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REGULAR ARTICLE

In vivo evaluation of Aggressiveness, pathogenicity and patulin accumulation by three *Penicillium expansum* strains isolated from Algerian apples

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ABSTRACT

Among the postharvest pathogens of apple fruit, *Penicillium expansum (P.expansum*) is considered to be one of the most common fungal pathogens worldwide. It is the causal agent of blue mold decay; the main serious economically disease of apple fruits, and the main producer of patulin (PAT). The principal objective of this work was to evaluate the aggressiveness and the PAT production of three isolated strains from apples produced in the East of Algeria. These strains were isolated then screened for their inhibitory activity against *Escherichia coli* and *Bacillus subtilis* (18 mm and 18 mm for S7; 10 mm and 11mm for S3), as well as their ability to cause blue rot (S4). The three strains S7, S3, and S4 (MN904448, MT023795 and MN904449, respectively) were identified as *P. expansum* by *ITS1*, *ITS4,* and β-tubulin gene sequences analysis. The *in vitro* ability to produce PAT was confirmed by Gas chromatography-mass spectrometry of strains' crude extracts cultured on YES (Yeast-Extract-Sucrose) medium. Indeed, these strains exhibited a great capacity to cause blue mold decay for all tested apples (26 to 52 mm lesion diameter), after nine days of incubation at 25°C. Apples rotten area analysis that was performed by UPLC analysis revealed that these three strains were able to produce PAT at different concentrations ranging from 0.57 to 469 µg/mL. Thus, these strains constitute a risk to consumer health because these amounts exceed the permissive level of PAT.

1. Introduction

Since chemicals are often present in our environment, food safety has become the major preoccupation (Carvalho, 2017). Food is one of the central products exposed to contaminants as pesticides, heavy metals, dioxins, mycotoxins, polycyclic aromatic hydrocarbons, drugs, and hormones (Traag et al., 2013; Pose-Juan et al., 2016). Mycotoxins are highly toxic compounds class, they are considered

as secondary metabolites secreted under specific environmental conditions by some fungi or molds, colonizing many foodstuffs (Barreira et al*.,* 2010; Zaied et al*.,* 2013). The most of these compounds are elaborated by the following three fungal genera; *Aspergillus, Penicillium,* and *Fusarium* (Abramson et al*.,* 2009; Anfossi et al*.,* 2016).

Pathogens cause postharvest losses of vegetables and fruits by their proliferation during harvest, transport, storage, and marketing (Spadaro et al, 2013; Abass et al*.,* 2014). Among the species of *Penicillium* genus, *P. expansum* is considered the most common contaminant of fruits and derived products and the major infectious agent of blue mould decay, the most important post-harvest apples disease in the world. The majority of the apple infections are induced by insects wounds, or injuries caused since harvest of the fruit to the processing houses (Amiri and Bompeix, 2005; Morales et al*.,* 2008). Moreover, the ability of *P. expansum* to grow even at low temperatures causes the infection development during storage periods of apples (Xu and Berrie, 2005; Baraldi et al*.,* 2003; Karaoglanidis et al*.,* 2011).

Furthermore, *P. expansum* is considered to be the main species producing the mycotoxin PAT. This unsaturated heterocyclic lactone, is currently elaborated by divers fungal species particulary those belonging to *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruit (Ritieni, 2003; Beart et al*.,* 2007; Pitt and Hocking 2009; Cao et al*.,* 2013; Karakose et al*.,* 2015). The main source of the mycotoxin PAT is rotten apples and apple-derived products. So, it has been detected at time in pears, grapes, apricots, blueberries, strawberries as well as peaches (Majerus and Kapp, 2002; Neri et al*.,* 2010; Elhariry et al*.,* 2011; Guo et al*.,* 2013), and it is principally secreted in decayed parts of the fruits (Cheraghali et al*.,* 2005; Puel et al*.,* 2010; Beltran et al*.,* 2014).

The occurrence of PAT in final fruit products as a natural contaminant reflects fruit quality used in the production, which is a worldwide problem (Funes and Reznik, 2009). This fungal toxin is easily transmitted from raw material to final fruit products due to its solubility in water, resistance to high temperature, and stability in acidic conditions as in fruit juice (Anderson et al*.,* 2008; Zaied et al*.,* 2013).

So, the main risk occurs is once unfit fruit is utilized in the production chain just like juices, and other processed products.

PAT consumption may cause acute symptoms such as vomiting, intestinal inflammation, convulsions, ulceration, edema, and agitation (Speijers, 2004; Raiola et al*.,* 2012). Furthermore, in chronic poisoning with PAT, the symptoms are manifested by neurotoxicity, genotoxicity, immunotoxicity, immunosuppressive, embryotoxicity, and teratogenicity (Wouters and Speijers, 1996; Moake et al*.,* 2005). Regarding the possible carcinogenicity, it was classified in group 3 (agents don't classified as carcinogen to humans) by International Agency for Research on Cancer (IARC) (Alshannaq and Yu, 2017).

Additionally, due to its potential toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) fixed the value of 0.4 µg/kg body weight/ day as provisional maximum tolerable daily intake (PMTDI) (WHO, 1995). On the other hand, Codex alimentarius have recommended PAT concentration of less than 50 µg/kg for apple products destined for human consumption and the European legislation has determined a maximum quantity of 50 µg/kg of PAT in apple juices, 25 µg/kg for apple puree, as well as 10 µg/kg for foods that are intended for infants, and young children (European Commission, 2006).

Regarding the danger caused by the presence of PAT in human food, several studies on the contamination of fruits by *P. expansum* and its capacity to produce PAT have been carried out (Marin et al., 2006; Reddy et al., 2010; Vico et al., 2014). However, very few studies concerning the role of PAT production in the aggressiveness and pathogenicity of *P.expansum* strains worldwide have been realized.

In Algeria, the greatest attention is paid to major mycotoxins (Aflatoxins and Ochrtoxins), whereas the study of PAT remains much neglected, that is why we have focused our study on this line of research.

For these previous raisons, the purpose of this investigation was to assess the aggressiveness and pathogenicity of three local *P. expansum* strains on apples and to study a potential of PAT accumulation *in vitro* and *in vivo* assays.

2. Materials and Methods

2.1. Apple fruits

The apple fruits utilized in this study are belonging

to Golden delicious variety obtained from Arris region located in the south-east of Batna locality (situated in East of Algeria) at 900 m of altitude, with 35° 33′ 0″ North, and 6° 10′ 12″ East.

2.2. Isolation of Fungi

Fungi were isolated from apple following the method of Harwing et al. (1973) and Paterson et al. (2003). Small pieces (1×1 cm) obtained from the tissues surrounding apples decay lesions were aseptically removed from the fruit. Three pieces were placed on fresh Malt Extract Agar (MEA: 20 g of glucose, 20 g of malt extract, 1 g of peptone and 20 g of agar in 1000 mL of distilled water) with Streptomycin (50 µg/L), then, the plates were incubated at 25°C till the apparition of colonies. Sheer cultures were acquired after successive subcultures by transplanting hyphal tips of each fungus to MEA. Isolates were preserved on MEA at 4°C.

2.3. Selection of isolates producing Mycotoxin

Fungal isolates were selected based on their antibacterial activity. The test was carried out according to the agar cylinder technique (Bramki et al., 2017; Bramki et al., 2019). *E. coli* and *B. subtilis* were reactivated in nutrient broth (5 g of meat extract, 10 g of peptone and 5 g of sodium chloride dissolved in 1000 mL of distilled water) and incubated at 37°C for 24 h.

Petri dishes containing nutrient agar (NA) were inoculated by 0.1 mL of each bacterial suspension. After drying for 5 min, disks of 5 mm diameter were obtained from extremity of 7 days old fungal colonies grown on MEA using a sterilized perforator**,** and deposited in center of NA containing the young bacterial culture. Plates were incubated at 37°C for 24 h, clear inhibition zones around the fungal disks were recorded.

