various solvents have not inhibitory activity against target pathogens (data not shown), while in the double Petri dish assays, the inhibition of mycelium growth and conidia germination was observed on all tested pathogens and was confirmed by preliminary assays on *Fusarium*, *Aspergillus*, *Alternaria*, *Cladosporium* and *Phialophora*. These effects could be attributed to the production of VOCs generated by LB8/99 strain. According to Vespermann et al. (2007), volatile substances are organic and inorganic compounds which are low in molecular weight (<300Da) and low in polarity, but high in vapour pressure and therefore easily volatilizable. In our survey VOCs from the LB8/99 strain were detected and identified with a HS-SPME-GC-MS, confirming the production of VOCs by *P. expansum*. The number of compounds detected in the headspaces of the fungi varies depending on species and not all present in the NIST library (comprising 147.000 compounds). After one day of incubation, LB8/99 strain produced an unknown substance followed by 1, 10-dimethyl-trans-9-decalol (geosmin) and phenethyl alcohol (PEA). Geosmin is the primary component of the musty, earthy odor associated with *P. expansum*. Its appearance coincides with the development of the blue-gray pigmentation typically observed in cultures of *P. expansum* on Czapeck agar (Mattheis and Roberts, 1992). It is also produced by *Aspergillus spp*. and *Chaetomium globosum* Kunze: Fr (Kikuchi et al. 1981), algae (Safferman et al. 1967), non-cyanobacteria and cyanobacteria (Scholer et al., 2002; Juttner and Watson, 2007). Geosmin is ineffective against fungi, Mattheis and Roberts (1992) suggested its use as an indicator of incipient losses due to *P. expansum* on apple in postharvest environment, in addition this VOC was found on grapes rotted by *B. cinerea* (La Guerche et al., 2005). For this reason we focused our experiments on PEA

detected in the profile of LB8/99 VOCs and widely reported as secondary metabolite of the endophytic fungus *Muscador albus* (Strobel et al., 2001) and yeasts such as *Kluyveromyces* species (Cathy et al., 1998) and *Candida intermedia* (Huang et al., 2011). PEA rose like odor is considered to be one of the most commercially important flavour molecules (Welsh et al., 1989) and presents interesting organoleptic characteristics influencing the quality of wine, distilled beverages, or fermented foods (Fabre et al., 1998). In our experiments, pure PEA resulted more effective against mycelial growth than conidia germination of tested pathogens, the highest concentration assayed (1230 mg/mL) inhibited completely both with expect for conidia germination of *P. expansum* that however was reduced by 90% respect to the control. Similar results were also obtained against some seed-borne fungi, in agreement with Dev et al. (2004) that found the MICs values of pure PEA in a range between 1410 and 1970 mg/mL. Mercier and Jiménez (2004) found nine VOCs produced by *M. albus* including PEA, inhibiting or killing some storage pathogens belonging to species of *Botrytis*, *Colletotrichum*, *Geotrichum*, *Monilinia*, *Penicillium* and *Rhizopus*. In a previous work the fungus, growing on colonised desiccated rye grain produced at least 28 VOCs and this mixture resulted more effective than fumigant agents used as single compound (Strobel et al., 2001); this could explain the higher efficacy of LB8/99 strain in double Petri dish assay, since more compounds probably produced an additive effect increasing its activity. This hypothesis was confirmed through quantitative analysis carried out using SPME-GC-FID. The higher average concentration of PEA released naturally from LB8/99 culture was 2130 times less than the maximum inhibitory concentration tested previously and proved to be ineffective alone against fungal pathogens growth. In *in vitro* trials, the VOCs produced by LB8/99 strain were more effective against *C. acutatum, B. cinerea* and *M. laxa* than *P. expansum* strains and a low sensitivity to secondary metabolites produced by the fungus belonging to the same genus could be hypotized.

The *in vivo* assay showed a strong competition between the two *P. expansum* strains: LB8/99 and P13, since on MEA plates amended with TBZ and inoculated with small pieces of rotted tissue taken from lesions of fruit inoculated with a conidia mixture of LB8/99+P13 strains, no conidia germination was observed revealing that the LB8/99 (TBZ-sensitive) was the strain responsible of decay (Fig.17B-C). The nature of inter- and

intraspecific competition between fungi is not well known, but both scramble competition (indirect) and interference competition (direct) via toxin production have been suggested by Kaya (2002).

CHAPTER 6

6. CONCLUSION

Greater consumption of low energy food “Fruits” reduce risk of global mortality. A new recent WHO/FAO expert consultation report on diet recommended intake of a minimum of 400 g of fruits and vegetables per day as part of national non communicable diseases (NCD) prevention especially cardiovascular diseases, cancer, obesity and type 2 diabete mellitus, but also as school health programs to increase fruit consumption overall among children in school (FAO/WHO, 2004). Apples, one of the most important fruits appreciated by the consumers worldwide, are not the only health-imparting food in that diet, but they make a vital contribution

However, between harvest and consumption both quantitative and qualitative apple fruit losses can occur due to diseases, disorders and progressive deterioration of fruit quality which can be considered as an obstacle to achieve this important aim, and enhanced interest to improve the control strategies to reduce postharvest fruit losses. As reported previously, postharvest losses of apple fruit are mainly due to fungal infections particularly *P. expansum*, the causal agent of blue mould rot.

In the past the application of the TBZ fungicide controlled *P. expansum* and extended the shelf life of fresh apple fruits, although growing health and environmental concerns over fungicide disposal and residue levels on fresh commodities and particularly the development of TBZ-resistant strains have considerably limited its use. *In vitro* assays, carried out to evaluate the effect of TBZ on 48 strains of *P. expansum,* have confirmed that TBZ induced resistance into 13 strains (27%). The higher percentage of sensible strains with respect data previously reported probably is related to the abandonment of the use of chemical fungicides a part by packinghouse operators to opt safer effective alternatives that pose no risk to human health or the environment. such as treatments based on heat, GRAS, disinfectants or the use of modified atmosphere, etc. According to the results obtained in this study, ULO levels did not affect directly the growth of *P.*

expansum strains *in vitro* but *in vivo*, reducing the rate of respiration, delayed fruit maturity and consequently decay, retaining quality traits and extend their storage life (Kader, 2002). However a monitoring program for early detection of reduced sensitivity to fungicides in *P. expansum* strains and to implement resistance management practices it is necessary. In this study a microtiter assay, specific for *P. expansum,* as preliminary quantitative screening was set up. Based on the results obtained in this assay, it is possible to simplify the monitoring procedure and apply this screening technique at higher discriminatory doses of 100 µg/mL and 200 µg/mL to split the resistant isolates into further subdivisions.

We noticed that TBZ resistance has been generally correlated with a single point mutation at codon 198 on the β-tubulin gene although it was absent in some resistant strains. More investigations are needed to elucidate other genes or molecular mechanisms involved in the resistance such as ATP- binding cassette (ABC), *PMR1* and *PMR5* .

Such monitoring would help to make proper decisions on resistance management programs such as the reduction in fungicide concentrations or the use of fungicides mixtures with different modes of action with periodic surveys for eventual development of resistant isolates or better an integrated control program combining biocontrol agents with fungicides or exogenous chemicals is recommended.

Another important result was obtained during this study, the potential use of the *P. expansum* strain LB8/99 as biofumigant. *In vitro* results on the toxic effect of VOCs produced by the strain on *B. cinerea*, *C. acutatum, M. laxa* and *P. expansum*, suggest the possibility of further exploitation under airtight conditions. To avoid the risk of fruit infection by *P. expansum* LB8/99 strain, a possibility is to grow it in a warmer environment and transfer the produced volatiles into the storage room without the direct contact with the fruits. More investigation are required to optimize biofumigation treatment. In addition lethal effects of PEA (the main VOC produced by LB8/99 strain) on storage pathogens suggests that the biofumigation could have widespread application in controlling microbial losses on other commodities than fruit such as seeds (Dev et al. 2004) and seedlings (Wan et al. 2008).

Moreover, future research will be directed toward studying the effect of VOCs treatments on fruit flavour and eventual risk of toxicity that can be induced by PEA, the major compound emitted by LB8/99 through organoleptic and chemical analysis.

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Bibliographic references:

Abanda- Nkpwatt, D., Krimm, U., Coiner, H.A., Schreiber, L., and Schwab, W. 2006. Plant volatiles can minimize the growth suppression of epiphytic bacteria by the pathogenic fungus *Botrytis cinerea* in co-culture experiments. Environmental and experimental botany, 56: 108-119.

