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RESEARCH ARTICLE

Hyper-proteolytic mutant of *Beauveria bassiana*, a new biological control agent against the tomato borer

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Abstract The world tomato production is threatened by the invasive tomato borer *Tuta absoluta*. Difficulties in managing this pest were imposed mainly by the development of resistance in strains treated with conventional chemical insecticides. Resistance problems were even reported to insecticides of natural origin, leading to search for other control alternatives. P2 is a spontaneous mutant of the entomopathogenic fungus *Beauveria bassiana.* It was previously selected from a local strain (P1) and was characterized as hyper-producer of extracellular proteases. Here, the insecticidal potential of P1 and P2 strains was evaluated against *T. absoluta* larvae under laboratory conditions. Both strains were effective but P2 showed stronger effect than P1; median lethal concentration of P2 is tenfold lower than that of P1. Enzymatic assay analysis showed that extracellular enzymes are differently expressed by the two strains, especially proteases and chitinases which are known as cuticle degrading enzymes. The major expressed subtilisin-like protease (SBP) was upregulated at the transcriptional level in P2 strain. Proteomic analysis revealed four SBP isoforms which are highly overexpressed in this strain compared to P1. Post-translational regulation, most probably phosphorylation, was further suggested to control the SBP protease expression in *B. bassiana* P1 and P2 strains. The enzymatic profile in the two strains might explain their different insecticidal potential against the tomato borer. This is the first report showing such efficiency of *Beauveria* strains against this dangerous pest. Particularly, P2 strain showed high virulence reaching almost total larval mortality within 5 days post-application. It thus should be recommended as a new tool for the biocontrol of *T. absoluta*.

Keywords *Beauveria bassiana* · Protease isoforms · *Tuta absoluta* · Biopesticide · Tomato borer · Biocontrol

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1 Introduction

The tomato leaf miner, or tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is considered as one of the most damaging tomato pest. It originated in South America and has recently spread to Europe and North African countries. It leads to serious losses, affecting up to 100 % of tomato crops (Campos et al. 2014; Pratissoli and Parra 2000). Larvae, which are the harmful stage, can feed on all parts of the plants (leaf, stem or fruit) and form mines within (Fig. 1). Several chemical insecticides such as abamectin, cartap and permethrin have been widely used against *T. absoluta* but later were abandoned because of widespread resistance (Siqueira et al. 2000). Other considerable problems were associated with the use of these conventional insecticides, such as environmental, ecological and human health effects. Furthermore, organically produced tomatoes