2.4. Identification of isolates; Penicillium S3, S4, and S7

2.4.1. Morphological identification

For preliminary identification of fungal isolates MEA medium was used. This step was essentially focused on the study of macroscopic morphological characters (growth rate, the appearance of the colonies, their color and its change over time, colonies upside color, presence or absence of exudates, diffusible pigment production, etc.) and microscopic features (nature of the filament, spore morphology and presence of particular structures, etc.)

2.4.2. Molecular identification

Screened strains of *Penicillium*, morphologically described, were then identified by molecular analysis. The total DNA was obtained from100 mg of the young culture mycelium of the three *Penicillium* isolates *S3, S4* and *S7* by scratching the plate using disinfected spatula. DNA molecules were released by lysis of the fungal cell wall and membrane, after adding of 500 µl of lysis tampon (400 Mm Tris/HCl, 150 mM NaCl, 1% SDS, 60 Mm EDTA, 2 mL H_2O ultrapure). The whole (mycelium+lysis buffer) was incubated at room temperature for one hour. After incubation time, 150 µL of potassium acetate (pH 4.8, solution composed of 11.5 mL glacial acetic acid, 60 mL of potassium acetate solution 5 M and 28.5 ml milliQ water) was added and the mixture was well mixed with vortex, and then centrifuged at 12000 rpm for 8 min. The supernatant was put in new tube containing 600 µL of isopropanol, the tube content was homogenized by shaking it up and down (10 times) and then conserved at -20°C overnight. Once the incubation time is over, isopropanol was removed by centrifugation (12000 rpm for 5 min at 4°C), and the pellet DNA was washed twice with 300 µL of 70% ethanol. Then, supernatant was thrown after centrifugation (12000 rpm for 5 min at 4°C), and the pellet was dried at «speed vac» at 40°C for 30 min. the DNA was resuspended in 50 µL of TE buffer (Tris-EDTA) and retained at -20°C (El Khoury, 2007).

The *ITS1* 5′-TCGGTAGGTGAACCTGCGG-3′ and *ITS4* 5′-TCCTCCGCTTATTGATATGC-3 primers were used for the amplification of Internal Transcribed Spacer (ITS) region of the strains S4 and S7 ribosomal DNA (White *et al*., 1990).

The Bt2a (5′ -GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2B (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) for the amplification of the β-tubulin gene of S3 strain ribosomal DNA (Glass and Donaldson, 1995).

For the amplification of the desired genes, the three steps of the PCR cycle take place at precise temperatures in a thermal cycler (Biometra, Germany). The reaction was performed in a final reaction volume of 24 µL consisting of : 2.5µl of 1X buffer (Promega), 1.5 µL of $Mgcl_2$(25mM), 0.5dNTP (10mM), 0.22µl of Taq DNA polymerase, 0.3µl of each primer (20 pmoles/µL, 17.8 µL of ultrapure H_2O, and 1 µL of genomic DNA. The stages of amplification process consisted of an initial denaturation at 95°C for 3min, succeeded by 30 cycles each cycle takes place as follows: denaturation at 95°C (30 s),

annealing at 55°C (30 s), extension at 72°C (1 min), and a final extension period of 15 min at 72°C. Once, the PCR was completed, the quality of the resulting products has been verified by electrophoresis in an agarose gel (1.5%). The amplified genes were sequencing using the same primers sited above and the obtained sequences were revised with the software Chromas pro. The obtained DNA sequences were compared to those previously published in Genbank database using the BLAST alignement program and the accession number was obtained.

2.5. Phylogenetic analysis

From the obtained sequences, a phylogenetic study was carried out. Sequences alignments were made by the "MUSCLE" program and the trees were built with MEGA7 software, according to the Maximum Likelihood (ML) statistical method. In addition, distance corrections were made by the TAMMURA, and NEI evolution model.

2.6. PAT production by Penicillium strains S3, S4, and S7

The S3, S4, and S7 strains were tested for PAT production *in vitro*. Strains were grown at 25°C on Malt Extract Agar (MEA) medium up to sporulation. A final concentration of 10^6 conidia/mL conidial suspensions were obtained using a haemocytometer after submerging colonies of *Penicillium* isolates in sterile distilled water supplemented with Tween 80, 0.005% (V/V). These spore suspensions served to inoculate (YES) medium yeast extract (2%)-sucrose (15%) in Erlenmeyers containing 100 mL of medium and incubated for 16 days at 25 C° (Harwing et al., 1973).

After this period of incubation, fungal biomasses were separated by filtration through Whatman paper No. 1, filtrates were extracted three times with equal volume of ethyl acetate. Solutions were later evaporated until total disappearance of the solvent using a rotary evaporator at 48°C and each extract was taken up in 2 mL of chloroform. At the end, the extracts were conserved below 5°C until used for TLC (thin layer chromatography).

2.7. Qualitative estimation of PAT in fermentation crude extracts

For qualitative estimation of PAT, extracts and PAT standard (NOVAKITS) (1 mg/mL) were deposited on silica gel TLC plates (DC Kieselgel 60 20×20cm). After spots drying, the plates were developed in the mobile phase consisting of toluene: ethyl acetate: 90% formic acid / 5:4:1 (v: v: v). When the TLC plates were completely dried, they were then submerged in 0.5% MBTH (3-methyl-2-benzothiazoline hydrazone hydrochloride) (Sigma) and dried for 15 min at 130°C. The spot corresponding to PAT is characterized by a yellow color in visible light and simultaneously as a yellow–orange fluorescence spot under long wavelength (366 nm) UV light. To better visualize the yellow-orange color of spots, the TLC plate was well pulverized with water: 90% formic acid (98:2 / v: v), and was allowed to dry at room temperature. The spot observation was performed under 366 nm UV light (Welke et al*.,* 2011).

2.8. GC-MS analysis for PAT detection in fermentation crude extracts

To confirm the ability production of PAT by S3, S4, and S7 strains *in vitro*, Gas chromatography (GC) Agilent Technologies 7890B (USA) coupled to mass spectrometry (MS) Agilent 240 ion trap mass selective detector was used for the detection and the identification of PAT. The GC is provided with an electronically controlled split/splitless injection port, the mass spectrometer was operated in electron impact ionization (EI, 70 eV) and the MS transfer line temperature was held at 280°C in full scan mode.

The separation was performed on Agilent HP-5MS column (30 m× 0.25 mm ×0.25 µm film thickness). A volume of one microliter was injected in the splitless mode at 280°C, helium was employed as carrier gas with a constant flow of 1 mL/min. The column oven was initially held at 50°C for 1 min and the temperature was increased to 200°C at 10°C/min. In one min, the temperature was raised to 325°C at 5 °C/min and then maintained for 1 min. The analysis time was estimated at 43 min (Kharandi et al*.*, 2013).

2.9. In vivo evaluation of aggressiveness and pathogenicity of S3, S4 and S7 Penicillium strains using apples

The three strains were cultured on (MEA) malt extract agar for 7 days at 25°C. Spores were harvested by adding distilled water with Tween 80 (0.005%, v/v), and 10^6 conidia/mL was obtained as a final concentration. Only healthy and undamaged apples were retained for these tests, fruits were sterilized with sodium hypochloride (NaClO, 2%), followed by washing with sterile distilled water for three times and leaved at room temperature. After

drying, thirty (30) microliters of conidial suspension of each strain were inoculated in the apple at a depth of 1 cm using a sterile syringe. Apples were incubated at 25°C in sterile plastic trays for 9 days (Reddy et al*.,* 2010).

2.10. Determination of PAT production in apples by the three strains

The detection of PAT produced *in vivo* by the three *Penicillium* strains was performed on decayed parts of apples used in the test described above. The samples were processed following the method of AOAC (2000) with some modifications.

After the incubation period, visibly necrosed section of each fruit was taken off from the apple fruit. The samples were weighed, ground and homogenized using mortar and pestle, then analyzed immediately.

