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Biological Control of Post-harvest Disease of Blue Mould (*Penicillium expansum*) of Pear Fruit by using Antagonist Microorganisms under Laboratory and Cold Storage Conditions

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Abstract

Penicillium expansum (Link) is one of the most important postharvest pathogens of stored pear fruit worldwide. It causes blue mold rot, a decay that can lead to significant economic losses during storage, which can also impact fruit destined for processing due to the production of carcinogenic mycotoxin patulin and citrinin. In the present study, microbial antagonist of *Trichoderma harzianum* have been isolated from mushroom compost and tested for antagonistic properties against *P. expansum*. Assays of biological control of *P. expansum* on pear fruits were carried out under cold storage condition artificially, by two different methods of inoculation. Significant differences (p<0.0001) were obtained in reducing the lesion diameters of blue mould on wounded pear fruits inoculated artificially either by Dipping or spray with *T. harzianum* in comparison with the control treatment. There were also significant differences between mean depth of infection in dipping treatment compared to spay method and control, whereas no significant differences were appeared between depth of infection in spray treatment compared to control. Furthermore, results obtained in this study demonstrated that *T. harzianum* would be an interesting microorganism to be used as a biocontrol agent and could be considered as one of the most promising alternatives for postharvest control of *P. expansum*. Therefore, integration of *T. harzianum* by artificially inoculation was effective and might be a safe strategy to control *P. expansum* infection in pear fruit, and will definitely become an internationally adopted practice.

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Keywords

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Introduction

The production of pears is a complex process involving orchard, storage and marketing phases. Modern postharvest technologies make the long-term storage of pears possible (Rosenberger, 1990; Batta, 2004; Barnes, 1979). Pear fruits are attacked by a wide range of pathogens (fungi or bacteria) in their postharvest phase (Snowdon, 1990; Monte, 2001; Vinãs, 2004; Batta, 2003). Postharvest diseases can be a limiting factor for the long-term storage of pear fruits and losses have been estimated to range from 10 to 30% of the harvested fruits per year (Snowdon, 1990), depending on cultivar and length of storage for fruit kept in controlled

atmosphere storage (Rosenberger, 1990; Snowdon, 1990), despite the use of modern storage facilities and techniques (Zhang et al., 2005 and 2008).

Blue mould decays caused by *P. expansum* Link is one of the major and most important post-harvest disease of pear fruits in the producer countries (Snowdon, 1990; Lima et al.,1998, Zhang et al., 2008) and is considered the most important decaying pathogen on stored pears worldwide (Pusey and Wilson,1984; Janisiewicz and Korsten, 2002; Rosenberger, 1990; Wilson et al., 1991). It causes also significant economic losses to the fresh-fruit during storage that impacts fruit destined for processing (Spotts, 1984; Zhang et al., 2008; Batta, 2004).

Besides its economic impact, *P. expansum* is also of potential public health significance since it produces the carcinogenic mycotoxin known to cause immunological, neurological, and gastrointestinal toxic effects in animal models (Batta, 2001; spotts, 1984). Further, exposure to high levels of patulin results in vomiting, salivation, anorexia, polypnea, weight loss, leukocytosis, erythropenia, and necropsy lesions of hemorrhagic enteritis in piglets (Rifai, 1969; Monte, 2001).

Patulin is a polar compound exhibiting antibiotic, mutagenic and immunotoxin properties (Batta, 2003), that cause acute and chronic toxicity (Janisie, 1987; Droby et al., 2009). Patulin can also induce the formation of cancerous tumors and to cause genetic mutations and embryonic developmental defects (Bisset, 1984; Batta, 2003). Furthermore, patulin causes impairment of kidney functions, oxidative damage, and weakness to the immune system. It also has a negative impact on reproduction in males via interaction with hormone production (Lima et al., 1998; Batta, 2001; Batta, 2003). The fungus can enter the fruit through natural openings, pedicel vascular system (Conway, 1992), lenticels, stem and the calyx ends (Holmes 2005; Snowdon, 1990), or directly through the cuticle and epidermis (Batta, 2001). Conidia of *P. expansum* infect pear fruits primarily through microwounds produced during fruit harvesting and handling (Conway et al., 1992; Janisiewicz et al., 2001).

Pear fruits infected in the field may not develop symptoms until stored. During storage, airborne conidia spread quickly from infected fruits that are always present in packing houses (Rosenberger, 1990; Sanderson and Spotts, 1995; Droby et al., 2009; Sharma et al., 2009; Zhang et al., 2005; Wilson and Pusey, 1985; Wilson et al., 1991). Symptoms of blue mould appear as light tan to dark brown (Sharm et al., 2009). The decayed tissue is soft due to maceration of the tissue by polygalacturonase enzyme which plays a significant role in *P. expansum* virulence (Sharma et al., 2009; Nunes et al., 2001).