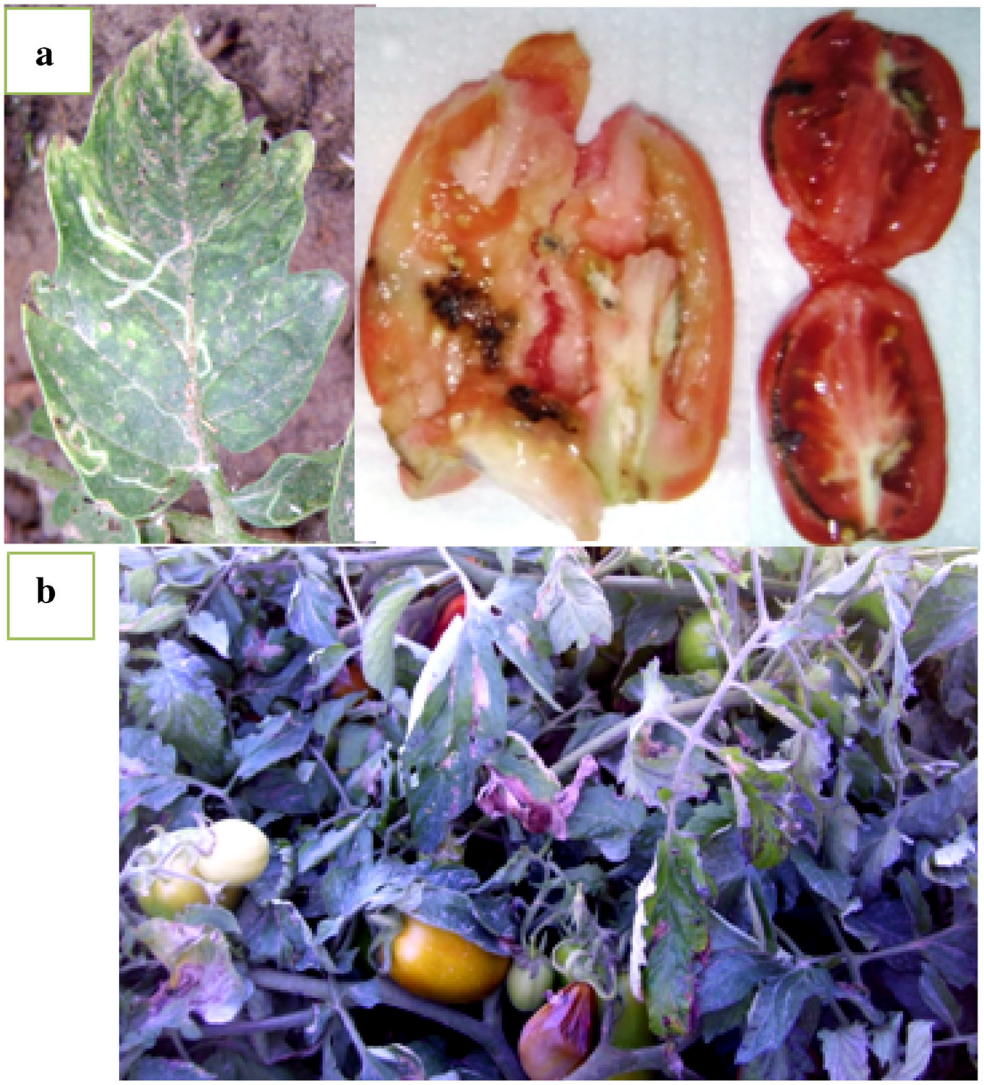

Fig. 1 *T. absoluta*, a dangerous insect pest to tomato plants and fruit. Tomato leaf and fruit damages. Larvae feed on the leaf mesophyll, ripe and unripe fruit creating mines and holes (**a**). Signs of damage on the tomato plant. Clear patches appear on the leaves affecting the photosynthetic capacity of plants and later become necrotic resulting in a subsequent reduction in the crop yield (**b**)

have been another factor leading to the use of bioinsecticides, mainly spinosad. However, reports of resistance counterpoint the potential for its further use (Campos et al. 2014). Another promising biocontrol strategy is the use of indigenous natural enemies. Various predators (i.e. *Nesidiocoris tenuis*, *Mimulus pygmaeus*) and parasitoids (i.e. *Trichogramma achaea*, *Neochrysocharis formosa*, *Bracon* sp.) species spontaneously attack *T. absoluta* in tomato crops worldwide. Some of these are already commercialized and have been employed successfully in integrated pest management strategies, i.e. the parasitoid *T. achaea* which is used by periodic inundative releases, individually and in association with mirid predators, i.e. *M. pygmaeus* and *N. tenuis* (Zappala et al. 2013). *Bacillus thuringiensis* is also efficient in controlling *T. absoluta* when pulverized in greenhouse and open-field tomato plants (González-Cabrera et al. 2011).

Entomopathogenic fungi are under intensive study since they are known to be non-toxic for animals and humans and to be safer for the environment than chemical products. They have been formulated for application in agricultural insect pest management systems with successful results.

The most common commercial mycoinsecticides and mycoacaricides on the market are based on *Beauveria bassiana* (33.9 %), *Metarhizium anisopliae* (33.9 %), *Isaria fumosorosea* (5.8 %) and *Beauveria brongniartii* (4.1 %) (Faria and Wraight 2007). These fungi infect the host insect by direct penetration of the insect cuticle which consists mainly of proteins and chitin, by producing extracellular enzymes directed to hydrolyse these cuticular components. Proteases and chitinases, which are the primary virulence determinants, were the most studied enzymes and their role in virulence is well documented (Fan et al. 2007; St. Leger et al. 1996). Furthermore, fungal virulence may attribute other factors and molecules including the hydrophobicity of conidia and the secretion of toxins (Ortiz-Urquiza et al. 2010). Few studies have investigated on the pathogenicity of *Beauveria* strains towards *T. absoluta*. Laboratory experiments showed that different isolates of this fungus, pulverized on eggs and larvae of

the tomato borer, were more virulent to eggs (Pires et al. 2010). Higher virulence was found when the first instar larvae were treated with *M. anisopliae*, compared to *B. bassiana* strains. However, no total efficiency was found using any strain (Inanli et al. 2012; Pires et al. 2010). Better results were obtained when *T. absoluta* larvae were fed with leaves treated with *B. bassiana* (Giustolin et al. 2001; Shalaby et al. 2013), reaching 100 % of total larval mortality (Klieber and Reineke 2015).

In this study, we evaluated two Tunisian strains of *B. bassiana* for their pathogenicity towards *T. absoluta* larvae. These strains, P1 and P2, were previously isolated and studied for their extracellular proteases production (Borgi and Gargouri 2014). Higher yields were found in P2, and the major expressed protease (subtilisin-like protease [SBP]), which is a serine subtilisin-like, was purified and the gene encoding this enzyme was sequenced (Borgi and Gargouri 2014). Here, the secretomes from both strains was compared via two-dimensional electrophoresis (2-DE) coupled to mass spectrometry analysis. These strains were also used for the assays of cuticle degrading enzymes.