10 g of the pulp was submitted to PAT extraction by three partitions of 10 mL of ethyl acetate, by blending vigorously for 3 minutes with a vortex. Then, the three organic phases were assembled and cleaned up with 10 mL of sodium carbonate solution (1.5%, w/v). The aqueous phase was removed from the organic phase using anhydrous sodium sulphate, and then the extracts were introduced in a silica gel column composed of a glass tube (200X10 mm) stuffed with 8 g of Silica gel (Silica gel 60 0.063-0.200mm) Merk KGaA, Germany.

PAT was extracted from the column with ethyl acetate. Once the solvent was completely evaporated, the extracts were dissolved in 100 µL chloroform for analyzing by TLC, and for HPLC analysis the extracts were dissolved in methanol.

2.11. Analysis of PAT using ultra-performent liquid chromatography (UPLC)

PAT analysis were carried out using ultra high performance liquid chromatography UHPLC (Dionex Ultimate 300) equipped with an auto-injector and UV detector. LC separation was realized at 30°C using C18 analytical column (Inertsil ODS-4) 5 µm, 4.6×250 mm. The mobile phases consisted with a multi step gradient of water (A) and acetonitril (B), at flow rate of 1 mL/min. The injection volume was 10 µL. The quantity of PAT found in the analyzed extracts was obtained after the measurement of the peak area at PAT retention time and the comparison with the relevant calibration curve (Chen et al., 2017)

PAT standard was provided by Novakits (Nantes, FRANCE), the preparation of PAT concentration solutions was performed by dissolving 1 mg of pure crystalline PAT in 1 mL of methanol. Dilutions obtained from the stock solution with methanol (500, 250, 125, 62.5, 31.25, 15.62, 7.81, and 3.9 µg/mL) were used to construct the calibration curve. All the solutions were conserved at +4°C; the experiments were realized twice.

2.12. Statistical analysis

The statistical analysis was performed using the EXCEL program (version 2007), and the differences in PAT levels between strains were analyzed. All the experiments were performed in duplicate. Data were expressed as mean ± standard deviation (SD).

3. Results

3.1. Isolation and identification of fungi

Eight isolates were obtained from apples (Golden delicious) produced in Aris, situated in Batna, in the East of Algeria. Three selected strains S3, S4 and S7 were screened for their substantial antibacterial potential. Both S3 and S7 were active with clear inhibition zones against *E. coli* (11 and 10 mm, respectively) and *B. subtillus* (18 and 18 mm, consecutively) Whereas, strain S4 that did not exert any bacterial growth inhibition, it was selected for its positive pathogenicity. The growth of the isolates S3, S4 and S7 is more or less rapid. These strains have dull green or slightly gray velutinous plane colonies with, sometimes, a very thin white margin and a pale reverse. Colonies that grew on MEA culture medium did not show a production of exudates or soluble pigments. Under an optic microscope, these strains present septed hyphae, and branched hyaline. The conidiophores bear terverticillary penicillary heads, and the conidia have a smooth dull green wall with an ellipsoidal shape, straight smooth straps, elongated phialides, and cylindrical ampouliforms (Figure 1).

3.2. Phylogenetic analysis

Based on β-tubulin, ITS1, and ITS4 gene sequences analysis; S3, S4 and S7 were identified as *Penicillium expansum* (MT023795, MN904448 and MN904449; respectively) and their relationship with other *Penicillium* strains was established by phylogenetic trees construction (Figure 2, 3).

3.3. Qualitative estimation of PAT in fermentation crude extracts

Once the incubation period was completed, and

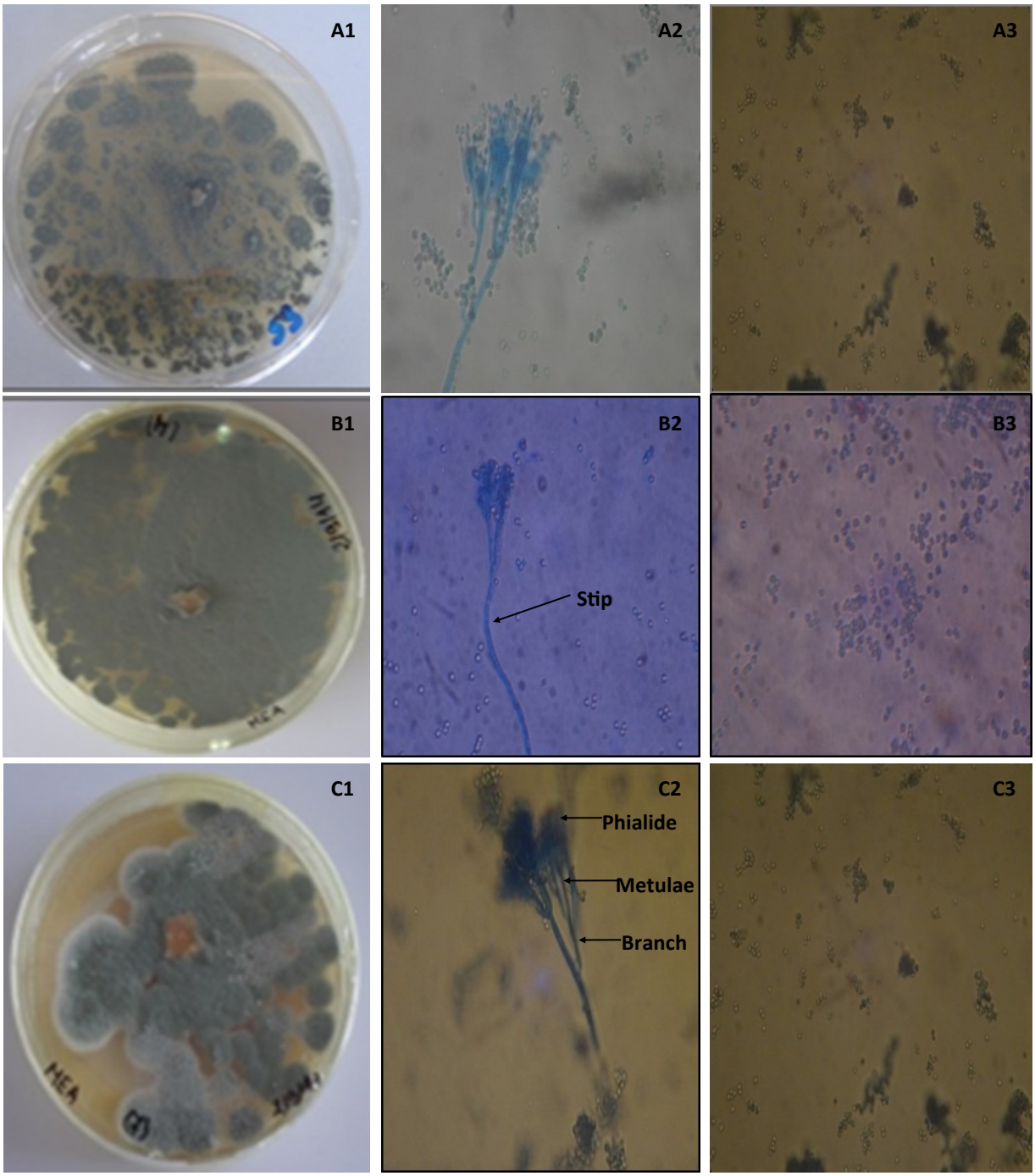


Figure 1: Macroscopic and microscopic (G×40) observations of the three *Penicillium* sp strains after 7 days of incubation; A1, B1 and C1 (Macroscopic appearance of S3, S4 and S7 strains); A2, A3, B2, B3, C2 and C3 (Microscopic observation of penicillary heads and conidia)

after the liquid extraction with ethyl acetate; the obtained extracts were analyzed by TLC. The ability of the three strains to produce PAT was observed by the presence of yellow spot under visible light, after treatment with MBTH 5%, and the presence of a yellow–orange fluorescence spot in UV light (long wavelength 366 nm) with Rf=0.4

3.4. GC-MS analysis for PAT detection in fermentation crude extracts

For more precision, Gas chromatography coupled to mass spectroscopy was used for detecting the mycotoxin PAT in extracts of *P. expansum* cultures. The GC-MS chromatogram spectra of the crude extracts revealed the appearance of many peaks and the component corresponding to the peak appeared at 13.856 min is PAT (4-hydroxy-4H-furo [3,2c]pyran-2(6H) one) (figure 4 and 5). The chemical formula, molecular weight, and exact mass of mycotoxin PAT are presented in Table 1.