Decayed tissue can be readily separated from the healthy tissue, blue or -green spore masses may appear on the surface of decayed area, starting at the infection site (Sanderson and Spotts, 1995; Sharma et al., 2009). In time, the entire fruit can become dry and mummified under moist conditions, a soft, wet mass (Lima et al., 1998; Holmes, 2005). Accurate diagnosis of postharvest diseases is the first step to implement relevant measures to control the problems (Snowdon, 1990; Rosenberger, 1990; Wilson et al., 1991).

During the post-harvest storage of fruits chemical control is commonly applied to reduce the incidence of blue mould in pears that are stored for long periods of time in storage (Sharma et al., 2009; Samules, 1996). However, due to the increasing concern for the environment , public safety , and demand for healthy food (Rosenberger, 1990; Lima et al., 1998), resulted in cancellation some of the most effective fungicides and develop alternative control method that should be both effective and economically feasible (Liske et al., 1996; Janisiewicz and Korsten, 2002).

Of these methods, biocontrol of postharvest fruit decay using antagonistic microorganisms has been considered a desirable method to synthetic fungicides. Bacterial and yeast antagonists have being commercially developed to control postharvest diseases on pome fruits (Janisiewicz and Marchi, 1992; Janisiewicz and Korsten, 2002; Zhang et al., 2007). The biological control of postharvest diseases by using antagonistic yeasts is one of the most promising alternatives to fungicides (Janisiewicz and Korsten, 2002; Zhang et al., 2005; Zhang et al., 2007; Droby et al., 2009).

Biocontrol of fruit diseases has shown great promise in recent years (Wilson and Pusey, 1985; Pusey and Wilson, 1984). Progress has been made particularly in postharvest of pome fruits (Janisiewicz, 1997). Blue mold and blue mold decay of apples and pears, caused by *Botrytis cinerea* and *P. expansum*, respectively, have been controlled by bacterial and yeast antagonists (Janisiewicz, 1998; Janisiewicz and Marchi, 1992; Lima

et al., 1998) have been obtained good results by applying antagonists on fruit surface (Janisiewicz, 1987). Some components of the microbial community present on the surface of fruit, such as bacteria and yeasts showed a significant antagonistic activity against *P. expansum* of blue mould on apple and pear fruits (Fravel, 2002; Janisiewicz and Korsten, 2002; Droby et al., 2009; Wilson and Pusey, 1985).

Among different microbial antagonists, *Trichoderma harzianum* Rifai have been investigated of special interest due to their general lack of antibiotic production (Samules, 1996; Rifia, 1969), rapid colonization of the fruit surface and wound sites (Monte, 2001).

Fungal antagonist of *T. harzianum* is an effective antagonist against other fungi especially *B. cinerea* on strawberry and apple fruits in similar emulsion formulations (Batta, 2001, 2003 and 2004), and has been shown to reduce postharvest decay of apples caused by *P. expansum*, especially when spores of the antagonistic fungus are formulated in an invert emulsion, water-in-oil type (Batta, 2004).

In addition, *T. harzianum* has been effectively used against pathogenic fungi, *P. expansum* on pear fruits under laboratory tests (Fravel, 2002; Batta, 2001). Finally, there is a gap between the small scale experiment and the commercial use of such methods in agriculture. This is partly because biological control is not yet fully developed and it has been difficult to obtain consistent I results over years (Fravel, 2002; Batta, 2003; 2004).

Objectives of the study

The objectives of this study were to investigate the effectiveness of antagonist *T. harzianum* against the blue mold of pear fruits caused by *P. expansum*.

Materials and methods

Fruits collection

In the 2015 growing season, Pears of the cultivars "Harme naska" were obtained from four storage facilities in Sulaimani. Pears were harvested at commercial maturity from Sharbajher region in Sulaimania, North Iraq. Pear fruits were selected for free of wounded, rots, and as much as possible homogeneous in maturity and size. After being immersed in a solution of 0.1% sodium hypochlorite (1% as chlorine) for 1 min, the samples were washed in flowing tap water and allowed to air dry at room temperature (20°C). The fruit were used after a short time of storage at 1°C (no longer than 3 months).

Isolation of pathogen *P. expansum*

Decayed pear fruits were collected during the storage period. Isolation of pathogen *P. expansum* were isolated from infected pear fruits of cultivars "Harme naska" that showed the typical symptoms of blue mould. Isolation of *P. expansum* from decayed pear fruits were performed by direct plating of the inner decayed tissue after surface sterilization with 70% ethanol and aseptically removal of skin. Tissue fragments were placed on potato dextrose agar (PDA) medium in Petri plates and incubated at room temperature in the dark for 5 days.