2 Materials and methods

2.1 Chemical reagents

All reagents and chemicals were obtained from either Sigma-Aldrich (USA/Steinheim, Germany), Biorad (München, Germany) or Amersham-Biosciences (Amersham, UK).

2.2 Fungal strains

B. bassiana P1 and P2 are laboratory strains, identified by sequencing the 18S ribosomal ribonucleic acid (rRNA) genes and comparing sequences with GenBank database via Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification was confirmed by the "Centraal Bureau voor Schimmelculture" of Holland (Borgi and Gargouri 2014). The P1 wild-type strain was deposited in the National Collection of Microorganisms of the Center of Biotechnology of Sfax (Tunisia) under the reference CTM10549 P1.

B. bassiana strains (P1 and P2) were cultured on Luria Bertani solid medium (5 g NaCl, 10 g bacto-peptone, 5 g yeast extract, 17 g agar and 1 l distilled water) at 30 °C for 12 days before being used in experiments. Aerial conidia were harvested in distilled water supplemented with 0.1 % Tween 80, filtered and counted using a haemocytometer. The filtrates were centrifuged, and the final concentrations were adjusted to 10^9 conidia/ml in the sterile water supplemented with glycerol 10 %.

2.3 Bioassays with *T. absoluta* larvae

A laboratory colony of *T. absoluta* was established from a Tunisian tomato field. This colony was maintained on tomato plants which were placed in pots and held in rearing plastic cages (50 cm × 50 cm × 50 cm) under laboratory conditions (25 ± 3 °C, 60 ± 5 % relative humidity with a natural photoperiod of ~16 h light/8 h dark). Emerged adults from pupae were fed on 10 % honey solution and tomato terminal buds and fresh leaves. After oviposition, *T. absoluta* larvae were used in the bioassays.

Ten conidial suspensions of *Beauveria* strains P1 and P2 (10^2 to 10^9 conidia/ml) were prepared by serial dilution and used for infection. Each suspension was spread onto larvae (second to fourth larval instars), resulting in 10 μl of conidial suspension/larvae. Control larvae were treated with sterile H_2O. For each experimental condition, 30 larvae were used. Each 10 larvae were transferred in individual Petri dishes containing 10 % honey solution and fresh leaves and incubated in an incubator at 26 ± 0.5 °C and 72 ± 5 % relative humidity with a natural photoperiod of ~16 h light/8 h dark. The number of dead larvae was recorded daily. All experiments were repeated three times.

2.4 Enzyme production and assays

Hydrolytic enzymes were produced on modified Mandels liquid medium (Borgi and Gargouri 2014), supplemented with 1 % of skimmed milk. Cultures were inoculated with 10^7 conidia/ml of P1 or P2 strain and incubated at 30 °C and 150 rpm. After different incubation times, cultures media were centrifuged at 6000 g for 10 min at room temperature and the supernatants were used for the assays as follows.

Protease activity was measured by the method of Kembhavi et al. (1993) using casein as substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine/ml in 1 min at pH 8 and 60 °C.

Chitinase activity assay was performed as previously described (Kim and Yang 2003). One unit of chitinase activity was defined as the amount of enzyme that released sugars equivalent to 1 mol of *N*-acetylglucosamine per hour at pH 5 and 50 °C.

β-Glucosidase was assayed with *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma) according to Saibi and Gargouri (2011), with one unit of activity being the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per minute at pH 5 and 60 °C.

β-galactosidase activity was monitored by measuring the increase in glucose concentrations over time using lactose as substrate at pH 6 and 60 °C (Nguyen et al. 2006). The glucose formed in the reaction was determined as described by Kunst et al. (1988). One unit of activity corresponds to the amount of enzyme that liberates 1 μmol of glucose per minute in the reaction conditions.

2.5 Two-dimensional electrophoresis

Six-day cultures were used to analyse extracellular proteins from P1 and P2 strains. Two hundred microgrammes of the extracellular supernatants (filtered through a 0.45-μm filter) was loaded on isoelectro focussing strips with a non-linear gradient of

ampholytes ranging from pH 3 to 10 using the isoelectro focussing cell system. After the IEF process, the strips were run in SDS-PAGE according to Laemmli (1970). Gels were stained with colloidal Coomassie blue G250. Gels were scanned using the GS-800 imaging densitometer (Bio-Rad) and analysed using the PDQuest software (version 8.0.1; Bio-Rad). Proteins making difference between P1 and P2 in 2-D profiles were manually excised. This experiment was repeated three times.

