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Determination and identification of the solanapyrones and new metabolites from *Ascochyta rabiei*

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Abstract

A simple, rapid, accurate and robust liquid chromatography / mass spectrometry (LC/MS) method was developed for the determination and quantification of the solanapyrones and new metabolites. Portions of the crude solvent extracts of culture filtrate from *Ascochyta rabiei,* agent of chickpea blight were also analysed for confirmation by combined LC with time-of-flight (TOF)-MS. Isolate Haypa 3 of *A. rabiei* was grown on Czapek Dox liquid medium supplemented with the inorganic cations for 7, 14 and 21 days. After the culture filtrates were extracted by solid phase extraction using C18 cartridge, toxins were eluted with acetonitrile (ACN). In addition to the three solanapyrones, a new metabolite in the culture filtrates of *A. rabiei* was identified by LC / electrospray ionization (ESI)-MS and LC/TOF-MS. The new metabolite was determined in the culture filtrate on 21st day *in vitro* growth of the fungus. The new metabolite eluting at 8.33 min was found to have an accurate mass of *m/z* 319.1603. The elemental composition calculator produced a formula based on the ion at *m/z* 319.1603 which indicated the formula of $C_{18}H_{22}O_5$.

Key words: chickpea, *Ascochyta rabiei*, phytotoxin, solanapyrone, LC/MS.

Introduction

According to FAO records in 2009, Turkey was the second largest producer of chickpea in the world after India. Total plantation field of chickpea was 454.928 hectares, production volume was 562.564 tons and rate of yield was 1.237 kg per hectare in Turkey in 2009 (FAO, 2010). In Turkey, different biotic and abiotic stresses negatively affect chickpea production and quality (Soran, 1977). Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. is one of the most damaging diseases of chickpea, which is common in many areas including the Middle East, the Mediterranean region, and North Africa (Nene, 1982; Nene et al., 1989). The fungus attacks all above-ground parts of plants, causing necrotic lesions which are circular on leaves and pods and elongate on petioles and stems. When stems and petioles are girdled, they usually break (Nene, Reddy, 1987). The disease may cause total yield loss if the environmental conditions are favorable (Singh, Reddy, 1990).

Many pathogenic fungi produce one or more toxic metabolites which are injurious to plants. It is thought that these toxins are playing an important role in plant diseases, because they can induce most or all of the disease symptoms (Yoder, 1980; Strobel, 1982). It is known that early symptoms of *A. rabiei* cause epinasty and loss of turgor in petioles and young branches (Alam et al., 1989). Later, the whole of the aerial part of the plant may dry out and die. It has been suggested that such symptoms could result from solanapyrones which are synthesized by the fungus during pathogenesis (Höhl et al., 1991). Solanapyrones A, B and C have been found in the culture filtrates and spore germination fluids of *A. rabiei* (Alam et al., 1989; Chen, Strange, 1991; Höhl et al., 1991; Latif et al., 1993; Chen, Strange, 1994; Kaur, 1995; Zerroug et al., 2007). In addition to the three solanapyrones, Chytochalasin D has been reported in liquid culture of *A. rabiei* (Latif et al., 1993; Shahid, Riazuddin, 1998). In the following years, Bahti and Strange (2004) found the new compound, which is named as demethylated solanapyrone A, in culture filtrates of *A. rabiei*. The solanapyrones have first been found in the culture filtrate of *Alternaria solani* (Ell. & Mart.) Jones and Grout, the causative agent of early blight of tomato and potato (Matern et al., 1978). The chemical structures of solanapyrones A, B and C were elucidated by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The solanapy-

rones are decalines with a pyrone moiety in which the alternatives of an ethanolamine or a methoxy group and an aldehydic or alcohol group give rise to the variation in structure found in the three compounds (Ichihara et al., 1983; Strange, 1997) (Fig. 1).

