

## Functional Secretomics of Phytotoxic Compounds of *Monosporascus cannonballus*

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### ABSTRACT

*Monosporascus cannonballus* and *M. eutypoides* are the causative agents of melons' root rot and vine decline, with little knowledge about their mechanisms of infection. *M. cannonballus* secretome was isolated and subdivided into two fractions based on molecular weight (smaller and larger than 10 kDa), and further separated via high voltage paper electrophoresis and SDS-PAGE, respectively. Large cell-free filtrates of the fungus were found to contain biologically active proteins that were further characterized via mass spectrometry and revealed to be  $\alpha$ -1,2-mannosidase and serine protease. Meanwhile, biochemical analyses of low molecular weight compounds were suggestive to be similar to marasmines. Both fractions were capable of inducing phytotoxicity, once infiltrated into the melon leaves. This is the first report of phytotoxic compounds isolated from *M. cannonballus* contributing to disease induction in melon plants.

**Keywords:** Biologically active compounds; *Cucumis melon*; Marasmine; Phytotoxin, Protein; Root rot and vine decline.

### INTRODUCTION

*Monosporascus* Root Rot and Vine Decline of melons (MRRVD) is an economically important disease worldwide (Troutman and Matejka, 1970; Watanabe, 1979; Reuveni *et al.*, 1983; Mertely *et al.*, 1991; Garcia-Jimenez *et al.*, 1994; Martyn *et al.*, 1994; Sales Junior *et al.*, 2004; Tsay and Tung, 1995; Martyn and Miller, 1996; Bruton and Miller, 1997a, b; Karlatti *et al.*, 1997; Aegerter *et al.*, 2000; Sarpeleh, 2008; Cohen *et al.*, 2012; Sarpeleh *et al.*, 2012), are induced by two species of this pathogen, namely, *M. cannonballus* (Pollack and Uecker, 1974) and *M. eutypoides* (Ben Salem *et al.*, 2013). It has been shown that the pathogenicity of *M. cannonballus* is mediated by root-infecting zoosporic

*Olpidium* spp. (Stanghellini and Misaghi, 2011; Aleandri *et al.*, 2017).

*M. cannonballus* causes a rapid plant wilt at fruiting stage, leading to total yield loss. Chlorosis and necrosis of crown leaves and secondary (feeder) root rot are typical symptoms of the disease. General chlorosis is first evident in crown leaves progressed into apical leaves and followed by progressive necrosis and collapse of the entire canopy 10-14 days before harvest. Brown spots are visible on roots, particularly at root junctions where the secondary and tertiary feeder roots are deteriorated (Sarpeleh, 2008). Symptom induction and development throughout the plants indicates the potential role of phytotoxins in interaction between *M. cannonballus* and melons.

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Phytotoxins have been shown to induce and develop typical disease symptoms in many plant-pathogen interactions (Sarpeleh et al., 2007). Phytotoxins, either Host Specific (selective) Toxins (HSTs) or host non-specific toxins, are pathogenic products that are mainly restricted to low molecular weight compounds and/or proteins and disturb the metabolism of the host at very low concentrations. They have the potential of movement in plants and hence can act far away from the infection site (Lucas, 1998).

The contribution of fungal phytotoxins in disease induction has been studied in many pathosystems (Walton, 1996; Manning and Ciuffetti, 2005; Strange, 2007; Horbach et al. 2009; Park et al., 2009; Stergiopoulos and de Wit, 2009), while similar reports are lacking for *M. cannonballus*. *Fusarium solani*, the causal agent of sudden death syndrome of soybean (*Glycine max* L.), was reported to produce a heat-unstable 17 kDa protein that contributes to necrosis (Jin et al., 1996). In tomato leaf mold, extracellular proteins (14 and 17 kDa) produced by *Cladosporium fulvum* were purified and shown to contribute to disease induction as virulence factors (Schottens-Toma and de Wit, 1988; Joosten and de Wit, 1988; Wubben et al., 1994; Lauge et al., 1997). Other Phytotoxic proteins such as cerato-ulmin (Yaguchi et al., 1993; Richards, 1993) and cerato-platanin (Pazzagli et al., 1999) have also been reported from *Ophiostoma novo-ulmi* and *Ceratocystis fimbriata* f. sp. *platani*, respectively. Additionally, *Rhynchosporium secalis*, the pathogen of barley leaf scald, produces necrosis inducing peptides of 3.8, 6.8 and 9.2 kDa (Wevelsiep et al., 1991). *Phytophthora cactorum*, a pathogen of strawberry, produces a unique Phytotoxic protein (PcF) with 5.6 kDa in mass, which induces leaf necrosis (Orsomando et al., 2001).