2.6 Protein identification by mass spectrometry

Spots were destained in 25 mM ammonium bicarbonate (NH_4HCO_3), 50 % (*v*/*v*) acetonitrile (ACN; VWR Chemicals) and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature. Proteins were digested by incubating each gel slice with 10 ng/μl of trypsin (T6567, Sigma-Aldrich) in 40 mM NH_4HCO_3, 10 % (*v*/*v*) ACN; rehydrated at 4 °C for 10 min; and finally incubated overnight at 37 °C. The resulting peptides were extracted from the gel in three steps: a first incubation in 40 mM NH_4HCO_3, 10 % (*v*/*v*) ACN for 15 min at room temperature and two incubations in 47.5 % (*v*/*v*) ACN and 5 % (*v*/*v*) formic acid (Sigma) for 15 min at room temperature. The three collected extractions were pooled with the initial digestion supernatant, dried in a vacuum centrifuge (SpeedVac; Eppendorf) and resuspended with 25 μl of 0.1 % (*v*/*v*) formic acid before performing the nano-LC-MS/MS analysis.

Peptide mixtures were analysed by online capillary nano-HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nano-spray LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Ten microlitres of each peptide extract were loaded on a 300 μm ID × 5 mm PepMap C18 precolumn (LC Packings, Dionex, USA) at a flow rate of 20 μl/min. After 5 min desalting, peptides were online separated on a 75 μm internal diameter × 15 cm C18 PepMapTM column (LC Packings, Amsterdam, The Netherlands) with a 5–40 % linear gradient of solvent B in 48 min (solvent A was 0.1 % (*v*/*v*) formic acid in 5 % (*v*/*v*) ACN, and solvent B was 0.1 % (*v*/*v*) formic acid in 80 % (*v*/*v*) ACN). The separation flow rate was set at 200 nl/min. The mass spectrometer operated in positive ion mode at a 1.8-kV needle voltage and a 4-V capillary voltage. Data acquisition was performed in a data-dependent mode alternating in a single run, an MS scan survey over the range *m*/*z* 300–1700 and three MS/MS scans with collision induced dissociation (CID) as activation mode. MS/MS spectra were acquired using a 2-*m*/*z* unit ion isolation window, a 35 % relative collision energy and a 0.5 min dynamic exclusion duration.

Mascot, MS Amanda and Sequest algorithms through Proteome Discoverer 1.4 Software (Thermo Fisher Scientific Inc., USA) were used for protein identification against the UniProt *B. bassiana* database (http://www.uniprot.org/taxonomy/176275 ; 22,152 entries). Two missed enzyme cleavages were allowed. Mass tolerances in MS and MS/MS were set to 2 and 1 Da, respectively. Oxidation of methionine, deamidation of asparagine and glutamine and acetylation of lysine and protein N-terminus were searched as dynamic modifications. Carbamidomethylation on cysteine was searched as static modification. Peptide validation was performed using Target Decoy PSM Validator, and only "high confidence" peptides were retained corresponding to a 1 % false positive rate at peptide level. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaín et al. 2014) via the PRIDE partner repository (http://www.ebi.ac.uk/pride/help/archive/about) with the dataset identifier PXD003287. Identification results are presented in supplementary data 1.

2.7 Statistical analysis

SPSS version 16.0 for Windows was used for all statistical analysis (SPSS Inc. 2008, USA). Cumulative larval mortality was corrected for natural mortality using Abbott's formula (Abbott 1925). Larval mortality and enzyme production data were statistically analysed using repeated measure ANOVA and the general linear model (GLM). Means were separated by Tukey's HSD test ($p = 0.05$). The median lethal concentration and time values were calculated using the Probit analysis.

3 Results and discussion

3.1 Evaluation of P1 and P2 strains for their virulence against *T. absoluta*

The tomato leafminer, *T. absoluta*, is a major pest insect which attacks solanaceous crops. Chemical control failed to overcome this pest because of resistance development, the mine-feeding behaviour of larvae and deficient spraying technology (Siqueira et al. 2000). In the search for other pest management, mycoinsecticides are an important tool among the new alternatives to be considered.

In this study, two strains P1 and P2 of *B. bassiana* were evaluated for their virulence towards *T. absoluta* larvae, under laboratory conditions. Larvae were chosen for treatments since it is the damaging stage affecting all parts of plant especially fruits. Bioassays were performed by topical application which is the conventional method used in biological control applications.