Following incubation, the plates were examined and fungal cultures were sub cultured and transferred to sterile PDA plates. Hyphal growth and morphology was examined with an Olympus BH2 microscope. Pure colonies were then used to obtain monosporic cultures using the streaking method. Monosporic cultures were preserved on slants in the refrigerator at 4°C during this study. Two isolate of *P. expansum* were verified and confirmed by Department of Plant and Environmental Science, University of Copenhagen, Denmark.

Morphological investigations

Isolates *P. expansum* grown on PDA were identified through their colony morphology, colour, growth habits and conidial and conidiophore morphology. Colony appearance, exudate production, pigmentation, and reverse colouration were assessed, and colony diameters were measured. Morphological identification methods were used to exam hyphal growth with an Olympus BH2 microscope.

Molecular identification of strain PE-L

In order to confirm the accuracy of morphological identification, the isolated strain was subjected to molecular analysis with fungus-specific universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Genomic DNA of the strain was obtained following the methodology previously described by Yang et al. (2006). Genomic DNA was

used for PCR amplification. All amplification reactions were carried out in volumes of 40 µl containing 2.0 µl of template DNA, 1 µl of each primer (20 µM), 5.0 µl of 5X PCR buffer, 5.0 µl of NaCl2 (25 mM), 2.0 µl of dNTPs (4 mM), 2.0 µl of BSA and 0.4 µl of Taq DNA polymerase (5 U/µl).

PCR was carried out using the following condition: initial denaturation at 94°C for 5 min; 37 cycles of denaturation (94°C for 1min), annealing (54°C for 1 min), and extension (72°C for 1 min); and a final extension step at 72°C for 7 min. A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture. PCR products were detected in 2% agarose ethidium bromide gels in 1×TAE buffer (Tris–acetate 40 mM and EDTA 1.0mM).

The ITS sequence was identified by searching databases using the BLAST sequence analysis tool. The extracted PCR products were sequenced by Sequenced by CATC-Biotic for sequestering (www.gatc.biotech .com)

Pathogenicity tests

Healthy pear fruits of the cultivar Harme naska at maturity stage were used to conduct Koch's postulates. Pears were washed and surfaced sterilized using 70% ethanol. A triangle shaped pear tissue fragment (15 mm length of each side) was taken out of the fruit. Mycelial disks with spores (6mm in diameter), were cut out from 7-day old cultures grown on PDA and placed in the wounds and topped with pear fragments.

Control fruit were treated the same way, but a sterile PDA disk was placed in each wound instead of mycelium. Four fruits for each treatment were inoculated on two sides per isolate and others were used as control. Following inoculation, the pears were stored at room temperature for 7 days. The pathogen was reisolated from the lesions that developed on inoculated fruits.

Preparation of conidia suspension of *P. expansum*

Conidia suspension were obtained by flooding the sporulating of the 7 days old cultures of *P. expansum* with sterile distilled water (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Janisiewicz and Marchi, 1992). The spore concentrations were determined using a haemocytometer and adjusted with sterile distilled water as required (1×10^6 conidia/ml).

Screening for antagonists

Isolation of *Trichoderma harzianum*

T. harzianum fungus was used as antagonistic in this study, since it has been used effectively against *P. expansum* (Vinas et al., 1998; Yu et al., 2007). *Trichoderma* strains were isolated from samples of mushroom compost by the method as previously described by Janisiewicz (1987). Four samples were collected from the surface of mushroom compost with visibly colonized by *Tichoderma*. Samples were taken from the compost plant used in the Mushroom production cells at College of Agriculture, Kirkuk University, Iraq from April to July 2015.

Samples were transported in sterile polyethylene bags to the laboratory and streaked on nutrient yeast dextrose agar(NYDA), then incubated at 28°C for one week. After incubation single colonies varying in morphological appearance were selected, purified and then stored on potato dextrose agar (PDA). Isolates of *T. harzianum* were sub cultured on (PDA) plates and incubated at 22ºC and 6 hrs of illumination per day for 10-14 days in order to obtain enough quantities of fungal conidia for inoculation.