The role of phytotoxins in disease induction was fully investigated in *Pyrenophora tritici-repentis*, the causal agent of wheat tan spot. The pathogen is a necrotrophic fungus, which induces the disease through the production of several

host selective toxins. Different races produce varieties of toxins acting alone or in combination as pathogenicity/virulence factors; defining the host range (Lamari and Bernier, 1989; Lamari et al., 1995; Ciuffetti and Touri, 1999; Strelkov and Lamari, 2003). Two proteinaceous toxins designated as Ptr ToxA (Tox A) and ToxB (Ciuffetti et al., 2010) (Different spelling in the list of references et al., 1998) are amongst the well-studied proteinaceous phytotoxins. Tox A is a host-selective toxin isolated from culture filtrates of *P. tritici-repentis* (Balance et al., 1989; Tomas et al., 1990; Touri et al., 1995) and from intercellular washing fluid of wheat infected with the fungus (Lamari et al., 1995). The toxin is a 13.2 kDa protein and the product of a single gene, which is only present in toxin-producing isolates (Balance et al., 1996; Faris et al., 1996; Ciuffetti et al., 1998). Transformation of a non-pathogenic isolate with Ptr ToxA clearly demonstrated that Ptr ToxA functions as a primary determinant of pathogenicity (Ciuffetti et al., 1997; Moffat et al., 2014; Manning and Ciuffetti, 2015). The toxin, localized to the chloroplasts, has shown to be active only in the presence of light (Manning and Ciuffetti, 2005). Ptr ToxB is produced by chlorosis-inducing pathotypes of *P. tritici-repentis* (Orolaza et al., 1995; Ali and Francl, 2001). The toxin is a 6.6 kDa protein (Orolaza et al., 1995; Strelkov et al., 1999; Strelkov et al., 2003) and the product of a single gene (Martinez et al., 2001).

The role of host specific and non-specific phytotoxins has been showed in symptom induction in barley plants infected with *Pyrenophora teres* (Sarpeleh et al., 2007, 2008, 2009; Ismail et al., 2014). Host specific proteinaceous toxins as well as low molecular weight compounds, belonging to a group of chemical compounds known as marasmines, were isolated from culture filtrates of the pathogen and showed to induce necrotic spots and extensive chlorosis in attached barley leaves, respectively (Sarpeleh et al. 2008, 2009; Ismail et al., 2014).

The role of toxins, however, has not been shown in *M. cannonballus*-melon interaction, despite the typical symptoms of the MRDVD that may be attributed to the secretion by the pathogen of these compounds. Hence, the objective of the present study was to detect and analyze if phytotoxic compounds secreted by *M. cannonballus* were potentially involved in symptoms onset in muskmelon.

## MATERIALS AND METHODS

### Fungal and Plant Materials

A pathogenic isolate of *M. cannonballus* (Sarpeleh *et al.*, 2012) was used for extraction of fungal secretome. Muskmelon ecotype (Zard-e-Garmsar) as well as cucurbit, wheat, barley and tomato seeds were grown at 28±2°C at 16/8 hours light/dark photoperiod on soil and used for the phytotoxicity analysis and host specificity of the secretome.

### Secretome Isolation and Identification

*M. cannonballus* was grown in five 250 mL flasks each containing 100 mL of Fries culture medium (Friis *et al.*, 1991). The cultures were incubated for 21 days at 28°C in dark without agitation. Fungal secreted compounds were separated from the culture media using previously established protocols (Friis *et al.*, 1991; Sarpeleh *et al.*, 2007). The medium containing fungus was harvested from the culture using a Buchner funnel and Whatman No. 1 filter paper followed by a 0.45-µm Millipore filter (Sartorius AG, Gottingen, Germany). The filtrates (50 mL) were passed through a YM-10 Amicon Centriplus filter (Millipore Corporation, Bedford, MA) with a 10 kDa exclusion to initially separate Low Molecular Weight Compounds (LMWCs). The concentrated retentates were washed several times with dH<sub>2</sub>O and concentrated again to 2 mL using the YM-10 filter

according to manufacturer instruction. The presence of proteinaceous compounds in high molecular weight retentates was preliminary detected through staining with Coomassie brilliant blue R-250 for 10 minutes at room temperature (22°C). Protein concentration was determined using Bradford protein assay (Bradford *et al.*, 1976).