Different concentrations of both strains conidia were tested on the daily larval mortality of *T. absoluta* (Table 1). Larval mortality caused by each strain increased proportionally with conidia concentration and time post-applications. Repeated measures ANOVA analysis showed a significant difference among treatments (at all concentrations) over the whole period of bioassay ($F = 74.9$; df = 1.51; $p < 0.001$). At each

Table 1 Cumulative percentage mortality of *T. absoluta* larvae treated with different concentrations of *B. bassiana* P1 and P2 conidia after different incubation times

Treatment	Concentration (conidia/ml)	Time after the application				*F* (df = 1.4)	*p* value
		1 day	2 days	3 days	5 days		
Control (water)	–	5a	6a	7a	8a	–	–
P1	10^2	0a	0a	0a	0a	15.974	0.016
P2		0a	0a	6.4a	13a		
P1	10^3	4.2a	5.3a	4.3a	5.4a	27.622	0.006
P2		8.4a	19.1a	24.7a	30.4a		
P1	10^4	12.6b	14.8b	23.6b	29.3b	33.158	0.005
P2		31.5b	35.1b	41.9b	48.9b		
P1	10^5	25.2c	35.1c	39.7c	47.8c	10.033	0.034
P2		50.5c	56.3c	63.4c	69.5c		
P1	10^6	45.2cd	48.9cd	56.9cd	68.4cd	18.881	0.012
P2		60cd	72.3cd	77.4cd	76cd		
P1	6.10^6	54.2de	51de	59.1de	68.4de	24.814	0.008
P2		68.4de	74.4de	86de	90.2de		
P1	10^7	53.6def	63.8def	74.1def	82.6def	64.204	0.001
P2		69.4def	76.5def	82.7def	91.3def		
P1	6.10^7	63.1ef	68ef	76.3ef	79.3ef	11.239	0.029
P2		74.7ef	84ef	93.5ef	96.7ef		
P1	8.10^8	65.2f	75.5f	80.6f	86.9f	15.662	0.017
P2		86.3f	92.5f	97.8f	98.9f		
P1	10^9	64.2f	73.4f	86f	91.3f	32.897	0.005
P2		84.2f	91.4f	93.5f	96.7f		

Results are means of three replicates for each conidial concentration. Cumulative mortality data were corrected using Abbott's formula. Means in a line followed by the same letter are not significantly different at the 0.05 level according to Tukey's HSD test

concentration, there was a statistically significant difference between P1 and P2 treatments. *F*or example, at 10^2 conidia/ml, $F = 15.9$; df = 1.4; $p = 0.016$; At 10^9 conidia/ml, $F = 32.8$; df = 1.4; $p = 0.005$ (Table 1). The concentration/time–mortality responses for each strain were fitted to regression lines, and the respective median lethal concentration and time values were obtained using the Probit analysis (Table 2). For all the regression lines, the chi-squared values were non-significant ($p > 0.05$), indicating a good fit of the regressions. At day 5 post-applications, the median lethal concentration of P1 was 10 times higher than that of P2 (1.22×10^5 and 1.21×10^4 conidia/ml, respectively). Moreover, at a concentration of 10^5 conidia/ml, the median lethal times were 5.8 days and only 1.03 day for P1 and P2, respectively (Table 2). The lower median lethal concentration and time found with P2 conidia treatment indicated the higher virulence of this strain towards *T. absoluta* larvae than P1. This is the first study showing such efficiency of *B. bassiana* in controlling *T. absoluta*, reaching 96.7 % of larval mortality using topical application of P2 strain (10^9 conidia/ml).

Pires et al. (2010) evaluated the entomopathogenic action of five *B. bassiana* isolates against eggs and larvae of *T. absoluta*. The tested isolates were pathogenic to eggs and larvae with a higher effect on eggs. However, the total mortality of the first instar larvae treated by the most virulent isolate of *Beauveria* (10^7 conidia/ml) reached no more than 37 % by the end of the period of larval development. The same report showed higher larval mortality using *M. anisopliae* conidia at the same concentration, reaching 56 %. Later, Inanli et al. (2012) found *B. bassiana* and *M. anisopliae* strains pathogenic on the first larval stage of *T. absoluta* with mortality of 12.5 and 91.67 %, respectively, 9 days after application.