Identification of *Trichoderma harzianum*

Identification was performed by using the identification keys provided by Rifai (1969) and Bissett (1984 and 1991). The potential antagonist isolate was identified by analysing the sequence of the as described by Kurtzman and Robnett (1998) and Barnett and Hunter (1972). Two techniques, visual observation on Petri dishes and micro-morphological studies in slide cultures, were used for identification of *Trichoderma* spp. Visual observations of the fungus *Trichoderma harzianum* were made by daily examining of cultures grown on (PDA) for 7-14 days. Morphological characteristics, colony morphology, colour, the mode of mycelia growth, conidia and conidiophore morphology of all isolates were observed and compared to a taxonomic key for the genus of *T. harzianum.*

For micro-morphological studies, a slide culture technique was used (Leathy and Colwell, 1990). Examination of the shape, size, arrangement and development of conidiophores and phial-ides provided a tentative identification of *T. harzianum.* Two isolates of *T. harzianum* were verified and confirmed by

Department of Plant and Environmental Sciences, University of Copenhagen University, Denmark.

Fungal strains of *T. harzianum* collected from the surface of mushroom compost used in the Mushroom production cells. This strain was identified on the basis of its morphological characteristics and the analyses of its nuclear ribosomal internal transcribed spacer (ITS) regions. The 7-day-old fungal mycelia grown in potato dextrose agar (PDA) were collected, and the fungal genomic DNA was extracted with a lysis buffer containing 200 mM Tris−HCl (pH 8.0), 25 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), 0.2 mM NaCl, and 5% sodium dodecyl sulfate (SDS) according to the protocol developed by (Chakraboty et al., 2010). The ITS regions were amplified from genomic DNA, using a pair of universal primers, ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′), for the region containing ITS1, ITS2, and the 5.8S rDNA.30

The polymerase chain reaction (PCR) was performed through the following cycle: initial denaturation at 94°C for 2 min, 35 cycles of 94°C denaturation for 1 min, annealing (54°C) for 1 min, 72°C extension for 1 min, and a final extension step at 72 °C for 7 min. The extracted PCR products were sequenced by Sequenced by CATC-Biotic for sequestering (www.gatc.biotech.com).

Macro-/Microscopic characteristics of *T. harzianum*

The colony colour was initially watery white and turned bright green to dark green and dull green with compact conidiophores throughout the Petri plates.

Preparation and application of spore suspensions of *T. harzianum*

The fungus *T. harzianum* was introduced as conidia according to technique described above by (Batta, 2004). Spore suspension were prepared by rubbing the surface of the 10-14 days old culture of *T. harzianum* with a loop suspending the obtained material in sterile distilled water . The concentration of conidia in the introduced suspension of *T. harzianum* was adjusted to 1×10^8 /ml using a haemocytometer.

Biological efficacy evaluation of *T. harzianum*

For testing biological efficacy of *T. harzianum* against blue mould on pear fruits three of the following treatments were used:

1. *T. harzianum* + *P. expansum*
2. *P. expansum* + sterile distilled water
3. Sterile distilled water only

Artificially inoculation of healthy pear fruits was made to determine the effectiveness of the *T. harzianum* against blue mould of *p. expansum* under cold storage condition. Two different methods, dipping and spraying were used to inoculate first healthy pears by *T. harzianum* and then pathogen *P. expansum* under cold storage conditions. Control treatment was performed with only a suspension of the pathogen *P. expansum*.

Inoculation of pear fruits

Dipping method: Pear fruits were initially rinsed with distilled water for a few minutes, then punctured with a sterile needle approximately 3mm deep at its base, within 45 min of wounding, pears were inoculated by spray with conidial suspension of *P. expansum* at concentration of (1×10^6 spores/ mL^{-1}) for 30 sec. The treated pears were kept at room temperature for 2 hrs. After that pear fruits were inoculated with conidial suspension of *T. harzianum* at concentration (1×10^8 spore/ mL^{-1}) for 30 sec. After treatment, Pear fruits were incubated in dark for 18-20 hrs at room temperature, then air dried and placed on polyethylene fruit pack and stored at room temperature. Three replicates were used for each treatment and replicate consisted of 32 fruits.

Spraying method: Surface sterilized pears with distilled water for a few minutes were punctured with a sterile needles approximately 3mm deep at its base, within 45 min of wounding , pears were inoculated by spray with 30 µl of conidial suspension of *T. harzianum* at concentration of (1x 10^6 spores/ mL−1) for 30 sec. After 2h, the inoculated pears were sprayed and inoculated with 30 µl of conidial suspension of *T. harizanum* at concentration (1x 10^8 spores/ mL−1) for 30 sec. After treatment ,Pear fruits were incubated in dark for 18-20 hrs at room temperature , then air dried and placed in plastic fruit pack and stored at room temperature. There were three replicate for each treatment with 32 pear fruits per replicate.

Control (P. expansum + sterile distilled water)/1: Wounds in control fruit received an equal amount of *P.*

expansum suspension (1×10^6 spores/ mL^{-1}) by the way as described above in spray and dipping method. The number of infected pears and there lesion diameters were recorded daily.