The solanapyrones have been determined by high-performance liquid chromatography (HPLC) (Chen et al., 1991; Hamid, Strange, 2000). These conventional methods based on C18 column separation with acetonitrile (ACN)-water (1:1) mobile phase with ultraviolet (UV) detection. They were not able to separate solanapyrones sufficiently for quantitative analysis and need optimization (Chen et al., 1991). Especially, solanapyrone B and solanapyrone C are coeluted so that they have the same retention time and it is rather difficult to separate them with UV detection. However, solvent optimization is still limited owing to the difficulty of monitoring the crossing over of peaks in different solvents and the large number of variables which affect the separation (Snyder, Kirkland, 1979). Höhl et al. (1991), therefore, used co-chromatography [TLC (thin layer chromatography), HPLC, MS and ^{1}H-NMR (nuclear magnetic resonance)] in order to identify solanapyrones in the culture medium of *A. rabiei*. Therefore there is a need of simple, rapid, accurate and robust analytical method for the separation. Liquid chromatography-mass spectrometry (LC/MS) and time-of-flight-mass spectrometry (LC/TOF-MS) as a routine analytical tool have become accessible for use in recent years. TOF-MS uniquely offers the possibility of providing accurate mass data at high sensitivity and can function across a wide mass range without loss in sensitivity. The possibilities of exploiting applications of LC/TOF-MS are really just becoming apparent, with publications appearing in the fields of emerging environmental contaminants (Ferrer, Thurman, 2003), estrogens in river sediments (Labadie, Hill, 2007) pesticides in fruit and vegetables (Ferrer et al., 2005), herbicides in olive oil (Garcia-Reyes et al., 2006), fungal metabolites and mycotoxins (Nielsen, Smedsgaard, 2003; Tanaka et al., 2006; Şenyuva et al., 2008).

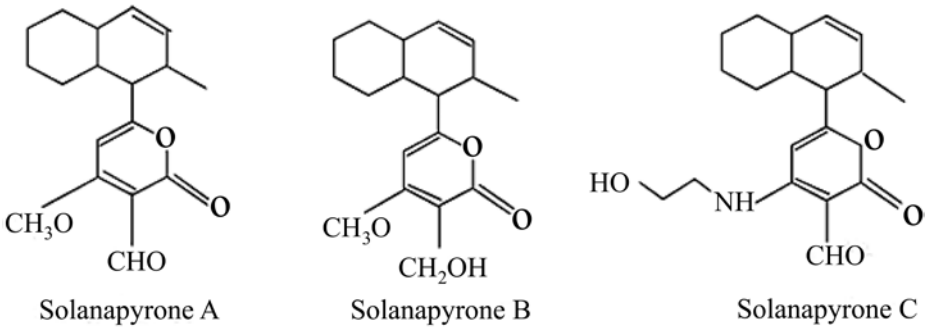

Figure 1. The structures of the solanapyrone toxins (Strange, 1997)

In this research we developed a simple, rapid, accurate and robust analytical method and used this technique to detect the presence of a new metabolite and the solanapyrones produced by *A. rabiei*. The identification was based on LC/MS analyses and the new metabolite was also confirmed by accurate mass measurements of LC/TOF-MS.

Materials and methods

The study was carried out in 2008 at University of Ankara and Ankara Test and Analysis Laboratory (ATAL), Turkey.

Reagents. ACN, formic acid (98%) and acetic acid (glacial) were analytical grade and obtained from "Merck" (Germany). Ultra-pure water was used throughout the experiments (Milli-Q system, "Millipore", USA).

Ascochyta rabiei isolate. Isolate Haypa 3 of *A. rabiei* obtained from the culture collection of the Department of Plant Protection, Faculty of Agriculture, University of Ankara. The isolate was grown on CSMDA (chickpea seed meal dextrose agar: chickpea meal 40 g, dextrose 20 g, agar 20 g, distilled water 1 l) at 22 ± 1ºC in a climate chamber with a 12 h light-photoperiod. Fungal isolate was stored as a suspension (10^7 spores ml^{-1}) in 10% glycerol at −80ºC for use in the future experiments.