Table 2 Median lethal concentration (LC_{50}) and time (LT_{50}) values following treatment of *T. absoluta* larvae with P1 and P2 conidia

Strain	LC_{50} values (conidia/ml) calculated after 5 days of treatment				LT_{50} values (days) using 10^5 conidia/ml			
	LC_{50} (95 % FL)	Slopes	Chi-square	*p* value	LT_{50} (95 % FL)	Slopes	Chi-square	*p* value
P1	1.22×10^5 (6.12×10^4–2.24×10^5)	0.4994 ± 0.062	0.052	0.974	5.8 (3.79–21.55)	0.8656 ± 0.2561	0.033	0.984
P2	1.21×10^4 (5.54×10^3–2.3×10^4)	0.4480 ± 0.0474	2.133	0.545	1.03 (0.17–1.64)	0.7235 ± 0.2501	0.200	0.905

Fiducial limits, Probit analysis parameters and statistical analysis were included for each value

Higher efficiency was shown by Shalaby et al. (2013): total larval mortality reached about 70 % when *T. absoluta* larvae were fed with leaves treated with *B. bassiana.*

In general, 5 to 10 days are required to kill an insect pest after application of a mycopesticide (Quesada-Moraga and Vey 2004). Increasing the speed of kill is the aim of recent researches to pursue the development of entomopathogenic fungi as commercial mycopesticides (Quesada-Moraga and Vey 2004). We showed that the death of 50.5 % of *T. absoluta* larvae treated with 10^5 conidia/ml of P2 was recorded after only 1 day. Higher concentration of conidia (8.10^8 conidia/ml) allowed increasing the larval mortality to 86.3 and 92.5 %, 1 and 2 days post-application. P1 strain exhibited lower virulence than P2; 91.3 % of larval mortality could be reached 5 days post-application of 10^9 conidia/ml (Table 1).

3.2 Regulation of enzymes associated with virulence

In this study, topical application method was used to mimic the natural mode of infection which does not require any specialized way of invasion. *B. bassiana* acts by direct penetration anywhere on the insect cuticle after the germination of conidia and the release of cuticle-degrading enzymes. Subtilisin-like serine proteases and chitinases are classified among these enzymes because they are involved in the penetration and digestion of insect cuticles; therefore, they are strongly associated with virulence (Fang et al. 2005; Fan et al. 2007; Joshi et al. 1995; Li et al. 2010). Besides, β-glucosidase, β-galactosidase, and *N*-acetylglucosaminidase activities from a strain of *B. bassiana* showed a specific activity against locusts (Quesada-Moraga and Vey 2004). For this reason, the study of secreted enzymes is an important field in research on filamentous fungi.

To find enzymatic correlation with the higher virulence of P2, we compared the production of extracellular protease, chitinase, β-glucosidase and β-galactosidase. As previously shown, P2 exhibited a protease activity, nine times more than P1, by day 10 (Borgi and Gargouri 2014). A high significant difference was shown over the whole period of production ($F = 688.5$; df = 1.4; $p < 0.001$) (Fig. 2a). Likewise, this strain over-produced at a lesser but significant extent a chitinase activity, approximately fourfold higher than P1 ($F = 858.1$; df = 1.4; $p < 0.001$) (Fig. 2b). β-Galactosidase was produced at the same yield ($F = 1.3$; df = 1.4; $p = 0.304$) in both strains, while P2 showed lower β-glucosidase activity than P1 ($F = 36.3$; df = 1.4; $p < 001$) (Fig. 2c, d). The overexpression of protease and chitinase is well demonstrated to be associated with increased virulence of *M. anisopliae* and *B. bassiana* strains (Fang et al. 2005; Fan et al. 2007; St Leger et al. 1996). Obviously, the increased pathogenesis of P2 strain to *T. absoluta* larvae is correlated with the increased levels of these enzymes.

In a previous report, we isolated the gene encoding a subtilisin-like protease (SBP) produced by P1 and P2 strains (Borgi and Gargouri 2014). Its sequence is available in GenBank with the accession no. KF562267.1. Subtilisins are among the most important determinants of entomopathogens virulence by degrading host cuticles, providing nutrition and inhibiting antimicrobial peptides (Bagga et al. 2004). These enzymes are well represented in the genomes of entomopathogenic fungi due to lineage-specific duplications (Gao et al. 2011; Li et al. 2010). It has been shown that SBP is highly homologous to subtilisins having a cuticle degrading capacity (Borgi and Gargouri 2014). Therefore, it was important to confirm if the higher virulence of P2 is correlated with a different SBP regulation compared to P1. The hyper-production of total extracellular protease in P2 might suggest an increased genic dose of the *sbp* protease gene in this strain. Southern blot analysis with genomic DNA digested with HindIII showed that *sbp* is a single-copy gene in both strains *B. bassiana* P1 and P2 (data not shown). This result implies that there is no duplication of the *sbp* gene in the P2 genome. Likewise, Fang et al. (2002) showed that a cuticle-degrading endoprotease (exhibiting 99 % of homology with SBP) was present as singly copy in the genome of *B. bassiana*. Instead, a transcriptional upregulation was suggested to be responsible of the protease hyper-production in P2 as the *sbp* transcript level in this strain was strongly increased versus P1 (data not shown).