Pear fruits were assessed for disease severity and incidence after 7, 14 and 21 days of inoculation at room temperature. Disease severity was evaluated by measuring lesion diameters (mm) at each evaluation period. Disease incidence was scored based on the number of rot spots that developed on each fruit. There were three replications of 32 fruits per treatment in a completely randomized design.

Controls (Sterile distilled water only)/2: The surface of pears were wounded with a sterile needles approximately 3 mm deep at the base, within 45 min pears were inoculated by spray and dipping in sterile distilled water. Pears were then air dried and placed on plastic fruit packs. After air-drying, the samples were stored at room temperature. The severity and incidence of decay pear fruits were recorded 7, 14 and 21 days after treatment.

Experimental design and analyses of data

The completely randomized design (CRD) was used in designing the experiments with four experimental treatments. Each treatment was replicated four times representing 32 pear fruits. Mean lesion diameter in each treatment was calculated for comparison and analysis. Data on lesion size and depth represent continuous variables and were analysed by analysis of variance assuming a normal distribution. Variances were stabilised by appropriate transformation of data if necessary. Number of lesions represents discrete variables since it was recorded how many infections occurred. Consequently, these data were analysed by logistic regression, assuming a Poisson distribution (corrected for over-dispersion when present). Hypotheses were rejected at $p<0.05$. All data were analysed by PC-SAS (release 9.4; SAS Institute, Cary, NC).

Results

The effect of treatment with *T. harzianum* on lesion size

There was a significant interaction between method (dipping/spray) and the treatments (R1, R2, R3, Control 1 and Control 2), $p<0.0001$. Therefore data were analysed separately for Dipping and Spray. Treatment with conidia suspension of *Trichoderma* + *P. expansum* was significantly different from treatment with *P. expansum* + sterile distilled water as control-1 treatment. Mean of lesion diameter within each column following different treatments were significantly different, $p<0.0001$ according to SAS program. This indicate that the antagonistic effect of *T. harzianum* against blue mould on pear fruits was significantly different compared control-1 + sterile distilled water. Significant differences ($p<0.0001$) were obtained in lesion diameter on wounded pear fruits inoculated artificially by dipping method compared to spray treatment and control -2 treatments (Table 1).

Finally, time appearance of lesions of blue mould on pear fruits was significantly different for dipping treatment compared to spray treatment. Therefore, longer time period was needed for the appearance of lesions of blue mould after artificially inoculation by dipping treatment than was needed for the treatment by spray (Fig. 1).

Table 1. Development of lesions of blue mould on pear fruits after treatment with conidia suspension of *T. harzianum* by dipping.

Treatment	Lesion size		No. of infections		Depth of infections	
R1	49.1	A	0.8	A	3.7	A
R2	88.5	B	1.6	B	2.8	A
R3	90.0	B	1.7	B	-	-
1 control	189.5	C	2.7	C	9.0	B
2 control	20.4	A	0.4	D	1.7	A
p-value	<0.0001		<0.0001		<0.0001	

Means followed by the same letter are not significantly different at $p<0.05$.