3.5. In vivo evaluation of aggressiveness and pathogenicity of S3, S4, and S7 strains using apples

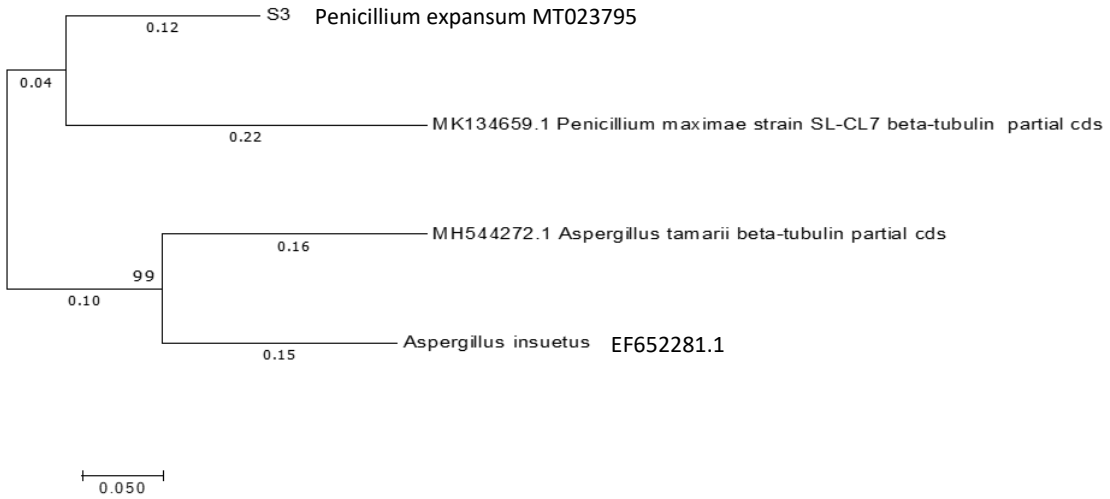


Figure 2: Molecular Phylogenetic Analysis of S3 strain beta tubulin sequences by Maximum Likelihood Method.

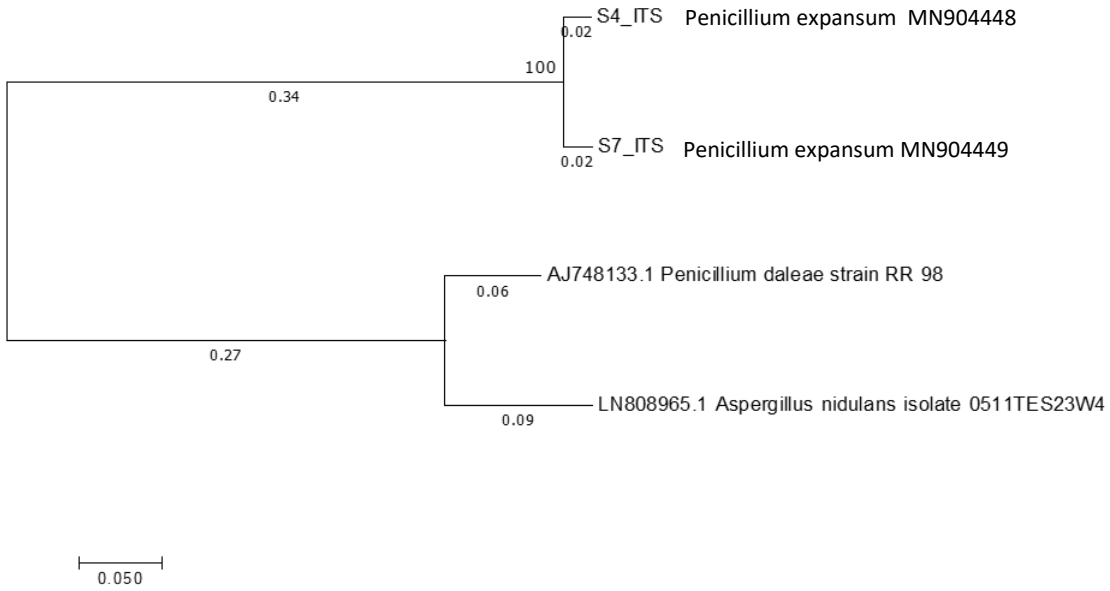


Figure 3: Molecular Phylogenetic Analysis of S4 and S7 strains ITS sequences by Maximum Likelihood Method. The 0.050 value indicates the scale (number or purcent of mutations between the sequences) of the tree.

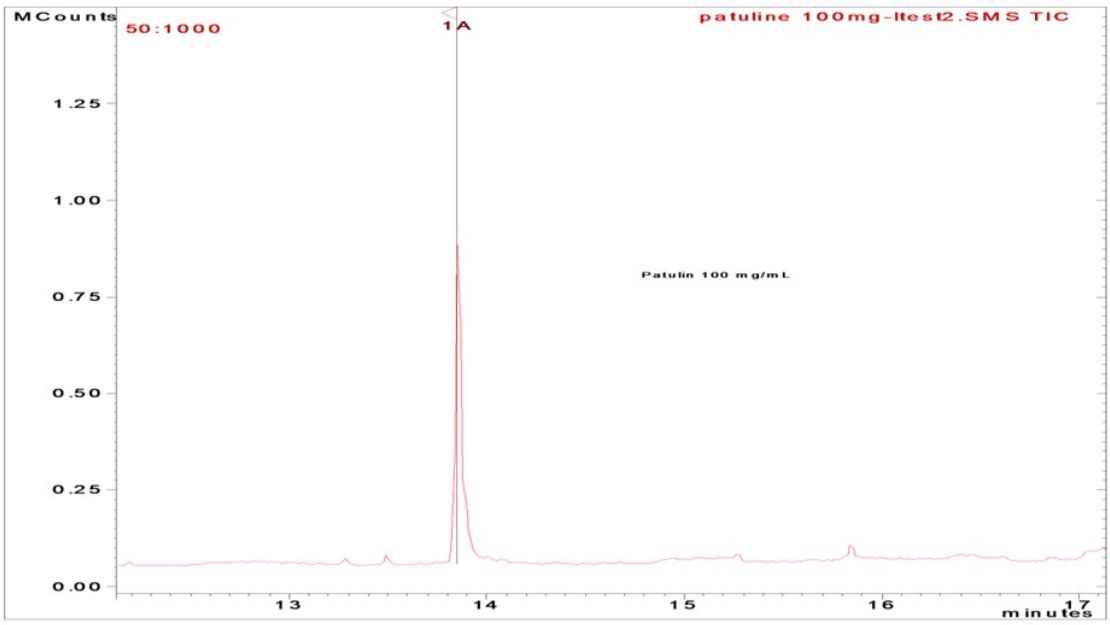


Figure 4: GC-MS chromatogram corresponding to the PAT standard (100 mg / mL).