Abramson, D. 1997. Toxicants of the genus *Penicillium*. In: D'Mello,J.F.P. (ed.), Handbook of Plant and Fungal Toxicants. CRC Press, Inc., Boca Raton, NY, pp, 303-317.

Acree, T.E., Lee, C.Y., Butts, R.M., and Barnard, J. 1976. Geosmin, the earthy component of table beet odor. Journal of Agricultural Food Chemistry, 24:430-431.

Albertini, C., Gredt, M., and Leroux, P. 1999. Mutations of the b-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. Pesticide Biochemistry and Physiology, 64: 17–31.

Allen, P.M., and Gottlieb, D. 1970. Mechanism of action of the fungicide thiabendazole, 2-(4'-thiazolyl) benzimidazole. Applied microbiology and Biotechnology, 20: 919-926.

AL-Taweel, A.M., EL-Deeb, K.S., and AL-Muhtadi, F.J., 2004. Chemical composition and antimicrobial activity of the essential oil of Kleinia odora. Saudi Pharmaceutical Journal, 12: 47–50.

Alves, I., Oliveira, N.G., Laires, A., Rodrigues, A.S, and Rueff, J. 2000. Induction of micronuclei and chromosomal aberrations by the mycotoxin patulin in mammalian cells: role of ascorbic acid as a modulator of patulin clastogenicity. Mutagenesis, 15: 229–234.

Amiri, A., and Bompeix, G., 2005. Diversity and population dynamics of *Penicillium* spp. on apples in pre-and postharvest environments: consequences for decay development. Plant Pathology, 54: 74-81.

Andersen, B., Smedsgaard, J and Frisvad, J. C., 2004. *Penicillium expansum* : Consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurence in fruit products. Journal of Agricultural and Food Chemistry, 52: 2421-2428.

Archbold, D.D., Hamilton-Kemp, T.R., Barth, M.M., and Langlois, B.E. 1997. Identifying Natural Volatile Compounds That Control Gray Mold (*Botrytis cinerea*) during

Postharvest Storage of Strawberry, Blackberry, and Grape, Journal of Agricultural and Food Chemistry, 45: 4032-4037.

Arthur, C.L., and Pawliszyn, J., 1990. Solid-phase microextraction with thermal-desorption using fused-silica optical fibers. Analytical Chemistry, 62: 2145–2148.

Atkinson, N. 1942. Antibacterial substances produced by moulds. 1. Penicidin, a product of the growth of a *Penicillium*. Australian Journal of Experimental Biology and Medical Science, 20: 287–288.

Baert, K., Meulenaer, B.D., Kasase, C., Huyghebaert, A., Ooghe, W., and Devlieghere, F., 2007. Free and bound patulin in cloudy apple juice. Food Chemistry, 100: 1278–1282.

Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., and Pratella, G.C., 2003. Studies on thiabendazole resi stance of *Penicillium expansum* of pears: pathogenic fitness and genetic characterization. Plant Pathology, 52: 362-370.

Baroffio, C.A., Siegfried, W., and Hilber, U.W., 2003. Long-term monitoring for resistance of *Botryotinia fuckeliana* to anilinopyrimidine, phenylpyrrole, and hydroxyanilide fungicides in Switzerland. Plant Disease, 87: 662–666.

Becci, P.J., Hess, F.G., Johnson, W.D., Gallo, M.A., Babish, J.G., Dailey, R.E, and Parent, R.A. 1981. Long-term carcinogenicity and toxicity studies of patulin in the rat. Journal of Applied Toxicology, 1: 256–261.

Ben-Arie, R. 1975. Benzimidazole penetration, distribution, and persistence in postharvest-treated pears. Phytopathology, 65: 1187-1189.

Benndorf, D., Mueller, A., Bock, K., Manuwald, O., Herbarth, O., and von Bergen, M. 2008. Identification of spore allergens from the indoor mould *Aspergillus versicolor*. Allergy, 63: 454–460.

Bergel, F., Morrison, A.L., Moss, A.R., Klein, R., Rinderknecht, H., and Ward, J.L. 1943. An antibacterial substance from *Aspergillus clavatus* and *Penicillium claviforme* and its probable identity with patulin. Nature, 152: 750.

Bernard, J.L., Franclet, J., Gaulliard, J.M., Giraud, M., Morgat, F., 1993. Maladies de pommes en fin de cycle et durant la conservation: résultats de l'enquête ANPP 1991–92. Phytoma la défense Des Végétaux, 455 : 50-52.

Bertrand, P.F., and Saulie-Carter, J.L. 1978. *Penicillium expansum* and *Botrytis cinerea* in the Mid-Columbia region of Oregon and Washington. Plant Disease Reporter, 62: 302-305.

Bertrand, P. F., and Saulie-Carter, J. L. 1979. Postharvest decay control of apples and pears after immersion dumping. Ore. State Univ. Exp. Stn. Spec. Rep.pp, 545.

Birchmore, R.J., and Forster, B. 1996. FRAC methods for monitoring the sensitivity of *Botrytis cinerea* to anilinopyrimidine fungicides. Bulletin OEPP/EPPO Bulletin. 26, 181–197.

Bolay, A., Crettenand, J., Gnaegi, F, and Schopfer, J.F, 1974. The control of grey rot of grapes. Revue Suisse de viticulture arboriculture horticulture, 6: 91-97:

Boyd, E.M. 1944. Patulin. Canadian Medical Association Journal, 50: 159.

Boysen, M., Skouboe, P., Frisvad, J., and Rossen, L. 1996. Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology: 142: 541-549.

Brackett, R.E and Marth, E.H. 1979. Ascorbic acid and ascorbate cause disappearance of patulin from buffer solutions and apple juice. Journal of Food Protection, 11: 864-866.

Bragulat, M.R., Martinez, E., Castella, G., and Cabanes, F.J., 2008. Ochratoxin A and citrinin producing species of the genus *Penicillium* from feedstuffs. International journal of Food Microbiology, 126: 43-48.

Brian, P.W., Elson, G.W, and Lowe, D. 1956. Production of patulin in apple fruits by *Penicillium expansum*.Nature, 178: 263-264.

Bridge, P.D., Hawksworth, D.L., Kozakiewicz, Z., Onions, A. H. A., Paterson, R. R. M., Sackin, M.J., and Sneath, P.H.A.A. 1989**.** Reappraisal of the terverticillate Penicillia using biochemical, physiological and morphological features. I. Numerical taxonomy. Journal of General Microbiology, *135*: 2941-2966.

Bridi, C. 2010. Mele, meno produzione in Europa, Prognosfruit 2010. Terra e Vita n°35. www.edagricole.it/immaginipdf (On line)

Brown, D.H., Matzuk, A.R., Ilves, I.R., Peterson, L.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Campbell, W.C., and Cuckler, A.C. 1961. Antiparasitic drugs. IV. 2-(4' -Thiazolyl)-benzimidazole, a new anthelmintic. Journal of the American Chemical Society, 83: 1764-1765.

Brown, G.E., Davis, C., and Chambers, M. 2000. Control of citrus green mold with Aspire is impacted by the type of injury. Postharvest Biology and Technology, 18: 57–65.

Bryk, H. 1997. Appearance and stability of Pezicula alba Gunth. resistance to benzimidazole fungicides. Journal of fruit and ornamental plant research, 5: 77-87.

Buckner, S. 2005. Good bugs vs. bad; beneficial micro-organisms gain grounds in disease battles. Grower, 38: 12 -14.

Bull, C.T., Stack, J.P., and Smilanick, J.L. 1997. *Pseudomonas syringae* strains ESC-10 and ESC-11 survive in wounds on citrus and control green and blue molds of citrus. Biological Control, 8: 81–88.

Bullerman, L.B. 2000. Mycotoxins. In: Robinson, R.K., Batt, C.A., Patel, P.D. (Eds.), Encyclopedia of Food Microbiology, Academic Press, London, UK, pp: 1512-1547.

Burda, K. 1992. Incidence of patulin in apple, pear and mixed fruit products marketed in new south wales. Journal of Food Protection, 10: 796-798.

Buttery, R. G., Guadagni, D.G., and Ling, L.C. 1976. Geosmin, a musty off-flavor of dry beans. Journal of Agricultural Food and Chemistry, 24: 419- 420.

Cabanas, R., Abarca, M.L., Bragulat, M.R. and Cabanes, F.J. 2009a. Comparison of methods to detect resistance of *Penicillium expansum* to thiabendazole. The Society for Applied Microbiology, Letters in Applied Microbiology, 48: 241–246.