3.3 Comparative extracellular proteomes of P1 and P2 via 2-DE/mass spectrometry analysis

The 2-DE coupled to LC-MS/MS was used to investigate all proteins differentially expressed in P1 and P2 secretomes. This study is useful to probe hyper-production of virulence-associated proteins, particularly to elucidate the SBP regulation at the translational level. The total extracellular proteins of P1 and P2 strains were fractionated using the 2-DE and 15 among the differently expressed ones were identified via mass spectrometry (Fig. 3). The comparison of the 2-DE maps revealed striking similarities. Almost all identified proteins were expressed in both strains but showed differences in abundance. The majority of these proteins are hydrolytic enzymes. The most abundant enzyme was the SBP identified in five different spots which correspond to the same molecular weight of around 30 kDa and pI varying from 3 to 10 (noted SBP 1 to SBP 5). It could be hypothesized that post-translational modification would be responsible for this electrophoretic behaviour. The most probable modification is phosphorylation as it greatly modifies the charge and faintly the weight of proteins. Only one of these isozymes, SBP 3, was equally expressed in both strains. However, the others were either exclusive to P2 (SBP 1 and SBP 4) or accumulated to significantly higher amounts in this strain compared to P1

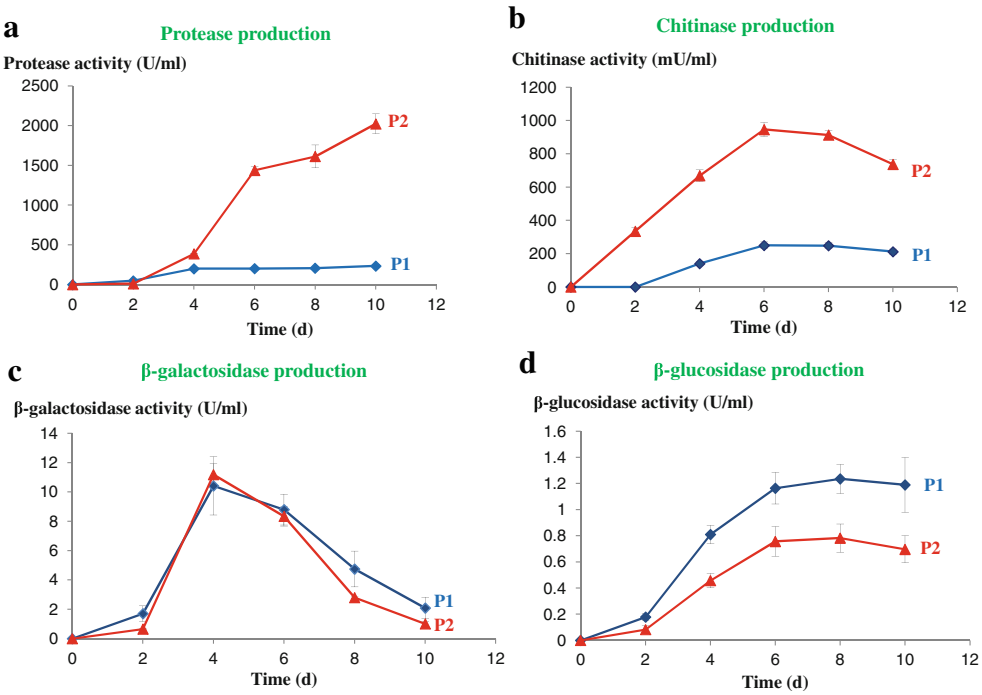

Fig. 2 Comparison of extracellular enzymes production by *B. bassiana* P1 (*blue*) and P2 (*red*). Values represent means ± SD of three determinations

(SBP 2 and SBP 5) (Fig. 3). These results suggested that the expression of SBP in *Beauveria* strains is under a double control: transcriptional and post-translational. Previous reports showed the occurrence of multiple subtilisin isoforms with different pI values and unique N-terminal sequences in the related entomopathogenic fungi *M anisopliae* (St Leger et al. 1994) and *Verticillium chlamydosporium* (Segers et al. 1999). It has been suggested that isoforms having the same molecular weight are post-translational variants or they represent gene families. Bye and Charnley (2008) found significant differences in substrate specificity and regulation of subtilisin-like proteases between isoforms of the same isolate and between different isolates of *Lecanicillium* spp. These isoforms, having pI values beween 6.48 and >9.47, included those sharing the same N-terminal sequence, which may have been derived from different transcripts from a single gene or from related genes.