The proteinaceous compounds (12 µl) were separated on a 15% Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Sambrook and Russell, 2001). The gel was fixed in a solution containing ethanol: acetic acid: water (3:6:1) for 15 minutes and stained overnight in 0.01% Coomassie Brilliant Blue R-250 (Sigma, St Louis, MO, USA). The protein bands were excised from the gel and peptide fingerprinting was carried out *via* MALDI-TOF-TOF at University of York.

Low molecular weight filtrates (40 mL) were concentrated to 5 mL using a rotary evaporator (Buchi Labortechnik, Flawil, Switzerland) at 40°C. Most of the salts were precipitated by storage of the concentrated filtrates at 4°C and removed by centrifugation at 6,000×g for 20 minutes. Remaining salts were precipitated using cold 70% methanol at pH= 3 in the presence of 2.48% formic acid (pH= 1.75). Supernatant was collected and concentrated to 2 mL using the rotary evaporator.

The partially purified low molecular weight compounds were analyzed by High Voltage Paper Electrophoresis (HVPE). HVPE was performed after spotting the filtrate on a sheet of Whatman No. 1 filter paper (28×10 cm). The paper was submerged in a 2.84% formic acid and 5.92% acetic acid buffer (pH= 1.75) and electrophoresis was carried out at 400V for 25 minutes in a HVPE device (LKB BROMMA, Buchler Instruments Inc., NJ, USA). Since many phytotoxic LMWCs are ninhydrin positive, the paper was stained with 0.2% ninhydrin in acetone and heated at 100°C for 2-3 minutes. Aspartic acid, as standard, was run on the HVPE side by side to the samples and the mobility of each



ninhydrin-positive compound in the paper was determined Relative to the mobility ( $R_m$ ) of aspartic acid.

### Secretome Bioassay

Fractions (150  $\mu$ L) of high (0.184  $\mu$ g mL<sup>-1</sup>) and low molecular weight were infiltrated in intact muskmelon leaves. In order to determine host range specificity, both fractions were infiltrated into muskmelon, cucurbit, wheat, barley and tomato leaves. The treated plants were kept at dark overnight and incubated further at 25°C under 16/8 hours photoperiod for 120 hours and monitored on a daily basis.

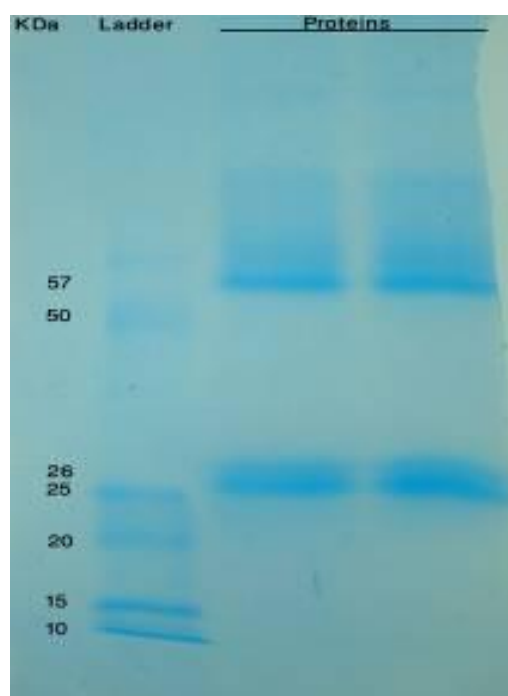
### Thermal and Enzymatic Degradation of Metabolites

In order to check the thermostability of the size-fractionated secretomes, proteins were heated at 40, 60, 80 and 100°C and LMWCs (150  $\mu$ L each) were heated at 100°C for 1 hour. Moreover, proteins (400  $\mu$ L) were treated with proteinase K (Life Sciences, UK) in sodium acetate buffer, pH= 7. The mixture was incubated at 37°C for 2 hours. Treated samples were injected into muskmelon leaves to test the bioactivity of fractions following heat and enzyme treatment. Control plants were injected with proteinase K, dH<sub>2</sub>O and diluted Fries Culture Medium (FCM).