Cabanas, R., Castella, G., Abarca, M.L., Bragulat, M.R and Cabanes, F.J. 2009b. Thiabendazole resistance and mutations in the β-tubulin gene of *Penicillium expansum* strains isolated from apples and pears with blue mold decay. FEMS, Microbiology Letters, 297: 189-195.,

Cargo, C.A., and Dewey, D.H. 1970. Thiabendazole and benomyl for the control of postharvest decay of apples. Hortscience, 5: 259–60.

Carpenter-Boggs, L., Loynacgan, T.E., and Stahl, P.D. 1995. Spore germination of *Gigaspora margarita* stimulated by volatiles of soil-isolated actinomycetes. Soil Biology and Biochemistry, 27: 1445–1451.

Cathy EF, Blanc PJ, Goma G. 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnology Progress 14, 270-274.

Chain, E., Florey, H.W., and Jennings, M.A. 1942. An antibacterial substance produced by *Penicillium claviforme*. British Journal of Experimental Pathology, 23: 202–205.

Chalutz, E., and Droby, S. 1997. Biological control of postharvest disease. In*:* Boland, G.J., and Kuykendall, L.D. (Eds.). Plant–microbe interactions and biological control. Marcel Dekker, Inc., New York., pp: 157-170.

Chand-Goyal, T., and Spotts, R.A., 1996. Control of postharvest pear diseases using natural saprophytic yeast colonists and their combination with a low dosage of thiabendazole. Postharvest Biology and Technology, 7: 51- 64.

Chapeland, F., Fritz, R., Lanen, C., Gredt, M., and Leroux, P. 1999. Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Bortytis cinerea* (*Botryotinia Fuckeliana*). Pesticide Biochemistry and Physiology, 64: 85–100.

Chen, D.H., and Ronald, P.C. 1999. A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Molecular Biology Reporter, 17: 53-57.

Chen, L., Ingham, B. H., and Ingham, S.C. 2004. Survival of *Penicillium expansum* and Patulin Production on Stored Apples after Wash Treatments. Journal of Food Science, 69 (8): 669-675.

Chen, H., Xiao, X., Wang, J., Wu, L.J., Zheng, Z.M., and Yu, Z.L. 2008. Antagonistic effects of volatiles generated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. Biotechnology Letters, 30: 919-923.

Ciegler, A., Detroy, R.W., and Lillehoj, E.B. 1971. Patulin, penicillic acid, and other carcinogenic lactones, pp: 409-434. In: Ajl, S., Ciegler, A., and Kadis, S., (ed.), Microbiol toxins, vol. VI. Academic Press Inc., New York.

Clinical and Laboratory Standards Institute (CLSI). 2002. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved Standard M38-A.Wayne, PA: Clinical and Laboratory Standards Institute.

Cole, M., and Wood, R.K.S. 1961. Pectic enzymes and phenolic substances in apples rotted by fungi. Annals of Botany N.S., 25: 435-452.

Cole, R.J., and Cox, R.H. 1981. Handbook of toxic fungal metabolites. New York (NY): Academic Press. pp: 152–263.

Cox, K.D., Quello, K., Deford, R.J., and Beckerman, J.L. 2009. A rapid method to quantify fungicide sensitivity in the brown rot pathogen *Monilia fructicola*. Plant Disease, 93: 328-331.

Davidse, L.C. 1986. Benzimidazole fungicides: mechanism of action and biological impact. Annual Review of Phytopathology, 24: 43-65.

Davidse, L.C., and Ishii, H. 1995. Biochemical and molecular aspects of the mechanisms of action of benzimidazoles, N-phenylcarbamates and N-phenylformamidoximes and the mechanism of resistance to these compounds in fungi. Pp: 305-322 in: Modern Selective Fungicides: Properties, Applications, Mechanisms of Action. Lyr, H., (ed.) Gustav Fisher Verlag, New York.

De Brabander, M.J., Van de Veire, R.M., Aerts, F.E., Borgers, M., and Janssen, P.A. 1976. The effects of methyl (5-(2- thienylcarbonyl)-1H-benzimidazol-2-yl)

carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured *in vitro*. Cancer Research, 36: 905–916.

De Koch, S. 1998. Control of postharvest decay of fruit by means of antagonistic micro-organisms. Ph.D. dissertation, University of Stellenbosch, Capetown, South Africa.

De Waard, M.A. 1997. Significance of ABC transporters in fungicide sensitivity and resistance. Pesticide Science, 51: 271–275.

Delp, C.J. 1988. Resistance management strategies for benzimidazoles . In Fungicide resistance in North America Delp, C.J. (ed.). American Phytopathgological Society (APS) Press, St. Paul, Minn., pp: 41-43.

Dennis, C., Webster, J., 1971. Antagonistic properties of species groups of *Trichoderma*. Part II. Production of volatile antibiotics. Transactions of the British Mycological Society, 57: 41–48.

Dev, U., Devakumar, C., Mohan, J., and Agarwal, P.C. 2004. Antifungal activity of aroma chemicals against seed-borne fungi. Journal of Essential Oil Research, 16: 496-499.

Deveraj, H., Radha-shanmugasundaram, K.R., and Shanmugasundaram, E.R.B. 1982. Neurotoxic effect of patulin. Indian Journal of Experimental Biology, 20: 230-231.

Dickschat, J., Martens, S., Brinkhoff, T., Simon, T.M., and Schulz, S. 2005. Volatiles released by a *Streptomyces* species isolated from the North sea. Chemistry and Biodiversity, 2: 837–865.

Domsch, K.H., Gams. W., and Anderson T.H. 1980. *Compendium of soil fungi*. Vol.1. Academic Press (London) LTD 24/28 Oval Road London NWI, pp: 859.

Doukas, E.G., Markoglou, A.N., and Ziogas, B.N. 2008. Biochemical and molecular study of triazole-resistance and its effect on ochratoxin production by *Aspergillus ochraceus* Wilh. 9th International Congress of Plant Pathology. ICPP,Torino, Italy.

Droby, S., Hofstein, R., Wilson, C.L., Wisniewski, M., Fridlender, B., Cohen, L., Weiss, B., Daus, A., Timar, D., and Chalutz, E. 1993. Pilot testing of *Pichia guilliermondii*: a biocontrol agent of postharvest diseases of citrus fruit. Biological Control, 3: 47-52.

Droby, S., Cohen, L., Daus, A., Weiss, B., Hover, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A. 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. Biological Control, 12: 97-101.

Dupont, J., Magnin, S., Marti, A, and Brousse, M. 1999. Molecular tools for identification of *Penicillium* starter cultures used in food industry. International Journal of Food Microbiology, 49: 109-118.

Eckert, J.W. 1988. Dynamics of benzimidazole-resistant Penicillia in the development of postharvest decays of citrus and pome fruits. In: Fungicide resistance in North America. Delp, C.J. (ed.). American Phytopatholigal Society (APS) Press, St. Paul, Minn.,pp: 31-35.

Eckert, J.W., and Ogawa, J.M., 1988. The chemical control of postharvest diseases: deciduous fruits, berries, vegetables and root/tuber crops. Annual Review of Phytopathology, 26: 433–469.

El-Ghaouth, A. 1997. Biologically- based alternatives to synthetic fungicides for the control of postharvest diseases. Journal of Industrial Microbiology and Biotechnology, 19: 160-162.

El-Ghaouth, A., Wilson, C.L., and Wisniewski, M. 1997. Antifungal activity of 2-deoxy-D-glucose on *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer*. Ultrastructural and cytochemical aspect. Phytopathology, 88: 772–779.

Engel, G., and Teuber, M. 1984. Patulin and other small lactones. In: Betina, V., (ed.) Mycotoxins: production, isolation, separation and purification. 1º ed. Netherland, pp: 290-314.

Errampalli, D. 2003. Evaluation of postharvest fungicides scholar, switch and biosave for control of blue mold of apples cv. Empire, during storage, 2001–2002. Pest Management Research Report, 41: 231–233.

Errampalli, D., 2004. Effect of fludioxonil on germination and growth of *Penicillium expansum* and decay in apple cvs. Empire and Gala. Crop Protection, 23: 811–817.

Errampalli, D., and Crnko, N. 2003. Fludioxonilalone or with cyprodinil affects thiabendazole-resistant *Penicillium expansum* on apples. In: 8th International Congress of Plant Pathology, 2-7 Feb.2003, Christchurch, New Zealand, Vol. 2.pp:.63.[Abstr.]

Errampalli, D., and Crnko, N. 2004. Control of blue mold caused by *Penicillium expansum* on apples 'Empire' with fludioxonil and cyprodinil. Canadian Journal of Plant Pathology. 26: 70–75.