Production and isolation of solanapyrones. The isolate of *A. rabiei* was grown on Czapek Dox medium supplemented with the inorganic cations ($ZnSO_4 \cdot 7H_2O$, 0.05 g l^{-1}, $CaCl_2 \cdot 2H_2O$, 0.1 g l^{-1}, $CuCl_2 \cdot 2H_2O$, 0.02 g l^{-1}, $CoCl_2 \cdot 6H_2O$, 0.02 g l^{-1}, $MnCl_2 \cdot 4H_2O$, 0.02g l^{-1}) (Bahti, Strange, 2004). After distribution to 250 ml Erlenmeyer flasks (30 ml per flasks) and autoclaving, each flask was inoculated with 30 µl spore suspension (10^7 spores ml^{-1}) of *A. rabiei* and incubated without shaking at 20 ± 1ºC in continuous light for 7, 14 and 21 days in a climate chamber. Fungal mycelia and spores were removed by filtration through Whatman No. 1 filter paper. Mycelial mass was dried at 70ºC (UNB 500 Oven – 108 lt, "Memmert GmbH + Co. KG", Germany) until constant weight. Solid phase extraction cartridges (SPE; 1 g C18, end-capped Isolute, "Alltech Chromatography", USA) were conditioned with methanol (10 ml) and then water (10 ml). The culture filtrates (10 ml) were passed through the column and after washing with water (5 ml), the solanapyrones were eluted with 2 ml ACN (HPLC grade) and stored in a deep freezer at −20ºC until required.

LC/electrospray ionization (ESI)-MS analysis. The LC/ESI-MS analyses for the screening and quantitation of solanapyrones A, B and C were performed by an Agilent 1100 HPLC system ("Waldbronn", Germany) consisting of a binary pump, an autosampler, diode array detector (DAD) system and a temperature controlled column oven, coupled to an Agilent 1100 MS detector equipped with ESI interface. The analytical separation was performed on a ACE 5 C18 (150 x 4.6 mm, 5 µm) ("Agilent Technologies", USA) using the isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid [A] and ACN [B], [A:B] [50:50; v:v] at a

flow rate of 0.8 ml min^{-1} and injection volume was 20 µl. UV-data obtained from DAD was monitored at 324 nm. MS data acquisition was performed in selected ion monitoring (SIM) mode using the interface parameters: drying gas (N_2) flow of 13 l min^{-1}, nebulizer pressure of 50 psig, drying gas temperatures of 350ºC, capillary voltage of 4 kV, fragmentor voltage of 55 eV, positive polarity. Monitored ions for the screening and quantification of solanapyrone A ($C_{18}H_{22}O_4$), solanapyrone B ($C_{18}H_{24}O_4$), solanapyrone C ($C_{19}H_{25}NO_4$) are 303, 287 and 332 respectively. Full scan analyses were performed in the mass range of 50–1500 for the identification of new metabolites.

Identification and confirmation of solanapyrones and the new metabolite by LC/TOF-MS analysis. All analytical work was performed using an Agilent 6210 TOF-MS coupled to an Agilent 1200 Series HPLC. The separation of solanapyrones and other metabolites was also carried out using an HPLC system (consisting of vacuum degasser, autosampler with thermostat, binary pump, and DAD system) equipped with a reversed-phase C18 column (ZORBAX Extent C18 (100 × 4.6 mm, 1.8 µm). The TOF-MS was equipped with a dual nebulizer electrospray source, allowing continuous introduction of reference mass compounds. The instrument was scanned from mass-to-charge ratio (*m/z*) 100 to 1000 for all samples at a scan rate of 1 cycle sec^{-1} in 9.429 transient/scan. This mass range enabled the inclusion of two reference mass compounds, which produced ions at *m/z* 121.050873 and 922.009798. The injected sample volume was 5 µl.