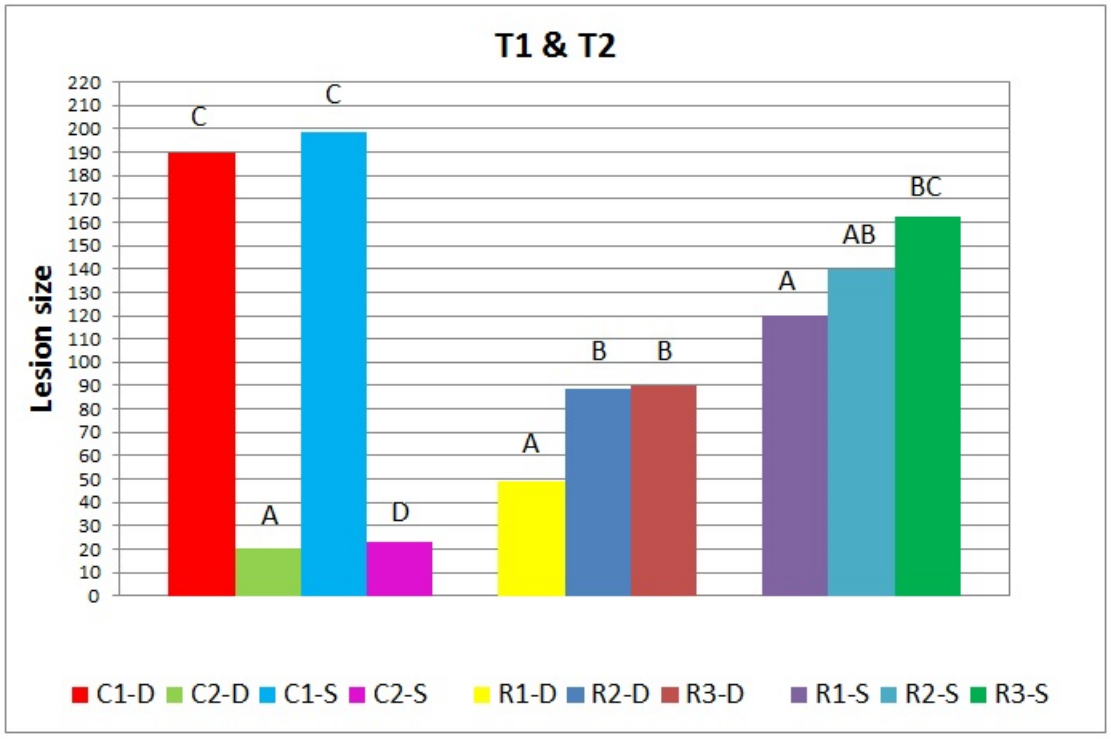

Fig. 1: Severity of blue mould on pear fruits inoculated artificially with *Penicillium expansum* by two different methods. Different alphabets indicate significant differences (p<0.0001) according to the SAS release 9.4; SAS Institute, Cary, NC). C1-D: *P. exapnasum* + Sterile distilled water; C2-D: Sterile distilled water only; C1-S: *P. expansum* + Sterile distilled water; C2-S: Sterile distilled water only (R1-D, R2-D, R3-D = Dipping treatment); R1-S, R2-S, R3-S = Spray treatment).

Effect of treatment with *T. harzianum* on number of infection

There was a significant interaction between method (dipping/spray) and the treatments (R1, R2, R3, Control 1 and Control 2), p<0.0059. Therefore data were analysed separately for Dipping and Spray (Fig. 2). There were significant (0.0059) differences in the number of infection between different methods used in artificially inoculation of pear fruits with *T. harzianum* + *P. expansum*. Mean of number infection in different columns were significantly different treatments. The highest (3.7) was obtained for spray method and lowest (0.8) for dipping method (Tables 1 and 2).

Finally, dipping treatment was found most effective compared to spray treatment, but was not significantly different compared to Control-2 (Table 1).

Table 2. Development of lesions of blue mould on pear fruits after treatment with conidia suspension of *T. harzianum* by Spray method.

Treatment	Lesion size		No. of infections		Depth of infections	
R1	120.3	A	2.4	A	12.3	A
R2	139.8	AB	3.5	B	4.0	B
R3	162.3	BC	3.7	B	-	-
1 control	198.3	C	3.1	AB	10.3	C
2 control	23.2	D	0.4	C	4.8	B
p-value	<0.0001		<0.0001		<0.0001	

Means followed by the same letter are not significantly different at p<0.05.