Errampalli, D., Brubacher, N.R., and DeEll, J.R. 2006. Sensitivity of *Penicillium expansum* to diphenylamine and thiabendazole and postharvest control of blue

mould with fludioxonil in 'McIntosh' apple. Postharvest Biology and Technology, 39: 101-107.

Escoula, L., Thomsen, M., Bourdiol, M., Pipy, B., Peuriere, S., and Roubinet, S. 1988. Patulin immunotoxicology: effect on phagocyte activation and cellular and humoral immune system of mice and rabbits. International Immunopharmacology, 10: 983-989.

European Commission. 2001. Commission Directive 2001 / 21 / EC of 5 March 2001 amending Annex I to Council Directive 91 / 414 / EEC concerning the placing of plant protection products on the market to include amitrole, diquat, pyridate and thiabendazole as active substances. Official Journal of European Communities, 69: 17–21.

Fabre, C.E., Blanc, P.J., and Goma, G. 1998. Production of 2-phenylethyl alcohol by *Kluyvermyces marxianus*. Biotechnology Progress, 14: 270-274.

FAO Statistical Database (FAOSTAT). 2005. Statistics on agriculture, nutrition, fisheries, forestry, food aid, land use and population. (Online) http://faostat. Fao.org/.

FAO/WHO. 2004. Report of a joint FAO/WHO workshop:fruits and vegetables for health, 1-3 september, kobe (Japan). WHO library cataloguing-in publication data, pp:46.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., and Savchuk, S.C. 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology Biochemistry, 37: 955–964.

Fiedler, K., and Schütz, E. 2000. Poster: Einfluss der Wachstumsbedingungen von Schimmelpilzen auf die Produktion von flüchtigen organischen Stoffwechselprodukten (MVOC) am Beispiel von *Penicillium expansum*. 8th. Kongress der Gesellschaft für Hygiene und Umweltmedizin Bonn, 29- 31March.

Fiedler, K., Schütz, E., and Geh, S. 2001. Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. International Journal of Hygiene and Environmental Health, 204: 111 -121.

Frac (Fungicide Resistance Action Committee), 2010. Pathogen risk list. Available from: www.frac.info. (Online)

Fravel, D.R., and Larkin, R.P. 1996. Availability and application of biocontrol products. Biol. Cul. Tests Control Plant Disease, 11: 1–7.

Frisvad, J.C. 1981. Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. Applied and Environmental Microbiology, 41: 568- 579.

Frisvad, J. C. 1992**.** Chemometrics and chemotaxonomy: a comparison of multivariate statistical methods for the evaluation of binary fungal secondary metabolite data. Chemometrics and Intelligent Laboratory Systems, 14: 253-269.

Frisvad, J.C., and Filtenborg, O. 1989. Terverticillate penicillia: chemotaxonomy and mycotoxin production. Mycologia, 81:837-861.

Frisvad, J.C., and Filtenborg, O. 1990. Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*. In Samson, R.A., and Pitt, J.I. (ed.). Modern Concepts in: *Penicillium* and *Aspergillus* Classification, pp: 373– 384. New York: Plenum Press.

Frisvad, J.C., Filtenborg, O., Lund, F., and Samson, R.A. 2000. The homogeneous species and series in subgenus *Penicillium* are related to mammal nutrition and excretion. In: Samson, R.A., and Pitt, J.I. (eds.). Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification, pp: 265-283. Harwood Academic Publishers, N.Y., USA.

Frisvad, J.C., and Samson, R.A. 2004. Polyphasic taxonomy of *Penicillium* species subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology, 49: 1-173.

Frisvad, J.C., and Thrane, U. 2002. Mycotoxin production by common filamentous fungi. In: Samson, R.A., Hoekstra, E.S., Frisvad, J.C., and Filtenborg, O. (Eds.), Introduction to Food- and Air Borne Fungi, 6th ed. Centraalbureau voor Schimmelcultures, Utrecht, the netherlands, pp: 321– 330.

Frisvad, J.C., Thrane, U., and Filtenborg, O. 1998. Role and use of secondary metabolites in fungal taxonomy. In: Frisvad, J.C., Bridge, P.D., and Arora, D.K. (eds). Chemical fungal taxonomy. Marcel Dekker, New York, pp: 289–319

Fujimara, M.K., Oeda, M., Inoue, H., and Kato, T. 1992. A single amino acid substitution in the β-tubulin gene of *Neurospora crassa* confers both carbendazim resistance and diethofencarb sensitivity. Current Genetics, 21: 399–404.

Gaucher, G.M., Lam, K.S., GrootWassingk, J. W. D., Neway, J., and Deo, Y. M. 1981. The initiation and longevity of patulin biosynthesis. Development of Industrial Microbiology, 22: 219-232.

Giraud, M., and Fauré, J. 2000. Assessment of *Penicillium* risk on pome fruit in storage. In: Proceedings of the 5th Workshop on Integrated Control of Pome Fruit Diseases, Fontevraud (France), 24–27 August 1999. Bordeaux: EFPP Group, 153–8. (International Organisation for Biological Control/ WPRS Bulletin 23.)

Gourama, H., and Bullerman, L.B. 1995. Detection of molds in foods and feeds: potential rapid and selective methods. Journal of Food Protection, 58: 1389-1394.

Gu, Y.Q., Mo, M.H., Zhou, J.P., Zou, C.S., and Zhang, K.Q. 2007. Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. Soil Biology and Biochemistry, 39: 2567–2575.

Gullino, M.L., Kuijpers, L.A.M., 1994. Social and political implications of managing plant diseases with restricted fungicides in Europe. Annual Review of Phytopathology, 32: 559–579.

Harrison, M., 1989. Presence and stability of patulin in apple products: a review. Journal of Food Safety, 9: 147-153.

He, G., Matsuura, H., Takushi, T., Kawano, S., and Yoshihara, T. 2004. A new antifungal metabolite from *Penicillium expansum*. Journal of Natural Product, 67: 1084-1087.

Henriquez, J.L. 2005. First report of Apple rot caused by *Neofabraea alba* in chile. Plant Disease, 89: 1360.

Hill, W.E. 1996. The polymerase chain reaction: applications for the detection of foodborne pathogens. Critical Reviews in Food Science and Nutrition, 36: 123– 173.

Hofstein, R., and Friedlender, B. 1994. Development of production, formulation and delivery systems. In: Proceedings of the Brighton Crop Protection Conference, Brighton, England, 21–24 November 1994, 3: 1273–1280.

Holloman, D.W., Butters, J.A., Barker, H., and Hall, L. 1998. Fungal β-tubulin, expressed as a fusion protein, binds benzimidazole and phenylcarbamate fungicides. Antimicrobial Agents and Chemotherapy, 42: 2171-2173.

Holmes, G.J., Edmunds, B. 2005. Bio-Save 11LP gets label for postharvest use in sweet potatoes. In: Toth, S.J. (Ed.),North Carolina PestNews,NC StateUniv, 20 (4). Available at: http://ipm.ncsu.edu/currentipm/05PestNews/05News4/fruitveg.html (Online).

Huang, H.C., Lin, R.H., Chang, C.J., Collmer, A., and Deng, W.L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpinpss secretion that are arranged collinearly with *Yersisnia ysc* homologs. Molecular Plant-Microbe Interactions, 8: 733-746.

Huang, R., Li, G.Q., Zhang, J., Yang, L., Che, H.J., Jiang, D.H., and Huang, H.C. 2011. Control of postharvest *Botrytis* fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. Phytopathology, 101: 859-869.

Janisiewicz, W.J. 1998. Biocontrol of postharvest diseases of temperate fruits: challenges and opportunities. In: Boland, G.J., and Kuykendall, L.D. (Eds.), Plant - Microbe interactions and biological control. Marcel Dekker, New York, USA, pp: 171-198.

Janisiewicz, W.J., and Marchi, A. 1992. Control of storage rots on various pear cultivars with a saprophytic strain of *Pseudomonas syringae*. Plant Disease, 76: 555-560.

Janisiewicz, W.J., and Jeffers, S.N. 1997. Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. Crop Protection, 16: 629-633.

Janisiewicz, W.J., and Korsten, L. 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology, 40: 411-441.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G, and Gibson, T.J. 1998. Multiple sequence alignment with clustal x. Trends in Biochemical Sciences, 23: 403-405.

Jones, A.L., and Aldwinckle, H.S., (eds.) 1991. Compendium of Apple and Pear diseases. The American Phytopathological Society, St. Paul, MN.

Jung, M.K., and Oakley, B.R. 1990. Identification of an amino acid substitution in the benA β-tubulin gene of *Aspergillus nidulans* that confers thiabendazole resistance and benomyl supersensitivity. Cell Motility and the Cytoskeleton, 17: 87–94.