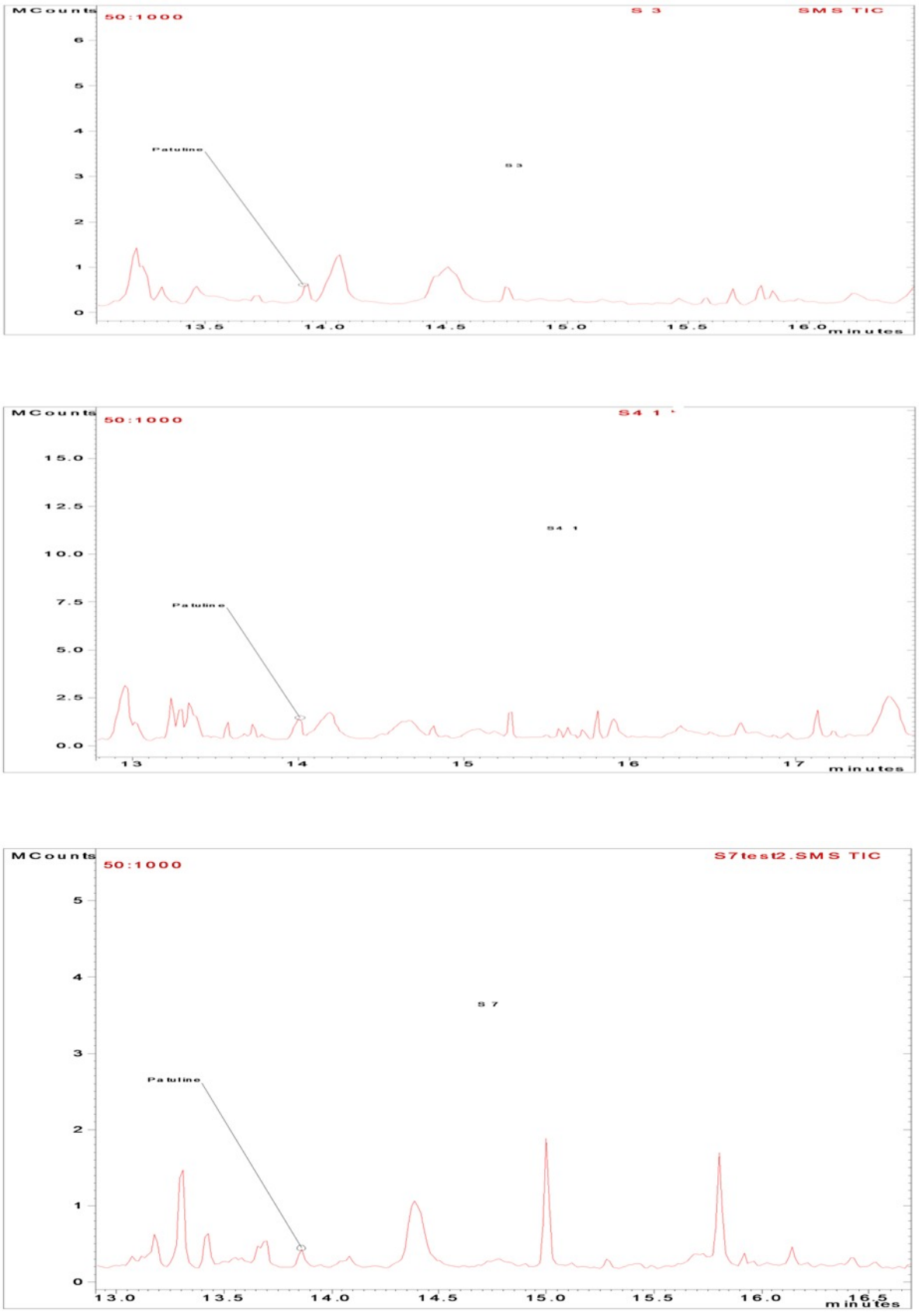


Figure 5. GC-MS chromatograms demonstrating the presence of PAT in the crude extracts of *S3, S4 and S7 P. expansum* cultures.

Figure 6. *In vivo* phatogenicity test of the three *P. expansum* strains on apples.

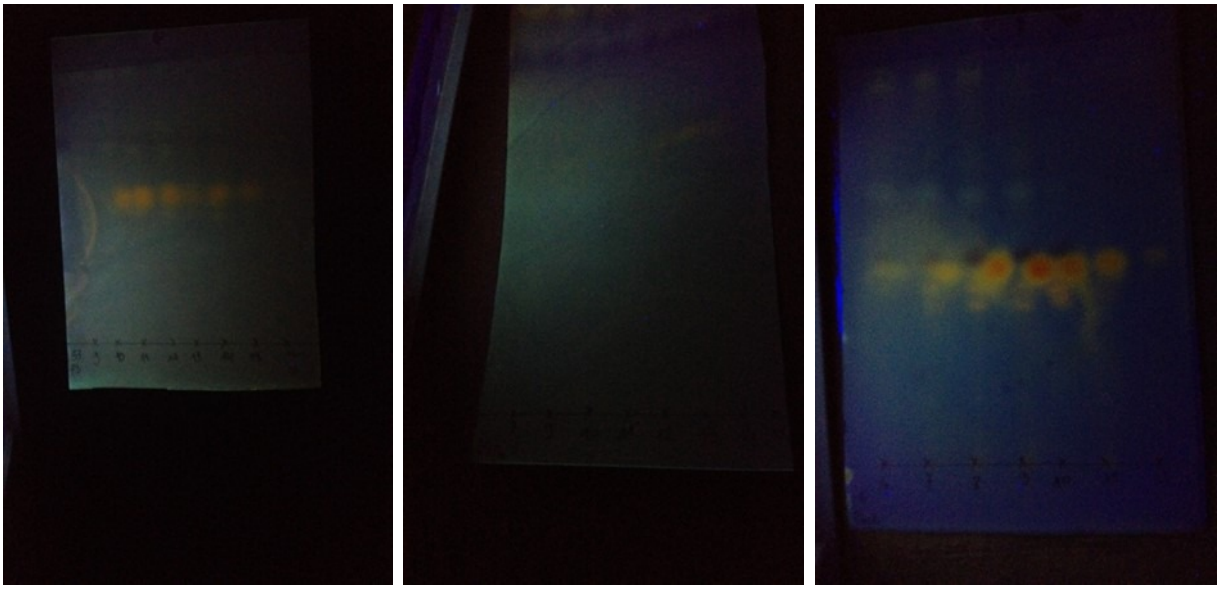


Figure 7. TLC characterization with intensities spots of PAT produced *in vivo* by S3, S4 and S7 strains at 366 nm.