The mobile phase in the HPLC analysis consisted of ACN [A] and 10 mM ammonium acetate in and aqueous solution of 0.1% formic acid [B], [A:B; 50:50; v:v]. The flow rate was 0.6 ml min^{-1}. The gradient elution started with 15% ACN and reached 100% ACN in 30 min. The column was washed with 100% ACN for 5 min. and equilibrated for 5 min between chromatographic runs. The optimum TOF-MS conditions are given in Table. The data recorded were processed with *Analyst-QS* software with accurate mass application.

Table. The standard operating conditions for liquid chromatography / time-of-flight-mass spectrometry (LC/TOF-MS) electrospray ionization (ESI) + ion mode

Parameter	
Capillary voltage	4000 V
Nebulizer pressure	50 psig
Drying gas	10 l min^{-1}
Gas temperature	350°C
Fragmentor voltage	150 V
Skimmer voltage	60 V
Octapole Radio Frequency	250 V
Mass range (*m/z*)	100–1000
Reference masses	121.050873, 922.009798

Method performance by spiking and analysis. The ability of the method to correctly identify solanapyrones was tested by spiking into the blank growth medium and analysis using the proposed method. Based on the above detection criteria, all three solanapyrones standards were completely recovered and correctly identified when spiked at 25–100 µg kg^{-1} into growth media, and analysed as described above. The mean recovery for each solanapyrone in the growth media ranged from 85% to 105%. Using positive electrospray, the accurate masses of protonated molecule ions and retention times were obtained in each case.

Results and discussion

In the present study, the solanapyrones A, B and C in culture filtrate of *A. rabiei* were analyzed by LC/ESI-MS. In the LC, solanapyones B and C were coeluted so that they had the same retention time (Fig. 2 a). However, solanapyrone A was well separated with UV detection, as has been reported by other researchers (Chen et al., 1991; Kaur, 1995). In ESI-MS analyses, protonated molecule ions were obtained by using positive electrospray in each case. The major peaks of the solanapyrone A and C were at *m/z* 303 and 332, respectively (Fig. 2 b and d). The major peak of solanapyrone B was observed at *m/z* 287 (Fig. 2 c). In SIM mode, therefore, ions used for quantification were selected as 303, 287 and 332 for solanapyrones A, B and C, respectively. The data from our study are compatible with previous study in which main fragment of solanapyrone B had a major peak at *m/z* 287 (Höhl et al., 1991).

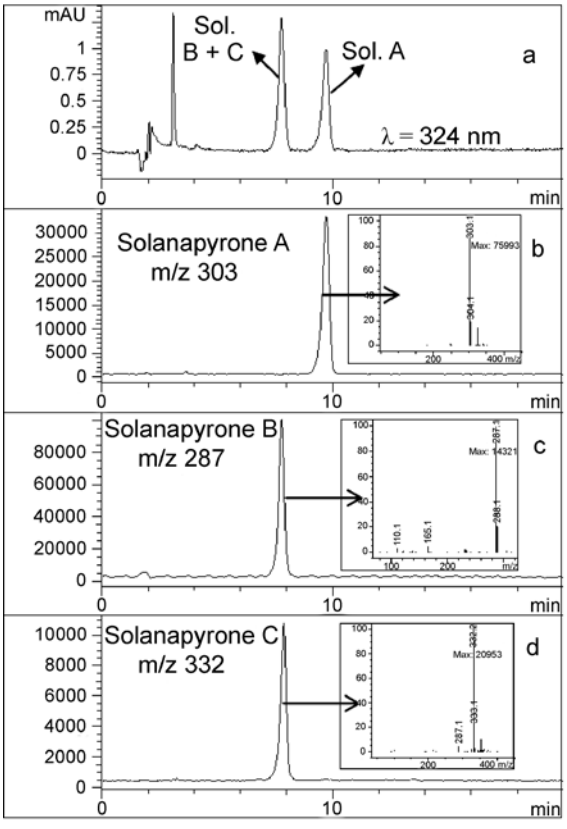

Figure 2. Separation of solanapyrones from a) separation of solanapyrone A, B and C by LC λ 324 nm; separation of solanapyrone A (b), B (c) and C (d) by LC/ESI-MS