Juttner, F., and Watson, S.B. 2007. Biochemical and ecological control of geosmin. Applied and Environmental Microbiology, 14: 4395-4406.

Kader, A.A. 2002. Modified atmospheres during transport and storage. In: post-harvest technology of horticultural crops. University of California agriculture and natural resources publication, 3311: 135-144.

Kai, M., Effmert, U., Berg, G., and Piechulla, B. 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Archives of Microbiology, 187: 351–360.

Kaiserer, L., Oberparleiter, C., Weiler-Gorz, R., Burgstaller, W., Leiter, E., and Marx, F. 2003. Characterization of the *Penicillium chrysogenum* antifungal protein PAF. Archives of Microbiology, 180: 204-210.

Karabulat, O.A., and Baykal, N. 2004. Integrated control of postharvest diseases of peaches with a yeast antagonist, hot water and modified atmosphere packaging. Crop protection, 23: 431-435.

Karaoglanidis , G.S., Markoglou, A.N., Bardas, G.A., Doukas, G.E., Konstantinou, S., and Kalampokis, J.F. 2011. sensitivity of *Penicillium expansum* field isolates to tebuconazole, iprodione, fludioxonil and cyprodinil and characterization of fitness parameters and patulin production. International Journal of Food Microbiology, 145: 195-204.

Katzman, P.A., Hays, E.E., Cain, C.K., Van Wyk, J.J., Reithel, F.J., Thayer, S.A., Doisy, E.A., Gaby, W.L., Carroll, C.J., and Muir, R.D. 1944. Clavacin, an antibiotic substance from *Aspergillus clavatus*. Journal of Biological Chemistry, 154: 475–486.

Kaya, H.K. 2002. Natural enemies and other antagonists. pp: 189–204. In: Gaugler, R., (ed.), Entomopathogenic nematology. CABI Publishing, Wallingford, Oxon, UK.

Kikuchi, T., Kadota, S., Surhara, H., Nishi, A., and Tsudaki, K. 1981. Odorous metabolites of a fungus, *Chaetomium globosum* Kinze ex. Fr.: identification of geosmin , a musty-smelling compound. Chemical and Pharmaceutical Bulletin, 29: 1782-1784.

Koch, A.L. 1985. Biology of industrial organisms. Menlo Park, CA, USA: Benjamin/Cummings Publishing Co.

Koenraadt, H., Somerville, S.C., and Jones, A.L. 1992. Characterization of mutations in the β-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Molecular Plant Pathology, 82:1348- 1354.

Koffman, W., Penrose, L.J., Menzies, A.R., Dabis, K.C., and Kaldor, J. 1978. Control of benzimidazole-tolerant *Penicillium expansum* in pome fruit. Scientia horticulturae, 9: 31-39.

Koffmann, W., and Penrose, L.J. 1987. Fungicides for the control of blue mold (*Penicilllium* spp.) in pome fruits. Scientia Horticulturae. 31: 225–232.

Koitabashi, M., Kajitani, Y., and Hirashima, K. 2004. Antifungal substances produced by fungal strain Kyu-W63 from wheat leaf and its taxonomic position. Journal of General Plant Pathology, 70: 124-130.

Kuhajek, J.M., Jeffers, S.N., Slattery, M., and Wedge, D.E. 2003. A rapid microbioassay for discovery of novel fungicides for Phytophtora spp., Phytopathology, 93: 46-53.

Kulakiotu, E.K., Thanassoulopoulos, C.C., and Sfakiotakis, E.M. 2004. Biological control of *Botrytis cinerea* by volatiles of 'Isabella' grapes. Phytopathology, 94: 924–931.

La Guerche, S., Chamont, S., Blancard, D., Dubourdieu, D., and Darriet, P. 2005. Origin of (-)-geosmin on grapes: on the complementary action of two fungi, *Botrytis cinerea* and *Penicillium expansum*. Antonie Van Leeuwenhoek, 88: 131-139.

Lai, C.I., Fuh, Y.M., and Shih, D.Y.C. 2000. Detection of mycotoxin patulin in apple juice. Journal of Food and Drug Analysis, 8: 85-96.

Laroche, A., Gaudet, D.A., Schaalje, G.B., Erickson, R.S., and Ginns, J. 1995. Grouping and identification of low temperature basidiomycetes using mating, RAPD and RFLP analyses, Mycological Research, 99: 297-310.

Larsen, T. O. & Frisvad, J. C. 1995. Chemosystematics of *Penicillium* based on profiles of volatile metabolites. Mycological Research**,** 99: 1167-1174.

Larsen, T.O., Frisvad, J.C., Ravn, G., and Skaaning, T. 1998, Mycotoxin production by *Penicillium expansum* on blackcurrant and cherry juice. Food Additives and Contaminant s, 15: 671- 675.

Lee, C.U. 1985. Tolerance of apple blue mould (*Penicillium expansum*) to various fungicides. Korean Journal of Plant Pathology, 1: 128-135.

Lennox, C. L., Spotts, R.A., and Cervantes, L.A. 2003. Populations of *Botrytis cinerea* and *Penicillium* spp. on pear fruit, and in orchards and packinghouses, and their relationship to postharvest decay. Plant Disease, 87: 639-644.

Leroux, P. 1996. Recent developments in the mode of action of fungicides. Pesticide Science, 47: 191-197.

Li, J.,Katiayar, S.K., and Edlind, T.D. 1996. Site-directed mutagenesis of *Saccharomyces cerevisae* β-tubulin: interaction between residue 167 and benzimidazole compounds. FEBS Letters, 385: 7–10.

Li, H.X., and Xiao, C.L. 2008. Baseline sensitivities to fludioxonil and pyrimethanil in *Penicillium expansum* populations from apple in Washington State. Postharvest Biology and Technology, 47: 239–245.

Lindroth, S., and Niskanen, A. 1978. Comparison of potential patulin hazard in homemade and commercial apple products. Journal of Food Science, 43: 446-448.

Llewellyn, G.C., McCay, J.A., Brown, R.D., Musgrove, D.L., Butterworth, L.F., Munson, A.E., and White, K.L. 1998. Immunological evaluation of the mycotoxin patulin in female B6C3F1 mice. Food and Chemical Toxicology, 36: 1107-1115.

Lund, F., and Frisvad, J.C. 1994. Chemotaxonomy of *Penicillium aurantiogriseum* and related species. Mycological Research, 98: 481–492.

Lyr, H. 1987. Modern selective fungicides: properties, applications, mechanisms of action. Gustav Fischer Verlag. New york (USA), pp: 595.

Ma, Z., Yoshimura, M.A., and Michailides, T.J. 2003. Identification and characterization of benzimidazole resistance in *Monilinia fructicola* from stone fruit orchards in California. Applied and Environmental Microbiology, 69: 7145-7152

Ma, Z., and Michailides, T.J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection, 24: 853–863.

Ma, Y., Chang, Z., Zhao, J., and Zhou, M. 2008. Antifungal activity of *Penicillium striatisporum* Pst 10 and its biocontrol effect on Phytophtora root rot of chilli pepper. Biological Control, 44: 24-31.

Magan, N., and Evans, P. 2000. Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of grain spoilage. Journal of Stored Product Research, 36: 319-340.

Marek, P., Annamalai, T., and Venkitanarayanan, K. 2003. Detection of *Penicillium expansum* by polymerase chain reaction. International Journal of Food Microbiology, 89: 139-144.

Mari, M., Bertolini, P., and Pratella, G.C. 2003. Non-conventional methods for the control of post-harvest pear diseases. Journal of Applied Microbiology, 94: 761–766.

Mari, M., Neri, F., and Bertolini, P. 2009. Management of important diseases in Mediterranean high value crops. Stewart Postharvest Review, 2: 1-10.

Mari, M., Gregori, R., Guidarelli, M., and Bertolini, P. 2010. Low Oxygen effect on *Penicillium expansum* mould in stored apple, Proc. 6th International postharvest symposium, Acta Horticulturae, 877: 1607-1612.

Markoglou, A.N., Vattis, K., Dimitriadis, K., Doukas, E.G., and Ziogas, B.N. 2008. Effect of phenylpyrrole resistance mutations on mycotoxin production by *Aspergillus carbonarius* and *Penicillium expansum*. 9th International Congress of Plant Pathology. ICPP, Torino, Italy.

Markoglou, A.N., Vitoratos, A.G., Doukas, E.G., and Ziogas, B.N. 2009. Phytopathogenic and mycotoxigenic characterization of laboratory mutant strains of Fusarium verticillioides resistant to demethylation inhibiting fungicides. DPG-BCPC 3rd International Symposium-Plant Protection and Plant Health in Europe, Berlin, Germany.