## RESULTS

### Secretome Isolation and Characterization

The secretome fraction larger than 10 kDa appeared to contain three major protein bands with relative mass of 26, 27 and 57 kDa (Figure 1). Peptide sequencing via mass spectrometry and MASCOT search results revealed that the 26 and 27 kDa proteins had the same sequence information and most



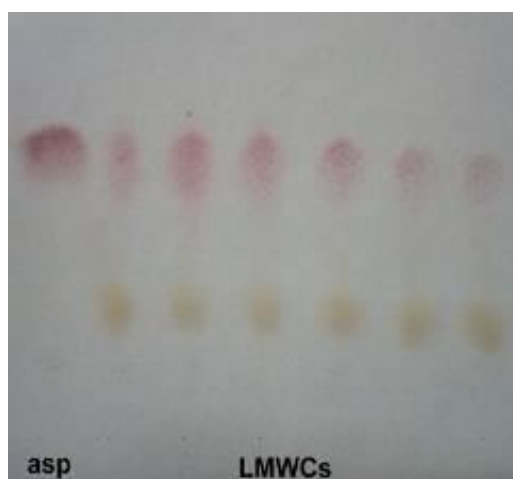
**Figure 1.** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of secretome fraction extracted from culture filtrates of *Monosporascus cannonballus* larger than 10 kDa. HMWCs produced three proteinaceous bands: 26, 27 and 57 kDa.

likely were the isoforms of an alkaline serine protease found in *Lecanicillium psalliotae* (accession No. AAU01968) and involved in cuticle degradation. The protein band with 57 kDa in size was identified as  $\alpha$ -1,2-mannosidase similar to *Phytophthora infestans* (T30-4) (accession No. XP\_002901095).

The smaller secretome fraction contained one ninhydrin-positive compound, which was visible on electrophoretogram and its Relative mobility ( $R_m$ ) was the same as aspartic acid (Figure 2).

### Secretome Bioassay

Both fractions induced phytotoxicity 120 hours after treatment of muskmelon leaves. The whole infiltrated area showed water-soaked and necrotic lesions with a chlorotic margin visible in some treated leaves.



**Figure 2.** Ninhydrin-positive compounds isolated from *M. cannonballus*. Low Molecular Weight Compounds (LMWCs) extracted from culture filtrates of *Monosporascus cannonballus* were run on high voltage paper electrophoresis and visualized using ninhydrin. The mobility of ninhydrin-positive compound was calculated relative to aspartic acid (asp).

Proteins induced brown necrotic lesions at injected area while LMWCs incited water-

soaking lesions; indicating their different role in pathogenicity process. No symptoms were evident on the leaves treated with diluted FCM as control (Figures 3 and 4).

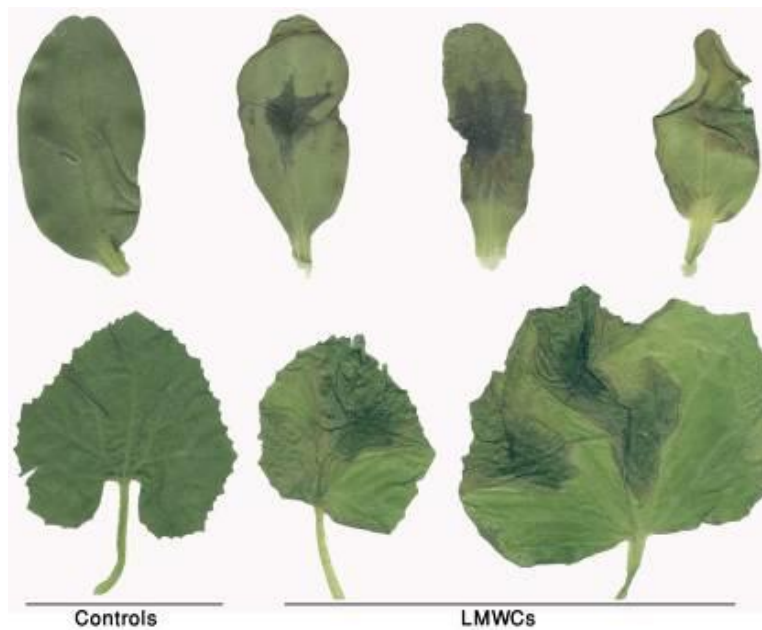
Host specificity of protein compounds present in the fraction bigger than 10 kDa induced necrotic symptoms only in muskmelon, cantaloupe, and cucurbit 120 hours post-injection, suggesting being specific on cucurbitaceous plants (Figure 5-A). In contrast, LMWCs induced water soaking and chlorosis symptoms on all injected leaves, except tomato, indicative of being non-host specific (Figure 5-B).