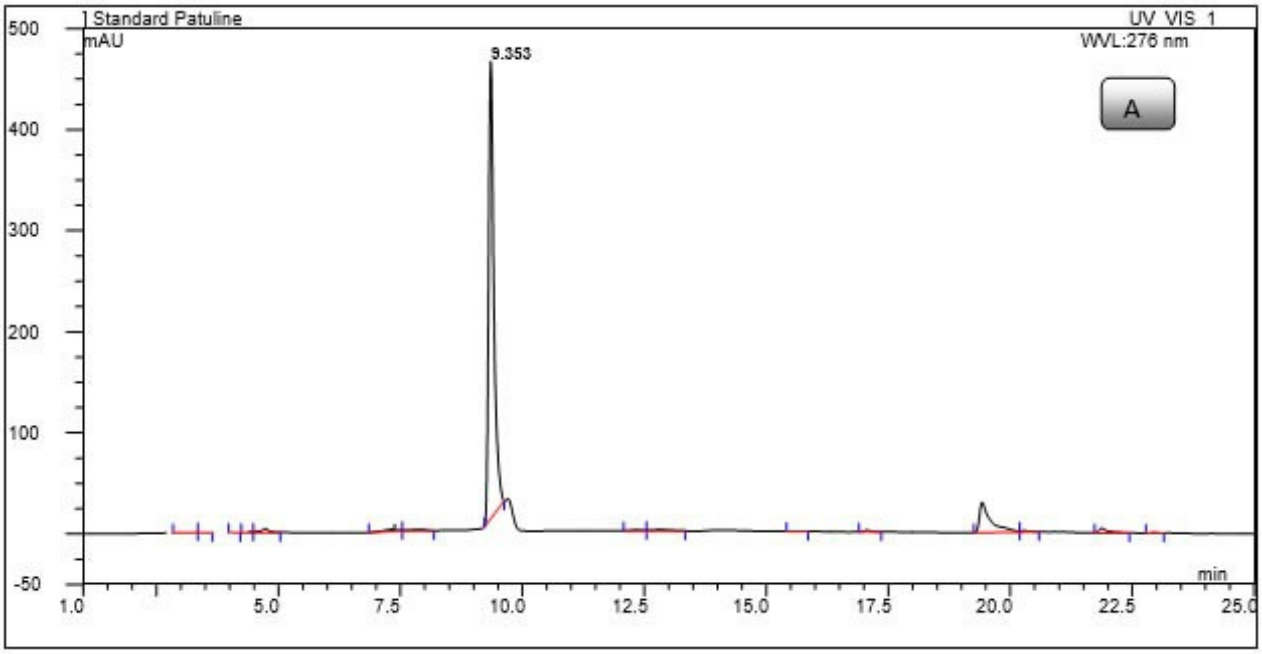
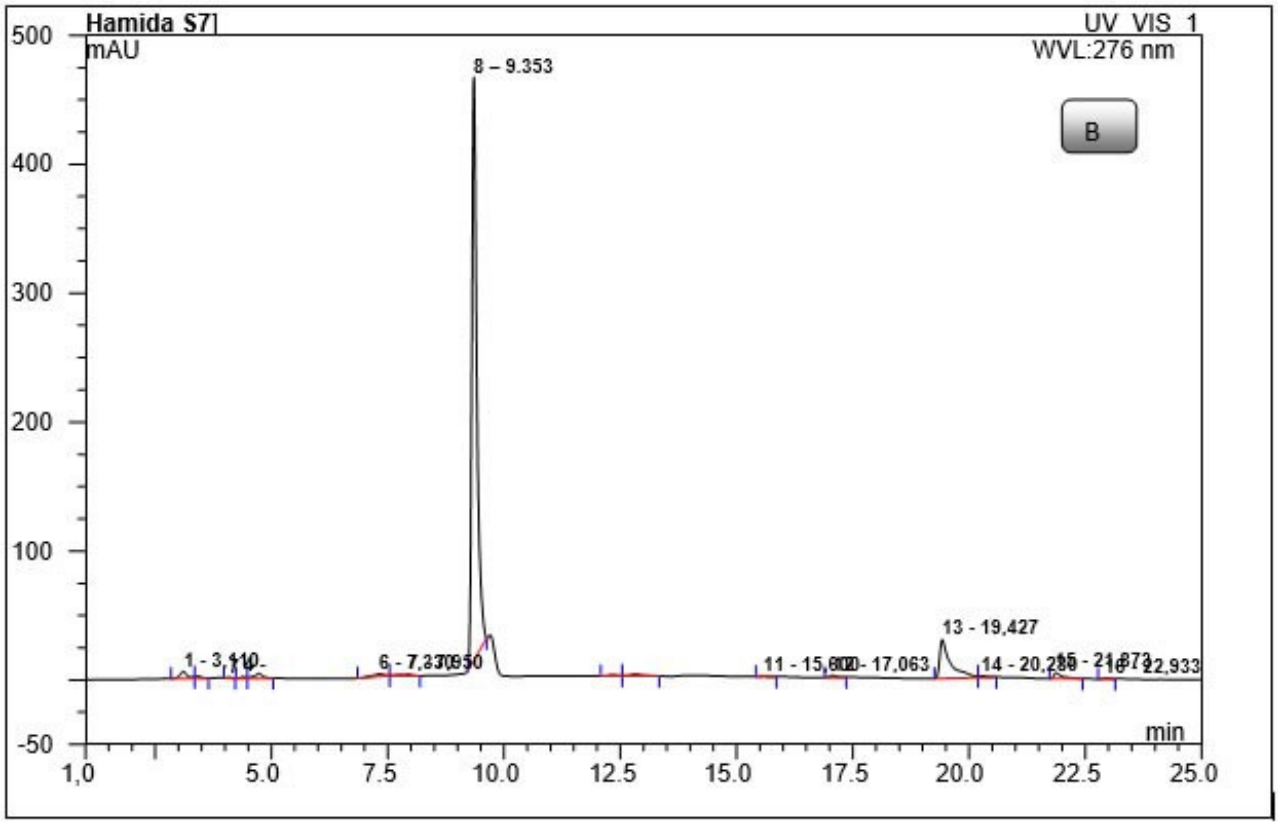


Figure 8 continued...



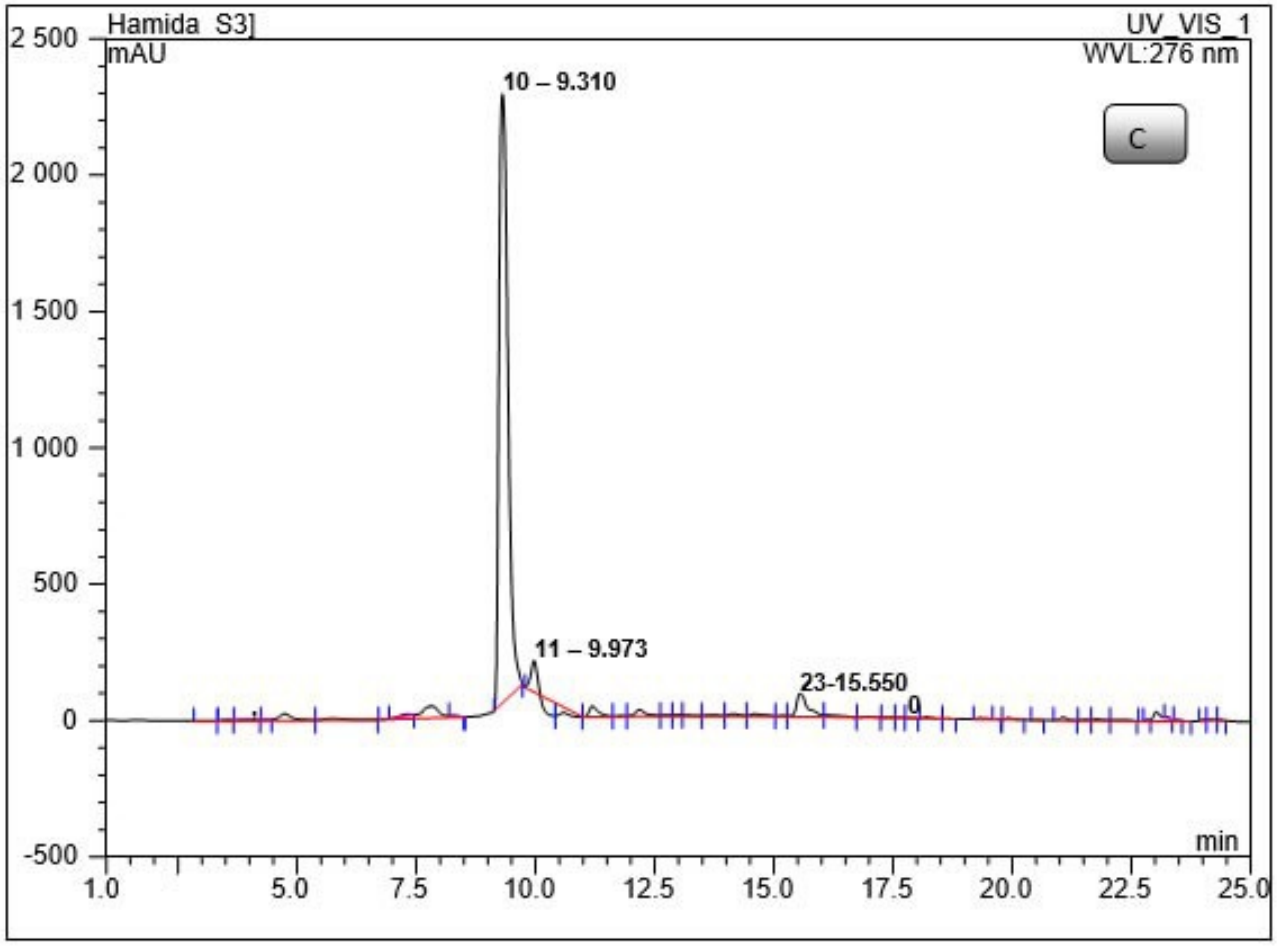


Figure 8 continued...