Massias, M., Rebuffar, S., Molho, L., Chiaroni, A., Riche, C., and Bodo, B. 1990, Expansolides A and B: tetracyclic sesquiterpene lactones from *Penicillium expansum.* Journal of the American Chemical Society, 112: 8112-8115.

Mattheis, J.P., and Roberts, R.G. 1992. Identification of geosmin as a volatile metabolite of *Penicillium expansum*. Applied and Environmental Microbiology, 58: 3170-3172.

McCallum, J.L., Tsao, R., and Zhou, T. 2002. Factors affecting patulin production by *Penicillium expansum*. Journal of Food Protection, 65: 1937-1942.

McKinley, E.R., and Carlton, W.W. 1991. Patulin. In: *Mycotoxins and Phytoalexins*; Sharma, R.P., and Salunkhe, D.K.,(eds.); CRC Press: Boca Raton, FL, pp: 191-236.

Menniti, A.M., Neri, F., Gregori, R., and Maccaferri, M. 2010. Some factors influencing patulin production by *Penicillium expansum* in pome fruits. Journal of the Science of Food and Agriculture, 90: 2183-2187.

Mercier, J., and Wilson, C.L. 1995. Effect of wound moisture on the biocontrol by *Candida oleophila* of gray mold rot (*Botrytis cinerea*) of apple. Postharvest Biology and Technology, 6: 9–15.

Mercier, J., and Jiménez, J.I. 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus.* Postharvest Biology and Technology, 31: 1–8.

Mercier, J., and Smilanick, J.L. 2005. Control of green mold and sour rot of stored lemon by biofumigation with *Muscodor albus*. Biological Control, 32: 401–407.

Moake, M.M., Radilla-Zakour, O.I., and Worobo, R.W. 2005. Comprehensive review of patulin control methods in foods. Comprehensive Reviews in Food Science and Food Safety, 1: 18-21.

Morales, H., Marin, S., Obea, L., Patino, B., Domenech, M., Ramos, A.J., and Sanchis, V. 2008a. Ecophysiological characterization of *Penicillium expansum* population in Lieida (Spain). International Journal of Food Microbiology, 122: 243-252.

Morales, H., Sanchis, V., Coromines, J., Ramos, A,J., and Marin, S. 2008b. Inoculum size and intraspecific interactions affects *Penicillium expansum* growth and patulin accumulation in apples. Food Microbiology, 25: 378-385

Morales,H., Sanchis, V., Usall, J., Ramos Antonio J and Marín, S. 2008c. Effect of biocontrol agents Candida sake and Pantoea agglomerans on Penicillium expansum growth and patulin accumulation in apples. International Journal of Food Microbiology, 122 : 61–67.

Morales, H., Marin, S., Ramos, A.J., and Sanchis, V. 2010. Influence of post-harvest technologies applied during cold storage of apples in *Penicillium expansum* growth and patulin accumulation: a review. Food Control, 21: 953-962.

Moyano, C., G´omez, V., and Melgarejo, P. 2004. Resistance to pyrimethanil and other fungicides in *Botrytis cinerea* populations collected on vegetable crops in Spain. Journal of Phytopathology, 152: 484–490.

Murphy, P.A., Hendrich, S., Landgren, C., and Bryant, C.M. 2006. Food Mycotoxins: An update.Journal of Food Science, 71: R51-R65.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K., and Hibi, T. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Applied and Environmnetal Microbiology, 64: 3983–3988.

Nakaune, R., Hamamoto, H., Imada, J., Akutsu, K., and Hibi, T. 2002. A novel ABC transporter gene, PMR5, is involved in multidrug resistance in the phytopathogenic fungus *Pencillium digitatum*. Molecular Genetics and Genomics, 267: 179–185.

Naqvi, S.A.M.H. 2004: Diseases of Fruits and Vegetables, Diagnosis and Management (Vol I). Kluwer Academic Publishers, New York, pp: 679.

Neumann, G.L., Winter, E.H., and Pittis, J.E. (1992). Pyrimethanil: a new fungicide. Brighton Crop Protection Conference, Pests and Diseases, 1: 395–402.

Oosterhuis, H.K. 1956. Antibiotica Uit Schimmels. Amsterdam (the Netherlands): Bijzonderheden. pp: 116.

Orbach, M.J., Porro, E.B., and Yanofsky, C. 1986. Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Molecular and Cellular Biology, 6: 2452–2461.

Ostry, V,, Skarkova, J., and Ruprich, J. 2004. Occurrence of *Penicillium expansum* and patulin in apples as raw materials for processing of foods - case study. Mycotoxin Research, 20: 24-28.

Paolesse, R., Alimelli, A., Martinelli, E., Di Natale, C., D'Amico, A., and D'Egidio, M. G. 2006. Detection of fungal contamination of cereal grain samples by an electronic nose. Sensors and Actuators, B: Chemical, 119: 425-430.

Papadopoulou-Mourkidou, E. 1991. Postharvest applied agrochemicals and their residues in fresh fruits and vegetables. Journal of the Association of official Analytical Chemists, 74, 743–765.

Peterson, S.W. 2000. Phylogenetic analysis of *Penicillium* based on ITS and LSU-rDNA sequences. In: Samson, R.A., and Pitt, J.I. (eds). *Classification of Penicillium and Aspergillus:* Integration of modern taxonomic methods: 163–178. Harwood Publishers, UK.

Paterson, R.R.M., Simmonds, M.S.J., and Blaney, W.M. 1987**.** Mycopesticidal effects of characterized extracts of *Penicillium* isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptora littoralis*. Journal Invertebrate Pathology, *50*: 124-133.

Paterson, R.R.M., and Kemmelmeier, C. 1989**.** Gradient high-performance liquid chromatography using alkylphenone retention indices of insecticidal extracts of *Penicillium* strains. Journal of Chromatography, *483*: 153-168.

Paucod, J.C., Krivobok, S., and Vidal, D. 1990. Immunotoxicity testing of mycotoxins T-2 and patulin on Balb/c mice. Acta Microbiologica et Immunologica Hungarica, 37: 143-146.

Pelloux-Prayer, A., Priem, B., and Joseleau, J. 1998. Kinetic evaluation of conidial germination of Botrytis cinerea by a spectrofluorometric method, Mycological Research, 102: 320-322.

Pettit, R.K., Weber, C.A., Kean, M.J., Hoffman, H., Pettit, G.R., Tan, R., Franks, K.S., and Horton, M.L. 2005. Microplate Alamar Blue Assay for *Staphylococcus epidermis* biofilm susceptibility testing. American society for microbiology,49 (7): 2612-2617.

Pianzzola, M.J., Moscatelli, M., and Vero, S. 2004. Characterization of *Penicillium* isolates associated with blue mould on apple in Uraguay. Plant Disease, 88: 23-28.

Pijls, C.F.N., Shaw, M.W., and Parker, A. 1994. A rapid test to evaluate in vitro sensitivity of *Septoria tritici* to flutriafol, using a microtiter plate reader. Plant Pathology, 43: 726-732.

Pitt, J.I. 1988. A Laboratory Guide to Common *Penicillium* Species, 2nd ed. CSIRO Division of Food Processing, North Ryde, New South Wales, Australia.

Pitt, J.I. 1991. Distribution of ubiquinones in *Penicillium* and related genera. Mycological Research, 95: 705-711.

Pitt, J.I. 1997. Toxigenic *Penicillium* species. In: Doyle, M.P., Beuchat, L.B., and Montville, T.J. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, DC, pp: 406-418.

Pitt, J.I., and Hocking, A.D. 1997. Fungi and Food Spoilage. Chapman & Hall, New york.

Pratella, G.C. 2000. Note di bio-patologia e tecnica di conservazione-trasporto dei frutti. La mela: terza parte. Frutticoltura, 10: 93-95.

Prusky, D., Bazak, M., and Ben-Arie, R. 1980. Tolerance of *Penicillium expansum* in Israel to postharvest fungicide treatments. Proc. 5th Congress. Mediterranean Phytopathological Union, 1: 158-159.

Prusky, D., Bazak, M., and Ben-Arie. 1985.Development, persistence, survival, and strategies for control of thiabendazole- resistant strains of *Penicillium expansum* on pome fruits. Phytopathology, 75: 877-882.

Qin, G., Tian, S.P., and Xu, Y. 2004. Biocontrol of postharvest diseases on sweet cherries by four antagonistic yeasts in different storage conditions. Postharvest Biology and Technology, 31: 51–58.