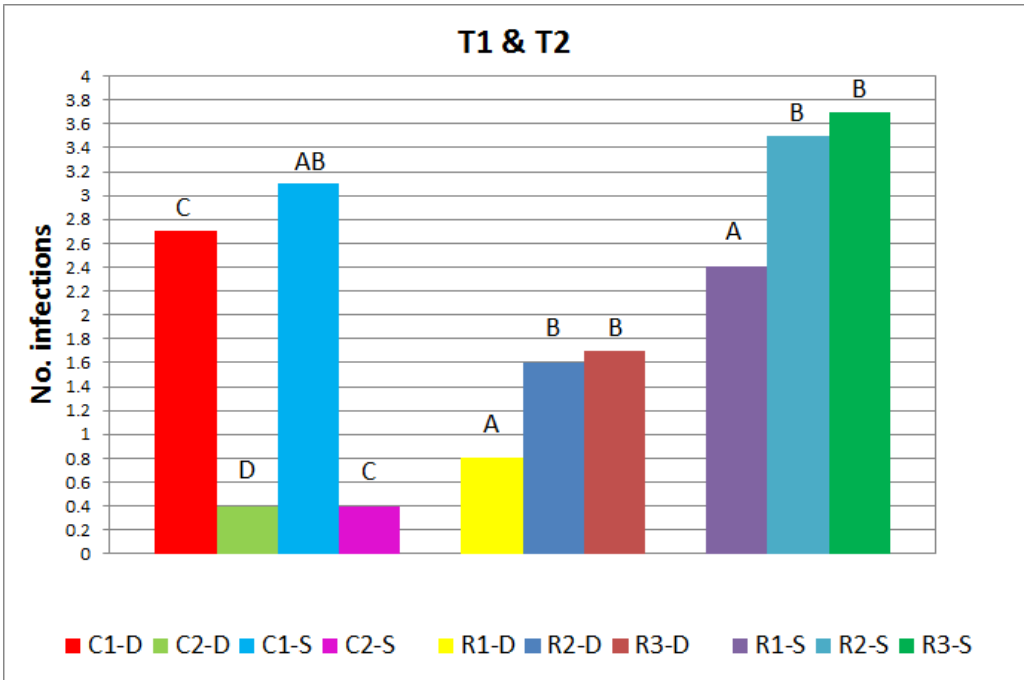

Fig. 2: Severity of blue mould on pear fruits inoculated artificially with *Penicillium expansum* by two different methods. Different alphabets indicate significant differences (p<0.0001) according to the SAS release 9.4; SAS Institute, Cary, NC). C1-D: *P. exapnasum* + Sterile distilled water; C2-D: Sterile distilled water only; C1-S: *P. expansum* + Sterile distilled water; C2-S: Sterile distilled water only (R1-D, R2-D, R3-D = Dipping treatment); R1-S, R2-S, R3-S = Spray treatment).

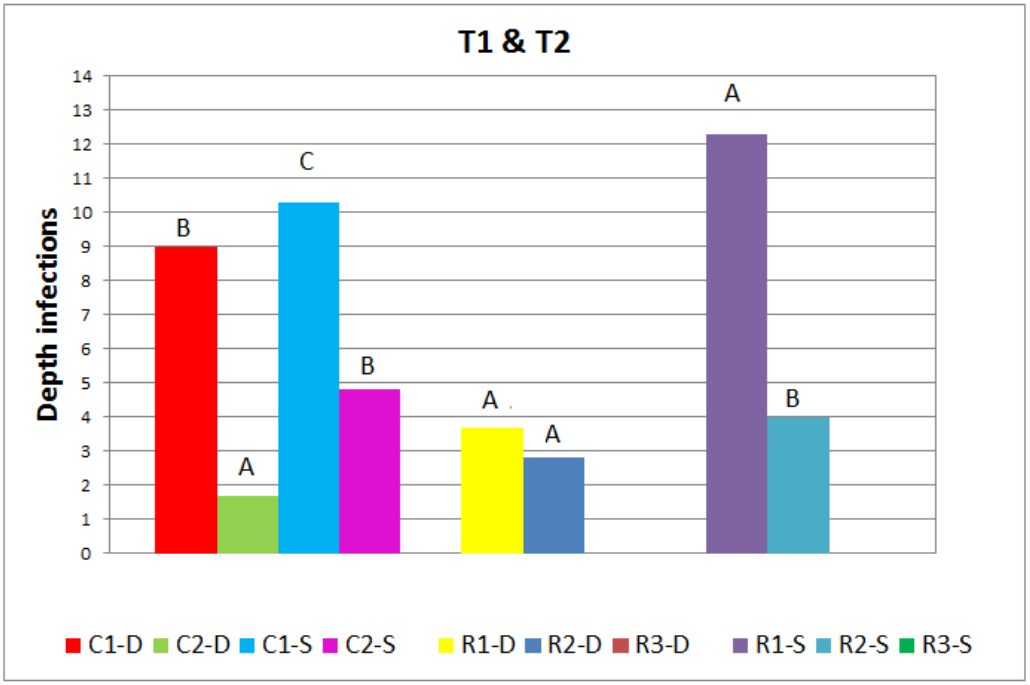

Fig. 3: Severity of blue mould on pear fruits inoculated artificially with *Penicillium expansum* by two different methods. Different alphabets indicate significant differences (p<0.0001) according to the SAS release 9.4; SAS Institute, Cary, NC). C1-D: *P. exapnasum* + Sterile distilled water; C2-D: Sterile distilled water only; C1-S: *P. expansum* + Sterile distilled water; C2-S: Sterile distilled water only (R1-D, R2-D, R3-D = Dipping treatment); R1-S, R2-S, R3-S = Spray treatment).

Effect of treatment with *T. harzianum* on depth of infection

There was a significant interaction between method (dipping/spray) and the treatments (R1, R2, R3, Control 1 and Control 2), $p<0.0001$. Therefore data were analysed separately for Dipping and Spray (Fig 3).

There were significant differences ($p<0.0001$) between mean depth of infection in dipping treatment compared to spay method and control, whereas no significant differences were appeared between depth of infection in spray treatment compared to control-2 (Tables 1 and 2). Finally dipping method was most effective treatment but not significantly different compared to control-2.