In the present study, isolate Haypa 3 of *A. rabiei* produced all three solanapyrone toxins in the liquid culture medium during different incubation periods (Fig. 3). On the 7th day, the concentrations of solanapyrone B (0.04 µg ml^{-1}) and C (0.12 µg ml^{-1}) were higher than those of solanapyrone A (0.01 µg ml^{-1}). Höhl et al. (1991) repor-

ted that the major toxin in fluids of germinating spores was solanapyrone C, and solanapyrone B was detected in trace amount on the 4th day along with solanapyrone C. In the same period, they determined that solanapyrone A was not found in culture filtrate and it was observed after 6th day of incubation. In fluids of germinating spores of *A. rabiei* there were present solanapyrone B and C, but solanapyrone A was not. Kaur (1995) determined that the maximum production of solanapyrones in culture media was observed after the onset of sporulation. Similarly, other researchers reported that the amount of solanapyrone A in the culture filtrates peaked on 14–16 day (Bahti, Strange, 2004; Zerroug et al., 2007). Results from our study showed that the concentration of solanapyrone A (21.34 µg ml^{-1}) in the culture filtrate of the fungus reached the highest concentration on the 14th day. In the same period, solanapyrone C was observed at concentration of 0.03 µg ml^{-1}, whereas no solanapyrone B was able to be detected. Similarly, solanapyrone B could also be absent on the 21st day of incubation. The concentration of solanapyrone A (1.02 µg ml^{-1}) rapidly decreased on the 21st day of incubation as compared with 14th day of incubation, but solanapyrone C (4.83 µg ml^{-1}) rose to maximal concentration (Fig. 3).

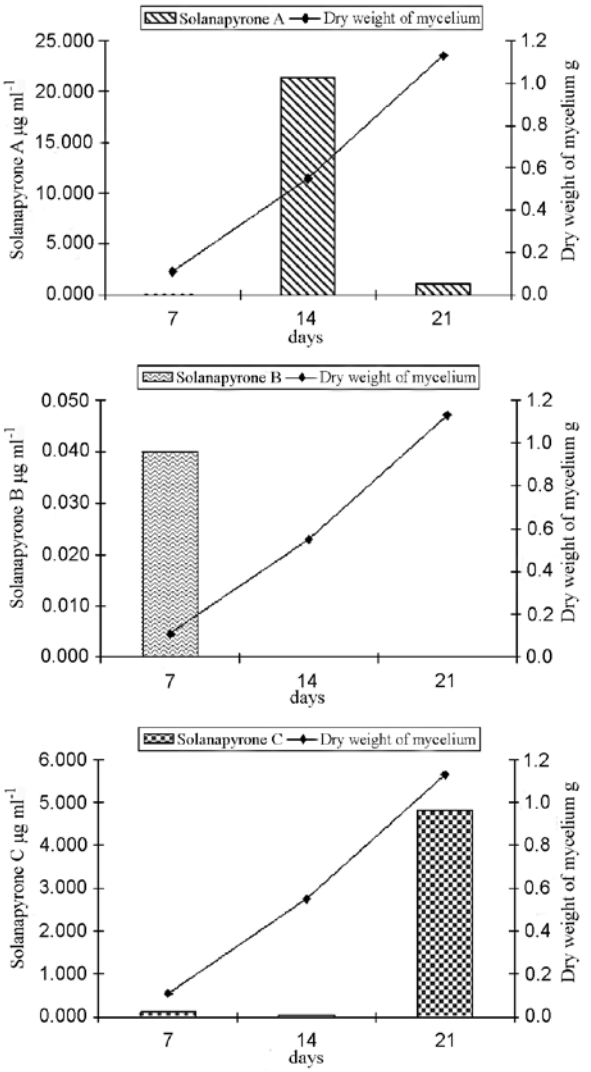

Figure 3. Production of solanapyrone A, B and C during *in vitro* growth of isolate Haypa 3 of *A. rabiei*