Rampersad, S.N. 2011. A rapid colorimetric microtiter bioassay to evaluate fungicide sensitivity among *Verticillium dahliae* isolates. Plant Disease, 95: 248-255.

Richards, K.L., Anders, KR., Nogales, E., Schwartz, K., Downing, K.H., and Botstein, D. 2000. Structure–function relationships in yeast tubulins. Molecular Biology of the Cell, 11: 1887–903.

Riley, R.T., and Showker, J.L. 1991. The mechanism of patulin's cytotoxicity and the antioxidant activity of indole tetramic acids. Toxicology and Applied Pharmacology, 109: 108-126.

Romano, M.L., Gullino, M.L., and Garibaldi, A. 1983. Evaluation of the sensitivity to several fungicides of post-harvest pathogens in North-western Italy. Meded. Fac. Landbouww. University of Ghent, 48: 591–602.

Rosenberger, D.A. 1990. Blue mold. In: Jones, A.L., Aldwinckle, H.S. (Eds.), Compendium of Apple and Pear Diseases. American Phytopathological Society Press, St. Paul, MN, pp. 54–55.

Rosenberg, D.A. 2003. Control of *Penicillium expansum* during apple harvest and storage. In: Patulin technical symposium. Feb 18-19, 2003; Kissimmee, Fla.; National Centre for Food safety and Technology; Summit,Ill.

Rosenberger, D.A., and Meyer, F.W. 1979. Benomyl-tolerant *Penicillium expansum* in apple packinghouses in eastern New York. Plant Disease Reporter, 63: 37–40.

Rosenberger, D.A., and Meyer, F.W. 1985. Negatively correlated crossresistance to diphenylamine in benomyl-resistant *Penicillium expansum*. Phytopathology, 75: 74–79.

Rosenberger, D.A., Wicklow, D.T., Korjagin, V.A., and Rondinaro, S.M., 1991. Pathogenicity and benzimidazole resistance in *Penicillium* species recovered from flotation tanks in apple packinghouses, Plant Disease, 75 : 712-715.

Rosenberger, D.A., Meyer, F.W., Ahlers,C.A., and Van Camp, K.L. 2002. Controlling *Penicillium expansum* in apples in postharvest applications of fludioxonil. Phytopathology, 92: S145.[Abstr.].

Rosslenbroich, H.J., and Stuebler, D. 2000. *Botrytis cinerea*-history of chemical control and novel fungicides for its management. Crop Protection, 19: 557–561.

Russell, P.E. 2004. Sensitivity Baselines in Fungicide Resistance Research and Management. FRAC Monograph No. 3. Crop Life International, Brussels, Belgium.

Safferman, R.S., Rosen, A.A., Mashni, C.I., and Morris, M.E. 1967. Earthy-smelling substance from a blue-green alga. Environmental Science and Technology, 1: 429-430.

Sanchez-Torres, P., and Tuset, J.J. 2011. Molecular insights into fungicide resistance in sensitive and resistant *Penicillium digitatum* strains infecting citrus. Postharvest biology and technology, 59: 159-165.

Sanderson, P. G., and Spotts, R. A. 1995. Postharvest decay of winter pear and apple fruit caused by species of *Penicillium*. Phytopathology, 85:103-110.

Santos, I.M., Abrunhosa, L., Venancio, A., and Lima, N. 2002. The effect of culture preservation techniques on patulin and citrinine production by *Penicillium expansum* Link. Letters in Applied Microbiology, 35: 272-275.

Sanzani, S.M. 2007. Activity and modeof action of selected phytoalexins in controlling blue mould and patulin accumulation on apples. Faculty of Agronomy, Department of Plant Protection and Applied Microbiology, University of Bari, Italy, PhD thesis, pp: 172.

Schmidt, L. S., Ghosoph, J. M., Margosan, D. A., and Smilanick, J. L. 2006. Mutation at β- tubulin codon 200 indicated thiabendazole resistance in *Penicillium digitatum* collected from California citrus packinghouses. Plant Disease, 90: 765-770.

Schöler, C.E.G., Gürtler, H., Petersen, R., Molin, S., and Wilkins, K.. 2002. Volatile metabolites from actinomycetes. Journal of Agricultural Food Chemistry, 50: 2615–2621.

Shapira, R., Paster, N., Menasherov, O.E.M., Mett, A., Salomon, R. 1996. Detection of aflatoxigenic molds in grains by PCR. Applied and Environmental Microbiology, 26: 3270– 3273.

Shewleft, R.L. 1986. Postharvest treatment for extending the shelf life of fruits and vegetables. Food Technology, 40: 70-80.

Sholberg, P.L., and Haag, P.D. 1996. Incidence of postharvest pathogens of stored apples in British Columbia. Cananadian Journal of Plant Pathology, 18: 81–85.

Sholberg, P.L., Bedford, K., and Stokes, S. 2005a. Sensitivity of *Penicillium* spp. And *Botrytis cinerea* to pyrimethanil and its control of blue and gray mould of stored apples. Crop Protection, 24: 127-134.

Sholberg, P.L., Harlton, C., Haag, P., Levesque, C.A., O'Gorman, D., and Seifert, K. 2005b. Benzimidazole and diphenylamine sensitivity and identity of *Penicillium* spp. that cause postharvest blue mould of apples using β-tubulin gene sequences. Postharvest Biology and Technology, 36: 41-49.

Sitton, J. W., and Patterson, M. E. 1992. Effect of high-carbon dioxide and low oxygen controlled atmospheres on postharvest Decay of Apples. Plant Disease, 76: 992–995.

Skouboe, P., Boysen, M., Pedersen, L.H., Frisvad, J.C., and Rossen, L. 1996. Identification of *Penicillium* species using the internal transcribed spacer (ITS) regions. In: Fungal identification techniques, pp : 160-164. Rossen, L., Rubio, V., Dawson, M.T., and Frisvad, J.C. (Eds.). European Commission, Brussels.

Skouboe, P., Frisvad, J. C., Taylor, J. W., Lauritsen, D., Boysen, M. and Rossen L. 1999. Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. Mycological Research, 103: 837-881.

Skouboe, P., Taylor, J.W., Frisvad, J.C., and Rossen, L. 2000. Molecular methods for differentiation of closely related *Penicillium* species. In: Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification, pp: 171-180. Samson, R.A., and Pitt, J.I. (Eds.). Harwood Academic Publishers, Amsterdam.

Smedsgaard, J., and Frisvad, J.C. 1996. Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. Journal of Microbiological Methods, 25: 5–17.

Smilanick, J.L., Denis-Arrue, R. 1992. Control of green mold of lemons with *Pseudomonas* species. Plant Disease, 76: 481–85.

Smilanick, J.L., Mansour, M.F., Gabler, M.F., and Goodwine, W.R. 2006. The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mould after harvest. Postharvest Biology and Technology, 42: 75-85.

Smith, C.M. 1988. History of benzimidazole use and resistance. In: Delp, C.J. (Ed.), Fungicide Resistance in North America. APS Press, St. Paul, MN, pp: 23-24.

Smith, E.E., Duffus, E.A., and Small, M.H. 1993. Effects of patulin on postimplantation rat embryos. Archives of Environmental Contamination and Toxicology, 25: 267–270.

Snowdon, A.L. 1990. Postharvest diseases and disorders of fruits and vegetables, Vol1. General introduction and fruits, CRC Press, Inc., Boca Raton, pp: 302.

Sommer, N.F., Fortlage, R.J., Buchanan, J.R. and Kader, A.A. *1981*. Effect of oxygen on carbon monoxide suspension of postharvest pathogen of fruits. Plant Disease, 65: 347-349.

Sommer, N.F. 1985. Strategies for control of postharvest diseases of selected commodities. In: Postharvest Technology of Horticultural Crops, Kader, A., Kasmire, R.F., Mitchell, F.G., Reid, M.S., Sommer, N.F., and Thompson, J.F. (eds), pp: 83-99, Cooperative Extension, University of California.

Sönnichsen, R., and Keller, R. 1997. Methodische Grundlagen zur Identifizierung der flüchtigen organischen Stoffwechselprodukte von Schimmelpilzen mittels GC-MSD und Thermodesorption ± Schriftenreihe des Institutes für Medizinische Mikrobiologie und Hygiene der Medizinischen Universität Lübeck, (R. Keller, eds.), Heft 1, pp: 161-192. Lübeck.

Spadaro, D., and Gullino, M.L. 2004. State of art and future perspectives of biological control of postharvest fruit diseases. International Journal of Food Microbiology, 91: 185–194.