Discussion

Different treatments were applied to pear fruits as described above, the fruits were wounded and inoculated with pathogens. They were stored at room temperature for different periods. The control fruits were not wounded but inoculated with the pathogen *P. expansum*. Treatment with the conidia suspension of *T. harzianum* has protected pear fruits from infection by the pathogen *P. expansum* of blue mould of the pear fruits treated by two different methods of inoculation resulted in differences in the level of blue mould disease incidence and severity as well as lesion of pear fruits.

Efficacy trials with the antagonist, *T. harzianum* showed a significant reduction of blue mould disease produced by *P. expansum*. This result is in agreement with that reported by Janisiewicz et al., 2001). The results of the present experiment showed that, the mean lesion diameter of the blue mould disease on the of infected pear fruits with *P. expansum* decreased significantly after artificially inoculation and treatment with conidia suspension of *T. harzianum*. In a previous study using the antagonist's *C. laurentii* and *M. pulcherrima*, both antagonists significantly reduced decay severity and incidence caused by *P. expansum* on apple and pear fruit (Wisniewski and Wilson 1992; Conway et al., 1992; Nunes et al. 2001).

The results of the present study demonstrate that *T. harzianum* showed a high efficacy in reduction of blue mould lesions produced by *P. expansum* on pear fruits. *T. harzianum* is an effective biocontrol agent against the post-harvest pathogen of *P. expansum* on pears, and could protect the fruits from blue mould decay under cold storage conditions. T. *harzianum* was used to assess its biological effectiveness against blue mould caused by the fungus *P. expansum* on pear fruits under laboratory conditions. *Trichoderma* spp. has evolved numerous mechanisms for attacking other fungi, these processes known as modes of action which are summarized in production of antibiosis (Agrios, 1997; Vinãs, 2004; Monte, 2001).

The minimum lesion size of blue mould disease of *P. expansum* was obtained on wounded pear fruits treated and inoculated artificially with *T. harzianum*, by dip method, but it was the maximum on unwounded pear fruits treated by spray method. This may be explained by the fact that *T. harzianum* is a strictly wound – parasite, so it can penetrate host tissues only through bruises and fresh wounds, especially in the fields through harvesting, handling, insects, and rodents (Barnes, 1979; Lisker et al., 1996). A similar significant effect was obtained in a previous study in controlling *P. expansum* on unwounded apple fruits (Batta, 2004) when these fruits were dipped for 30 in formulated *T. harzianum* conidia before being inoculated by *P. expansum* compared to the wounded fruits. This indicates the importance of this type of treatment in protecting apple fruits from blue mold infection for long time at postharvest stage without refrigeration (Batta, 2004).

Significant differences were obtained between means of percent reduction in lesion diameter treated with conidia suspension of *T. harzianum* relative to sterile distilled water (control treatment). This could be explained by the disruption of the host fungus cell wall by direct parasitism of *Trichoderma* (Goldman and Goldman, 1998; Monte, 2001) or by the metabolic activities of the antagonist that may change the radial growth and spore formation of the target fungi (Vinãs, 2004). The exact mechanism by which this antagonist inhibits spore germination of pathogen is not yet clear. Despite that it was not possible to show the production of antifungal metabolites in the culture medium of *T. harzianum* used in this study.

Conclusion

Results of this study show that the conidia suspension of *T. harzianum* has successfully protected pear fruits from blue mould of *P. expansum* at room temperature. However, further experiments are recommended to test

the suspension conidia *T. harzianum* against *P. expansum* decay of pear fruits stored under various conditions and using emerging technologies such as controlled atmosphere.

It is concluded that *T. harzianum* is interesting microorganism to be used as a biocontrol agent and at present we are evaluating mixtures of the fungi with compost with the aim to improve the control of decay in pears. There is no antifungal compound at cultural conditions established in the present study, but it is believed that the production of antifungal by this fungus would be an important tool for controlling postharvest pathogens. Development and implementation of this new technology will require greatly accelerated research. Operative research among laboratories is needed to develop products and procedures that are effective. The effect of certain types of fungi that are used during pear storage is also recommended for investigation along with the *T. harzianum* containing emulsion treatments. Full-scale commercial evaluation is needed to demonstrate the value of these treatments to the pome industry. Finally, since the present study constitutes the first trial to use the antagonistic fungus *T. harzianum* against *P. expansum*, it may be considered as the first step towards using *T. harzianum* in biocontrol of *P. expansum* commercially or, at least, in the disease management programs. However, further experiments are recommended to be conducted before this commercial use such as confirmation of the fungus efficacy against *P. expansum* under natural conditions of fruit storage and marketing; the side-effects (if any) of the formulation when applied under natural conditions should be also investigated.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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