As mentioned by previous researchers, these results show that production of the solanapyrones is limited to a certain stage of growth period. It was also reported that production of the solanapyrones was affected by composition of nutrients in culture medium (Chen, Strange, 1991; Höhl et al., 1991; Latif et al., 1993; Chen, Strange, 1994; Kaur, 1995). Moreover, Chen and Strange (1991) found that the amounts of the solanapyrones secreted by *A. rabiei* highly increased in Czapek Dox medium supplemented with zinc, manganese, calcium, cobalt and cupper. Latif et al. (1993) and Shahid et al. (1998) determined that *Ascochyta rabiei* produced chytochalasin D as well as solanapyrone A, B and C in culture medium when it was grown in chickpea seed extract medium. In the following years, the new compound in culture filtrates of *A. rabiei* was reported by Bahti and Strange (2004), which was identified as demethylated solanapyrone A. The researchers determined that the demethylated solanapyrone A was formed as a result of the reaction of solanapyrone A with residual ammonium sulphate. In the present study, we detected a new metabolite in culture filtrates of *A. rabiei*. The new metabolite was determined in culture filtrate after 21 days in vitro growth of the fungus. It had a much longer retention time than solanapyrone A, whose retention time was 10.083. When its UV spectrum was compared to that of solanapyrone A by DAD, the differences between them were clearly showed (Fig. 4).

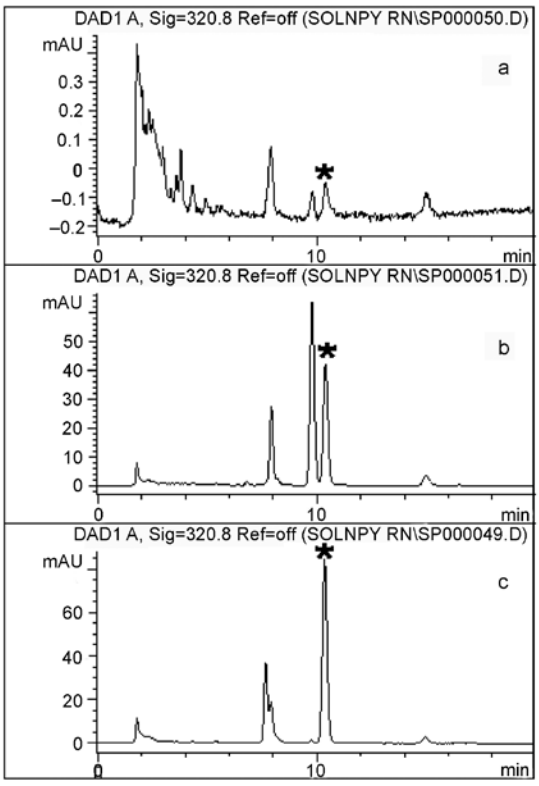

Figure 4. Separation of solanapyrones and the new metabolite (*) by LC-DAD on 7th (a), 14th (b) and 21st (c) days of incubation of isolate Haypa 3 of *A. rabiei*

Moreover, in LC/TOF-MS analyses we detected that the peak eluting at 8.37 had an accurate mass of *m/z* 319.1603. The elemental composition calculator produced

a formula based on the ion at *m/z* 319.1603. In order to narrow down the choices, the isotope ion at *m/z* 319.1603 should be consistent with the number of carbons in the new molecule and naturally occurring abundance of the ^{13}C isotope. This formula was then automatically translated into a Web connection search with NIST, ChemIndex, and Medline. The search results did not indicate the structure of the formula which may be identified by other techniques. Data of mass spectrometric analysis showed that it had the molecular formula $C_{18}H_{22}O_5$ (Fig. 5).