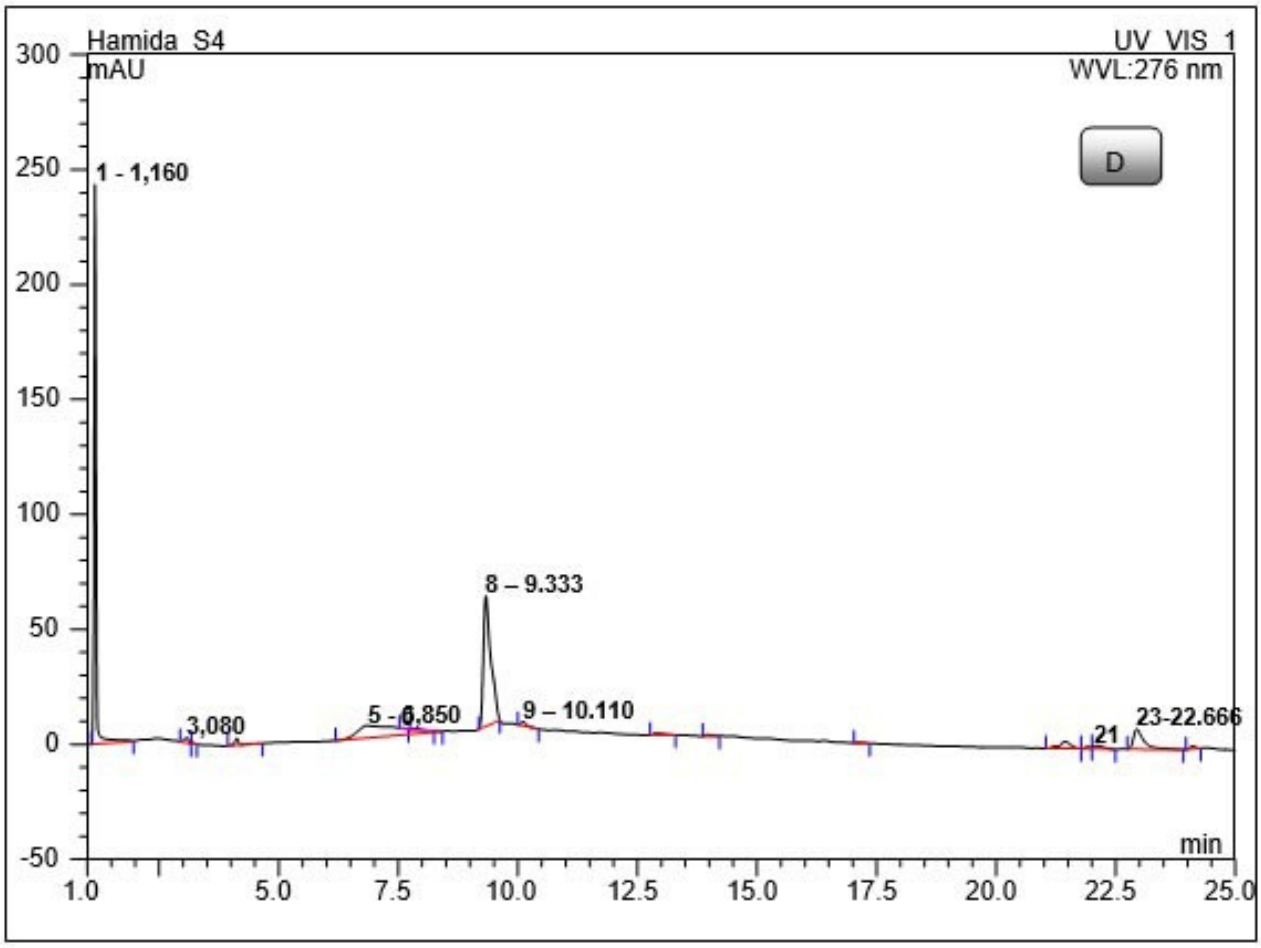


Figure 8: UPLC chromatograms: (A) PAT standard (1mg/ml); (B, C and D) PAT in inoculated apples with the three strains, *in vivo test*.

Pathological analysis of *Penicillium* strains (*S3, S4, and S7)* during 9 days of artificial infection on apple fruits is shown in figure (6). All strains caused soft, circular, aqueous, and brown-colored lesions. Furthermore, blue-green spores' characteristic was presented in the inoculation sites. These symptoms are identical to those of blue mold rot of post -harvest apple. The strains S3, S4, and S7 of *P. expansum* induced infectious decay on apples at 25°C and PAT production.

These strains produced large lesions ranging from 26 mm to 52 mm on golden delicious apples. The infection gradually increased to reach the diameter of 26 mm for S4, 47 mm for S3, and 52 mm for S7. Strain S4 induced lesion of smaller diameter compared to the other two strains. Lesions development increased progressively as function of time for all strains and after the incubation period, the lesion diameter exceeded half of fruit.

Besides, after extraction of PAT from infected tissues, obtained fractions from the column chromatography were analyzed by TLC and the PAT was shown as yellow spot as has been demonstrated above. Indeed, it has been revealed that all *P.expansum* strains were able to producing PAT *in vivo* at 25°C with different intensities (figure 7), and the most significant spot intensity was noted by strain S7. Otherwise, the spots of obtained fractions from the extraction of apple tissues infected with the strain S4 were the lowest compared to strains S3 and S7. In UPLC analysis, PAT determination was carried out by comparing the retention time of the peaks areas presented in extracts with PAT standard (figure 8). The retention time of PAT in the operating conditions mentioned above, was found about 9.35 ± 0.24 min.

Thus, the obtained results from the fractions analysis by *in vivo* test revealed the appearance of chromatograms having the same retention time of PAT standard (figure 8).

Calibration curve was built using each PAT concentration. It was linear in range of 3.9 to 500 µg/mL of PAT and the correlation coefficient is 0.996 which showing a good calibration.

According to the UPLC results, the three *P. expan-*

sum strains secreted PAT with different quantities. The highest amount of PAT produced *in vivo* was shown by strain S7 (0.436 ± 0.04 mg/mL) at 25°C, while strain S3 produced PAT at concentrations lower than those developed by S7 isolate (0.349±0.01 mg/mL). Conversely, the lowest concentrations of PAT were detected in fractions corresponding to isolate S4 (0.002±0.001 mg/mL), which confirms the previous results obtained by TLC.

4. Discussion

In the forties, PAT was named as one of the antibiotics developed by the genus, *Penicillium* (Sanzani et al., 2012). Thus, it was used with success against pathogens of colds and bronchitis (Snini, 2014). PAT inhibits the growth of Gram+ and Gram- bacteria.

Although, in 1960s, various researches demonstrated PAT toxicity in animals and higher plants, this led to its reclassification to the mycotoxin group (Tannous, 2015). While, PAT is generated by various genera and species, it is principally related with *P. expansum*, the responsible pathogen for blue decay of pome fruits, especially apples (Moake et al., 2005; Sanzani et al., 2009).