### Thermal and Enzymatic Degradation of Metabolites

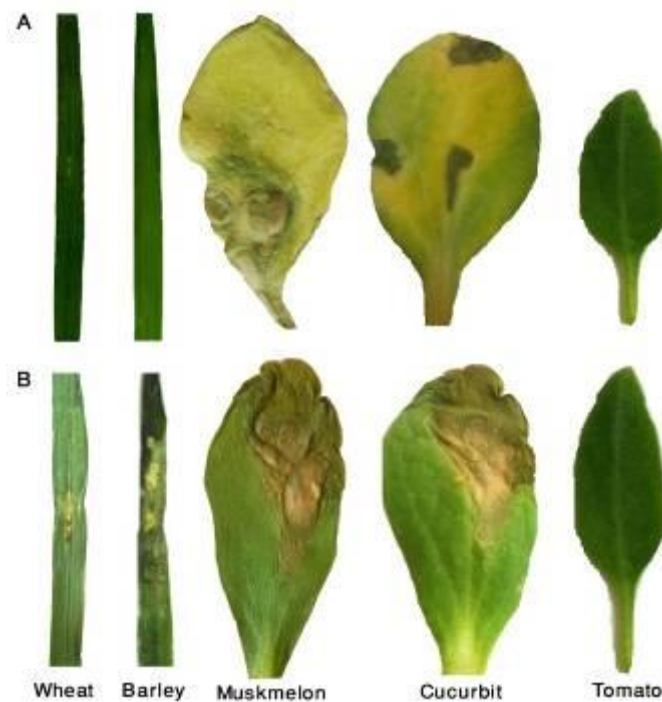
The proteins were heat and protease labile with no toxicity over muskmelon leaves (Figures 6-A and 7). In contrast, LMWCs was heat stable that induced water soaking lesion in infiltrated leaves (Figure 6-B).



**Figure 3.** Biological activity of different proteins extracted from culture filtrates of *Monosporascus cannonballus* upon their injection into muskmelon leaves and the plants were kept at conditions described in the text. Control leaves were injected with diluted Fries culture medium.

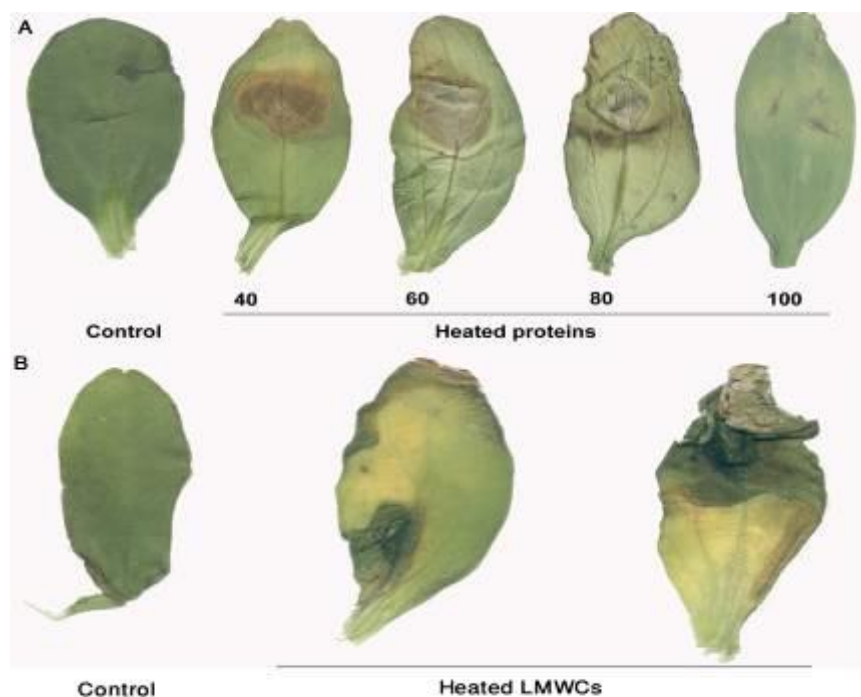


**Figure 4.** Biological activity of Low Molecular Weight Compounds (LMWCs) extracted from culture filtrates of *Monosporascus cannonballus* partially purified and injected into muskmelon leaves. The plants were kept at dark overnight and incubated further under 16/8 hours photoperiod for 120 hours. Control leaves were injected with diluted Fries culture medium.