Spiegel, J., and Stammler, G. 2006. Baseline sensitivity of *Monilinia laxa* and *M. fructigena* to pyraclostrobin and boscalid. Journal of Plant Diseases and Protection, 113 (5): 199–206.

Spotts, R.A., and Cervantes, L.A. 1986. Populations, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp. and *Mucor piriformis* in packinghouses. Plant Disease, 70: 106-108.

Spotts, R.A., and Cervantes, L.A. 2001. Disease incidence-inoculum dose relationships for *Botrytis cinerea* and *Penicillium expansum* and decay of pear fruit using dry, airborne conidia. Plant Disease, 85: 755-759.

Spotts, RA., Cervantes, L.A. 1993. Filtration to remove spores of *Penicillium expansum* from water in pome fruit packinghouses. Tree Fruit Postharvest Journal, 4: 16–8.

Spotts, R.A., Holmes, R.J., and Washington, W.S. 1988. Sources of spores and inoculum concentration related to postharvest decay of apple and pear. Australian Journal of Plant Pathology, 17: 48– 52.

Stammler, G., and Speakman, J. 2006. Microtiter Method to Test the Sensitivity of *Botrytis cinerea* to Boscalid. Journal of Phytopathology, 154: 508–510.

Stinson, A..M., Zidack, N.K., Strobel, G.A., and Jacobsen, B.J. 2003. Mycofumigation with *Muscodor albus* and *Muscodor roseus* for control of seedling diseases of sugar beet and Verticillium wilt of eggplant. Plant Disease, 87: 1349–1354.

Stockwell, V.O., and Stack, J.P. 2007. Using *Pseudomonas* spp. for integrated biological control. Phytopathology, 97: 244–249.

Stott, W.T., and Bullerman, L.B. 1975. Patulin: a mycotoxin of potential concern in foods. Journal of Milk and Food Technology, 38: 695-705.

Strange, R.N., and Scott, P.R. 2005. Plant disease: a threat to global food security. Annual Review of Phytopathology, 43: 83-116.

Strobel, G.A., Dirkse, E., Sears, J., and Markworth, C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology, 147: 2943-2950.

Strobel, G.A., Kluck, K., Hess, W.M., Sears, J., Ezra, D., and Vargas, P.N., 2007. Muscodor albus E-6, an endophyte of Gauzuma ulmifolia, making volatile antibiotics: isolation, characterization and experimental establishment in the host plant. Microbiology, 153: 2613-2620.

Subramanian, C.V. 1983. Hyphomycetes Taxonomy and Biology. Academic Press, London.

Sugar, D., and Spotts, R.A. 1999. Control of postharvest decay in pear by four laboratory grown yeasts and two registered biocontrol products. Plant Disease, 83: 155-158.

Sunesson, A.L., Nilsson, C.A., Carlson, R., Blomquist, G., and Andersson, B. 1997. Production of volatile metabolites from *Streptomyces albidoflavus* cultivated on gypsum board and tryptone glucose extract agar-influence of temperature, oxygen and carbon dioxide levels. The Annals Occupational Hygiene, 41: 393-413.

Svendsen, A., and Frisvad, J.C. 1994. A chemotaxonomic study of the terverticillate penicillia based on high performance liquid chromatography of secondary metabolites. Mycological Research, 98: 1317-1328.

Sydenham, E.W., Vismer, H.F., Marasas W.F.O., Brown N., Schlechter M., Westhuizen L., and Rheeder J.P. 1995. Reduction of patulin in apple juice samples-influence of initial processing. Food Control, 6: 195-200.

Taniwaki, M.H., Hocking, A.D., Pitt, J.I., and Fleet, G.H. 2001. Growth of fungi and mycotoxin production on cheese under modified atmospheres. International Journal of Food Microbiology, 68(1-2): 125-33.

Tepper, B.L., and Yoder, K.S. 1982. Postharvest chemical control of Penicillium blue mold of Apple. Plant Disease, 66: 829-831.

Thomas, J.H., Neff, N.F., and Botstein, D. 1985. Isolation and characterization of mutations in the beta-tubulin gene of *Saccharomyces cerevisiae*. Genetics, 111: 715–734.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The Clustal X windows interface flexible strategiesfor multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 24: 4876-4882.

Thompson, J.R., and Latorre, B.A. 1999. Characterization of *Botrytis cinerea* from table grapes un chile using RAPD-PCR, Plant Disease, 83: 1090-1094.

Vespermann, A., Kai, M., and Piechulla, B. 2007. Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Applied and Environmental Microbiology, 17: 5639-5641.

Vinas, I., Usall, J., and Sanchis, V. 1991. Tolerance of *Penicillium expansum* to postharvest fungicide treatments in apple packinghouses in Lerida (Spain). Mycopathologia, 113: 15-18.

Vinas, I., Vallverdu, N., Monllao, S., Usall, J., and Sanchis, V. 1993. Imazalil resistant *Penicillium* isolates from Spanish apple packinghouses. Mycopathologia, 123: 27-33.

Wady, L., Bunte, A., Pehrson, C., and Larsson, L. 2003. Use of gas chromatography-mass spectrometry/solid phase microextraction for the identification of MVOCs from moldy building materials. Journal of Microbiological Methods, 52: 325–332.

Wady, L., and Larsson, L. 2005. Determination of microbial volatile organic compounds adsorbed on house dust particles and gypsum board using SPME/GC-MS. Indoor Air, 15: 27–32.

Wan, M., Li, Guoqing., Zhang, J., Jiang, D., and Huang, H.C. 2008. Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. Biological Control, 46: 552–559.

WAPA-world apple and pear association. 2011. WAPA releases European apple and pear crop forecasts. www.**wapa**-association.org *(Online).*

Watkins, C., Erkan, M., Nock, J., Beaudry, R., and Moran, R. 2005. Harvest date effects on maturity, quality, and storage disorders of 'Honeycrisp' apples. HortScience, 40: 164-169

Welsh, F.W., Murray, W.D., and Williams, R.E. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. Critical Reviews in Biotechnology, *9*: 105-169.

Wheeler, J.L., Harrison, M.A., and Koehler, P.E. 1987. Presence and stability of patulin in pasteurised apple cider. Journal of Food Science, 52: 479-480.

WHO and Joint FAO/WHO Expert Committee on Food Additives (JECFA). 1998. Position paper on patulin (prepared by France), 30th session. The Hague (the Netherlands): WHO and Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Wilson, D.M., and Nuovo, G.J. 1973. Patulin Production in Apples Decayed by *Penicillium expansum*. Applied Microbiology, 26: 124-125.

Wilson, C.L., and Wisniewski, M.E. (eds). 1994. Biological Control of Postharvest Diseases of Fruits and Vegetables—Theory and Practice. CRC Press, Boca Raton, Florida, pp: 182.

Xiao, C.L., and Boal, R.J. 2009. Preharvest application of a boscalid and pyraclostrobin mixture to control postharvest gray mould and blue mould in apples. Plant Disease, 93: 185-189.

Yang, L., Xie, J., Jiang, D., Fu, Y.,, Li, G., and Lin, F. 2008. Antifungal substances produced by *Penicillium oxalicum* strain PY-1 potential antibiotics against plant pathogenic fungi. World Journal of Microbiology and Biotechnology, 24: 909-915.

Yurdun, T., Omurtag, G.Z., and Ersoy, O. 2001. Incidence of patulin in apple juices market in Turkey. Journal of food protection, 64: 1851-1853.

Zhang, S.Y., Panaccione, D.G., Gallegly, M.E., and Zhang, S.Y. 1997. Metalaxyl stimulation of growth of isolates of *Phytophtora infestans*, Mycologia, 89: 289-292.

Zhou, G., Whong, W.Z., Ong, T., and Chen, B. 2000. Development of a fungal-specific PCR assay for detecting low-level fungi in an indoor environment. Molecular and Cellular Probes, 14: 339– 348.

Zhou, T., Northover, J., Schneider, K.E., and Lu, X. 2002. Interaction between *Pseudomonas syringae* MA-4 and cyprodinil in the control of blue mould and gray mould of apple. Canadian Journal of Plant Pathology, 24: 154-161.

Zhou, T., Yu, H., and Errampalli, D. 2005. Strategies for biological control of fungal diseases of temperate fruits. In: Chincholkar, S.B. (Ed.), Management of Fruit and Vegetable Diseases, vol. IV. Biological Control. Kluwer Academic, Netherlands (in press).

Zureik, M., Neukirch, C., Leynaert, B., Liard, R., Bousquet, L., and Neukirch, F. 2002. Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey. British Medical Journal, 325: 411–414.