Recently, it has been demonstrated that the Cdc14 phosphatase has a hub role in asexual development multiple stress responses and virulence in *B. bassiana*, by regulating protein expression and many phosphorylation events (Wang et al. 2013, 2016). Phosphoproteomic analysis revealed 14 phosphorylation motifs from all the identified phosphorylation sites as potential substrates of various protein kinases targeted by Cdc14 (Wang et al. 2016). We found that 9 of these motifs exist in 14 phosphorylation sites predicted in the SBP protease sequence (GenBank accession no. KF562267.1), including 1 site SphxxE, 2 RxxTph, 1 SphP, 1 TphP, 3 RxSph, 1 RxxSph, 1 DSphxxE, 3 SphxP and 1 site TphxxP (Sph, Tph and x denote a phosphorylated serine, a phosphorylated threonine

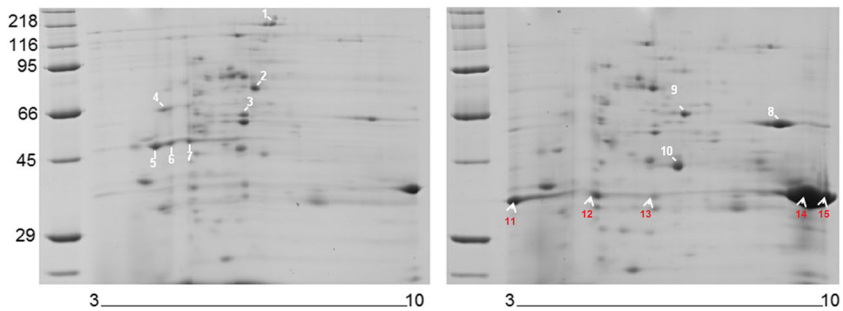

Fig. 3 Two-dimensional electrophoresis analysis. Extracellular proteins of P1 (on the *left*) and P2 strains (on the *right*). *Numbers* and *arrows* indicate the position of identified proteins. *1* β-glucosidase; *2* arabinofuranosidase; *3* tripeptidyl peptidase; *4* carboxypeptidase Y; *5*, *6* and *7* major allergen Mal f*1*; *8* carboxypeptidase Y; *9* α-amylase; and *10* chitinase. *Arrows* from *11* to *15* denote SBP protease isoforms, named SBP 1 to SBP 5, respectively. Proteins from 1 to 8 and from 9 to 15 are upregulated in P1 and P2, respectively, except for 13 which is expressed at the same level in both strains. The pH range is indicated below the gel. Molecular weight marker in kilodalton is loaded on the *left*. This experiment was repeated three times

and a random amino acid residue, respectively). However, neither expression regulation nor phosphorylation of Pr1 proteases has been detected as events associated with Cdc14 in the study of Wang et al. (2016). This could be due to the time chosen by these authors (3-day-old culture samples) which was so early to identify all differently regulated proteins in fungi, especially post-translational modifications such phosphorylation of extracellular proteins, as suggested by the authors.

Figure 3 shows that a chitinase, a carboxypeptidase Y (spot 8) and an α-amylase were equally present in higher quantities in P2 strain. In P1 strain, four enzymes were found upregulated which are a β-glucosidase, an arabinofuranosidase, a carboxypeptidase Y (spot 4) and a tripeptidyl peptidase. Two isoforms of the major allergen Mal f1 were exclusive to P1 and another was over-expressed compared to P2.

A previous study showed by comparative transcriptome analysis that growth in culture containing insect cuticles does not mimic the environment experienced on the insect surfaces. Secreted proteins, particularly proteases, were highly more induced on insect cuticle surfaces (Gao et al. 2011). As the experiments of this study were performed in vitro, further experimentation is required to demonstrate the production, accumulation and localization of individual SBP protease isoforms and chitinase in insects during infection processes.

4 Conclusion

Difficulty in managing the tomato borer led to the spread of this dangerous specie in most of the countries, threatening the current world tomato production. Mycoinsecticides are among the best choices to control this pest. Here, we showed a high insecticidal potential of *B. bassiana* P1 and P2 strains towards *T. absoluta* larvae and significant better efficiency of P2 than P1. This allows the recommendation of P2 for the application in the management of the tomato borer. P2 is a mutant hyperproducer of extracellular proteases and chitinase, compared to the wild-type strain P1. We have identified four isoforms of the SBP, which were over-expressed in P2. Differently expressed isoforms were suggested to be the result of different transcriptional and translational regulation in both strains, and this might explain the higher virulence of P2 towards *T. absoluta*. These results showed that the profile of fungal extracellular enzymes, especially proteases, could be considered as a fingerprint reflecting pathogenicity towards insects; enzymatic assays may contribute to the screening of the most virulent strains.

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Compliance with ethical standards

Conflict of interest The authors that they have no conflict of interest.

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