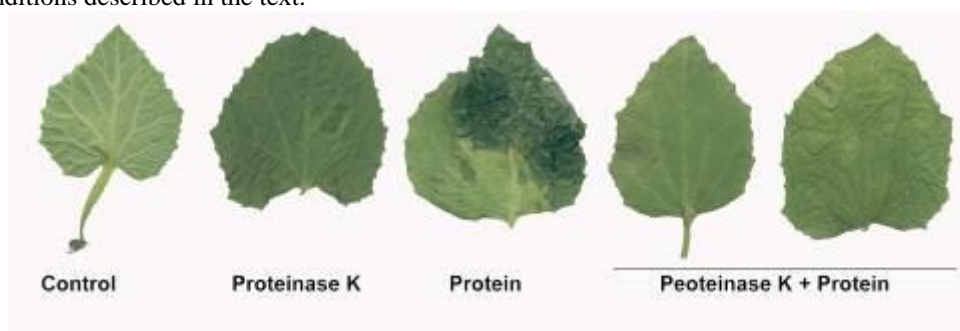


**Figure 5.** Host specificity of proteins and Low Molecular Weight Compounds (LMWCs) extracted from *Monosporascus cannonballus*. The intact leaves of different plant species were infiltrated with proteins (A) and LMWCs (B) extracted from culture filtrates of *M. cannonballus*. The plants were kept at conditions described in the text for up to 120 hours. Mock injected plants were treated with diluted Fries culture medium.





**Figure 6.** Phytotoxicity test of heated proteins (A) and Low Molecular Weight Compounds (LMWCs) (B) extracted from culture filtrates of *Monosporascus cannonballus*. Proteins were heated at 40, 60, 80 and 100°C and LMWCs at 100°C for 1 hour. The samples were injected in intact leaves of muskmelon plants and kept at conditions described in the text.



**Figure 7.** Effect of proteinase K on the phytotoxicity of proteins incubated at 37°C for 2 hours and injected into muskmelon leaves.

## DISCUSSION

In the present study, both proteins and LMWCs were isolated from culture filtrates of a pathogenic isolate of *M. cannonballus*, with each shown to induce phytotoxicity in a muskmelon leaf toxicity assay. Proteins were heat and protease labile, identified as serine proteases and  $\alpha$ -1,2-mannosidase.

LMWCs suggested to be consistent with those described previously from other fungal pathogens and were probably marasmines because of their behavior in HVPE and heat resistance (Poplestone and Unrau, 1973; Friis *et al.*, 1991; Weiergang *et al.*, 2002; Sarpeleh *et al.* 2009).

Both Proteins and LMWCs induced biological activity in affected leaf tissues in very small quantities. The role of proteins in



symptom induction was confirmed because heat-treated proteinaceous metabolites did not induce the symptoms and treatment with protease decreased their phytotoxicity. Additionally, the stability of proteinaceous metabolites under heat also was determined by incubating the samples at 40, 60, 80 and 100°C for 60 minutes. The loss of activity that occurred with these treatments followed a pattern fairly typical for protein denaturation. Given the very small quantities of proteins needed to induce symptom development, it is likely that highly active and mobile proteinaceous toxin was present among these filtrates and its impact on disease development was significant.

A different role is suggested for each of the proteins and LMWC compounds in pathogenicity process. Proteins and LMWC induced necrotic and water-soaking lesions in affected leaf tissues. The main symptom induced by proteins was evident as brown necrotic lesions at injected area (Figure 3), while LMWCs caused water-soaking lesions (Figure 4). Similarly, the two proteinaceous phytotoxic compounds detected in culture filtrates of *P. tritici-repentis*, Ptr ToxA and Ptr ToxB, induced different symptoms on infiltrated wheat leaves. Ptr ToxA caused necrosis in wheat cultivar susceptible to the necrogenic isolates of the pathogen, while Ptr ToxB caused extensive chlorosis but not tan necrosis in attached wheat leaves (Lamari and Bernier, 1989; Strelkov and Lamari, 2003; Strelkov et al., 1999; Tomas and Bockus, 1987; Orolaza et al., 1995). In the interaction between *P. teres* and barley, Sarpeleh and coworkers (2007) showed similar findings. Proteinaceous and low molecular weight compounds isolated from culture filtrates of *P. teres* induced brown necrotic spots and extensive chlorosis, respectively. In the present study, proteins and LMWCs each induced specific symptoms, suggesting their individual role in symptom induction in a *M. cannonballus*-melon pathosystem.