Besides, isolates obtained from apple fruits in this study have macroscopic and microscopic characteristics typical of those reported for *P. expansum*, causal agent of apple decay (Anderson et al., 2004; Frisvad & Samson, 2004; Pitt & Hocking, 2009; Vico et al., 2014). These isolates were retained for their antibacterial activity against *E. coli* and *B. subtilis* growth; the same test was carried out by Larous et al. (2007). The morphological identification is not reliable to determine the species shown the resemblance of both macro and microscopic features between the species. Thus, their unequivocal identification often necessitates another method such as molecular identification (Sanzani et al, 2013). In our present study, the ITS region (Internal Transcribed Spacer) and β-tubulin gene of the ribosomal DNA were amplified with ITS1 and ITS4; Bt2a and Bt2b universal primers. ITS is the most widely sequenced fungal DNA region, and the available universal primers (Schoch et al, 2012). More recently, intron-rich sequences of the β-tubulin gene have been used for discrimination within genera such as *Penicillium* or *Aspergillus* (Samson et al., 2004).

Furthermore, the corresponding DNA amplification of the tested isolates with previous primers confirmed that the screened isolates were belonging to *P. expansum*. Vico et al. (2014) have identified *Penicillium spp* strains isolated from blue mold decay of apple as being *P. expansum*, by amplifying ITS regions. As well, Hammami et al. (2016) could identify 23 strains of *P. expansum* from undamaged apples and apples based food, with the same primers. Yin et al. (2017) have also identified blue mold *Penicillium* species isolated from stored fruits using ITS and β-tubulin primers. The results of our molecular identification were approximately similar to those found by Tas et al. (2015).

The capacity to produce PAT *in vitro* by *P. expansum* strains isolated in this work was carried out on YES medium under 25°C. This culture medium proves favorable for the generation of secondary metabolites, and has been adopted in precedent studies (Aderson et al., 2004; Laarous et al., 2007; Elhariry et al., 2010; Vansteelandt et al., 2012).

Other mycotoxins such as citrinin, requefortin C, communesin B, and expansolides A as well as B were produced by *P. expansum* in YES. The production of PAT in this medium by the three strains of *P. expansum* was detected using TLC. It was also characterized by a yellow color in visible, yellow-orange under UV and Rf=0.4 after treatment with MBTH, a chemical component very reactive for PAT detection and plays the chromogen role. Similar results have been described by Martin et al. (2002) and Welke et al. (2009).

To confirm the presence of PAT in the crude extracts, gas chromatography-mass spectrometry (GC-MS) was adopted. This technique is provided with precision and reproducibility in the separation and the identification of biomolecules. Rodríguez-Carrasco et al. (2012) has demonstrated the presence of PAT and other mycotoxins in wheat semolina by GC-MS. Furthermore, Kharandi et al. (2013) determined the presence of PAT in apple juice with the same technique. Furthermore, Hamza et al. (2015) analyzed the various metabolites produced by *P. expansum* in liquid culture, and identified twenty eight compounds by GC-MS. In the present work, the EI mass spectrum of PAT contained an abundant molecular ion at *m/z* 110 (base peak), 126, 136, and 154 (M+), corresponding to PAT. The same mass spectra were obtained for the PAT contained in our three samples, comparable results were also reported by Roach et al. (2002).

The potential of *P. expansum* isolated strain to produce PAT was investigated in apples (Golden deli-

cious). *In vivo* pathogenicity test showed that screened isolates were capable of causing blue mold at 25°C after 9 days of incubation. The infection was light brown and tissue was soft and aqueous, this result corroborates with the pathogenicity test realized by Vico et *al*. (2014). In the current investigation, the infection increased gradually up to a diameter of 50 mm. After the incubation time, lesion dimension progressed to be more than the half of an apple. The aggressiveness test of *P. expansum* strains against apples by the artificial inoculation with spores of pathogens, have been already realized by Harwing et al.(1973), Watanabe (2008), Neri et al.(2010), Louw and Korsten (2013) and Baviskar and Dekate (2016). The diameter of the inoculated part differs from one study to another; Reddy et al. (2010) obtained the largest lesion diameter (39.1 mm) after 9 days of incubation at 25°C, furthermore, Marin et al. (2011) achieved a diameter of 40 mm between 7-8 days at 20°C, while Tannous (2015) attained different diameters on diverse varieties of apples. Presumably, various factors were involved in the aggressiveness of each isolate towards its host.

In the present work, analysis of PAT production by the isolated strains in the rotted area was performed by UPLC. The strain S7 was successful in secreting a very remarkable amount of PAT (0.436 ± 0.04 mg/mL), followed by strain S3 which also produced a good concentration (0.349 ± 0.01 mg/mL) at 25°C. These values are significantly greater than that established by the Commission of the European Communities for solid apples products (25 µg/ kg). On the other hand, strain S4 revealed much lower amounts compared with the other two strains. Celli et al. (2009) showed the natural contamination of apples by PAT with concentrations up to 120.40 mg/kg.

Therefore, Singh and Sumbali (2008) have obtained PAT concentrations ranging from 11.75 to 67.50 mg/kg produced by *P. expansum* strain on apples. Abramson et al. (2009) got a PAT concentration of 100 µg/mL in YES culture. Reddy et al. (2010) examined the pathogenicity of several *P. expansum* strains on apples and other fruits (Apricots, kiwis, peaches, and plums) and they have obtained a PAT production of 800 µg/kg on apples. In the same year, Neri et al. (2010) have recorded accumulations of PAT between 1.7 and 33.9 µg/m. Moreover, Baviskar and Dekate (2016) demonstrated a production of PAT by different strains of *P. expansum* between 28 and 35 mg / kg. Recently, Coton et al. (2020) reported varying concentrations of PAT produced by three *P. expansum* strains (300-50.000 ng/g). In the same way, variations in PAT production are often observed among strains as shown in our findings.

The amount of produced PAT changes from one strain to another and it is a specific to the strain. Garcia et al. (2011) suggested that different factors affect mycotoxin production such as temperature, water activity, substrate aeration, inoculum concentration, isolate age, genetic information, physiological state of mold, etc. In this research, *P. expansum* strains isolated from apples produced in the Eastern of Algeria were tested for both blue mold decay and PAT production. The pathogenicity tests performed with the three pathogens are in perfect agreement with the results of *in vivo* PAT production tests. Indeed, strain S7 that produced the largest lesions. It is considered as a pathogenic for apples and the most virulent because it has induced the blue rot disease as well as produced the largest amount of PAT *in vivo*, correspondingly.

The strain S3 is similar to the strain S7, but in less remarkable way. In contrast, strain S4 caused blue rot infection but with a lower degree compared to the other two strains and produced, elsewhere, a very small amount of PAT which makes it the least virulent among these three pathogens.

According to Yoder's (1980) definition, pathogenicity is a qualitative term that refers to the capacity of a fungus to cause disease; while virulence is a quantitative term that refers to the amount or extent of the disease. The role of PAT in the aggressiveness of *P. expansum* has been the subject of some investigations. Sanzani et al. (2012) showed that PAT may be associated with the virulence and pathogenicity of *P. expansum*, by testing mutant strains and wild strains *in vivo.*

They have demonstrated that mutant type strains could produce PAT but in a minimal level compared to wild strains. Besides, the incidence and the severity of infection induced in apples inoculated with mutant strains were inferior to those infected with wild-type strains. According to Snini et al. (2014), PAT can be considered as a factor of virulence and not of pathogenicity. This may explain by the fact that strain S4 is pathogenic because it developed and has caused apple tissues maceration. In contrast, the presence of small amounts of PAT produced by this strain decreased the rate of symptom development. Our results are in the same di-

rection of those obtained by Snini et al. (2016).

We conclude from this study that apples produced in eastern Algeria can be colonized by the pathogen *P. expansum* and subsequently produce different amounts of PAT at 25°C which exceeded by times permissive levels. Also, the existence of potentially generating PAT species does not mean perforce the presence of PAT. Nevertheless, PAT production is commonly but not exclusively related to blue apple decay, mainly, caused by *P. expansum*.

This finding indicates that the ingestion of juices produced with apples rotten areas may constitute a risk of human exposure to PAT. Therefore, it is advisable to remove apple tissue surrounding the decayed part, and this can be a good preventive method for industry using apple fruits to decrease the contamination of the final product by PAT.

Conflict of interests

The authors have not declared any conflict of interest.

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