Proteins with phytotoxic activity were identified in culture filtrates of *M. cannonballus* as serine proteases and  $\alpha$ -1,2-

mannosidase. In similar studies, several proteinaceous compounds were detected from fungal plant pathogens with phytotoxic activity (Joosten and de Wit, 1988; Schottens-Toma and de Wit, 1988; Lamari and Bernier, 1989; Tomas et al., 1990; Wevelsiep et al., 1991; Lamari et al., 1995; Jin et al., 1996; Ciuffetti and Touri, 1999; Pazzagli et al., 1999; Orsomando et al., 2001; Strelkov and Lamari, 2003). Proteinaceous compounds like ABC transporters, cellulase, and chitinase from *P. teres*, the causal agent of barley net blotch, demonstrated to have role in symptom induction during interaction between barley and the pathogen (Sarpeleh et al., 2007, 2008). Similarly, present results suggest that the proteins with phytotoxic activity isolated in this study (serine proteases and  $\alpha$ -1,2-mannosidase) may have a similar identity and role in disease induction during the interaction between melon and *M. cannonballus*. Serine proteases are reported from a variety of organisms with different roles (Hedstrom, 2002). Such enzymes extracted from *Fusarium solani* f. sp. *eumartii* (Olivieri et al., 2002) and *Phytophthora infestans* (Tian et al., 2005) have been shown to contribute to pathogenicity process through degradation of pathogenesis-related proteins. However, the mechanism of  $\alpha$ -1,2-mannosidase has remained elusive in pathogenicity. Since there is no report of retaining linkages of mannosyl residues to any biomolecules within plant cells, this would be an interesting venue to pursue.

Previous studies isolated LMWCs from *M. cannonballus* including Dehydroxyarthrinone, monosporascone, monosporascol A, demethylcerdarin, 2H-benzo[f]isoindole-4,9-dione, and azamonosporascone (Piggott, 2005), while the phytotoxicity of these compounds have not been reported. In this study, one LMWC was isolated, which induced phytotoxic activity in muskmelon leaves. The compound reacted positively with ninhydrin and its Relative mobility (Rm) was the same as aspartic acid. This evidence suggests that



the compound may belong to marasmines, a group of phytotoxic and ninhydrin-positive compounds reported previously from other fungal species (Poppstone and Unrau, 1973; Smedegaard-Petersen, 1976; Bach *et al.*, 1979; Friis *et al.*, 1991; Weiergang *et al.*, 2002; Sarpeleh *et al.*, 2009). Further investigations are required to precisely identify the compound.

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## سکرتومیکس کارکردی ترکیبات سمی مونوسپوراسکوس کنوبالوس بر علیه گیاه

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### چکیده

مکانیسم عمل مونوسپوراسکوس کنوبالوس و مونوسپوراسکوس یوتیپویدس (عامل پوسیدگی ریشه و زوال بوته خربزه و طالبی) به خوبی شناخته نشده است. سکرتوم گونه کنوبالوس جدا و به دو قسمت بر اساس وزن ملکولی (کوچکتر و بزرگتر از 10 کیلوالتون) تقسیم و توسط الکتروفورز کاغذی با ولتاژ بالا و ژل پلی اکریلامید حاوی سدیم دودسیل سولفات جدا گردیدند. عصاره‌های عاری از سلول قارچ پروتیین‌ها فعالی داشت که توسط اسپکترومتر جرمی توالی یابی گردیدند و مشخص شد که الف-1 و 2 مانوسیداز و سرین پروتیناز هستند. از طرف دیگر آنالیزهای بیوشیمیایی ترکیبات با وزن ملکولی پایین مشابه ماراسمین‌ها بودند. هر دو قسمت زمانی که بر روی گیاه تیمار شدند منجر به گیاه‌سوزی گردیدند. این اولین گزارش ترکیبات گیاه‌سوز جدا شده از مونوسپوراسکوس کنوبالوس است که باعث القای بیماری در گیاهان جالیزی می‌گردد.