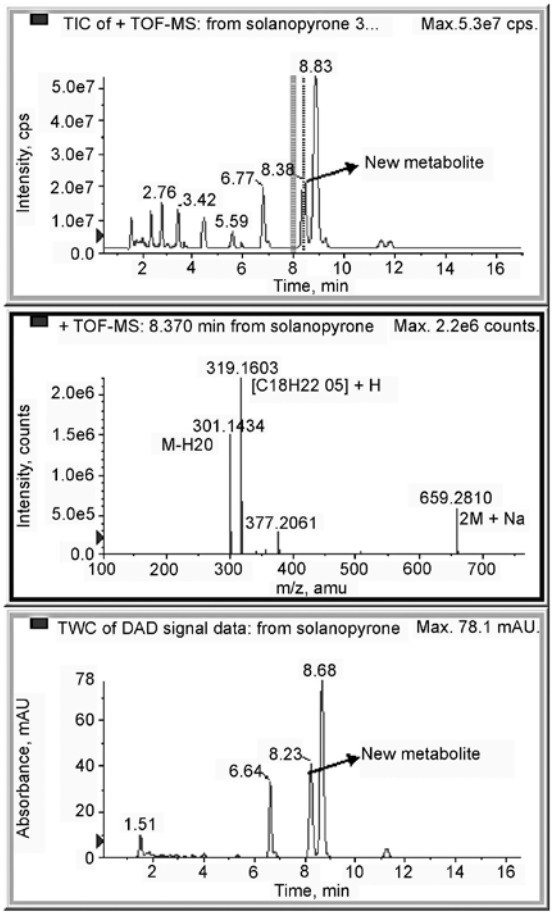

Figure 5. Identification of new metabolite by LC/TOF-MS isolate Haypa 3 of *A. rabiei*

Conclusions

1. Our studies showed that solanapyrones B and C are coeluted and it is rather difficult to separate them with UV detection. However, LC/MS provides sufficient separation for the solanapyrones.

2. Isolate Haypa 3 of *A. rabiei* used in the study produced all three solanapyrones in the culture medium. The solanapyrones production of the isolate varied considerably during the three different growth periods. Moreover, solanapyrones A and C were detected in culture medium on 7th, 14th and 21st days of growth period, but solanapyrone B could not be detected in culture medium on 14th and 21st days of growth period.

3. During growth periods of 21 days, the isolate also produced a new metabolite which has an accurate mass of *m/z* 319.1603. The mass accuracy of the TOF-MS has provided discrimination of the target metabolite from the three solanapyrones, but there is a need to identify the new metabolite by other chromatographic techniques such as ^{1}H-NMR. The studies should be continued to identify biological pathway, gene structure and toxigenity of the metabolite on the plant varieties.

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Solanapironų ir naujų metabolitų iš *Ascochyta rabiei* nustatymas bei identifikavimas

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[3]Turkijos mokslinių ir technologinių tyrimų tarybos Ankaros tyrimų ir analizių laboratorija

Santrauka

Solanapironams bei naujiems metabolitams identifikuoti ir jų kiekiui nustatyti sukurtas paprastas, greitas, tikslus bei patikimas skystosios chromatografijos ir masės spektrometrijos (LC/MS) metodas. Analizuotas žalio tirpalo ekstraktas iš *Ascochyta rabiei* kultūros filtrato, kuris yra avinžirnio dėmėtligės sukėlėjas. Rezultatams patvirtinti taikytas kombinuotas LC ir (TOF)-MS („time-of-flight“) metodas. *A. rabiei* izoliatas *Haypa 3* buvo 7, 14 ir 21 dieną augintas *Czapek Dox* skystoje terpėje, papildytoje neorganiniais katijonais. Ekstrahavus kultūros filtratus, naudojant C18 kasetę, toksinai buvo išplauti acetonitrile (ACN). Taikant LC, elektropurškimo jonizacijos (ESI)-MS ir LC/TOF-MS metodus, *A. rabiei* kultūros filtratuose, be trijų solanapironų, buvo identifikuotas naujas metabolitas. Kultūros filtrate naujas metabolitas nustatytas 21-ą dieną, grybą auginant *in vitro*. Naują metabolitą plaunant 8,33 min, nustatyta tiksli jo masė *m/z* 319.1603. Elementinės sudėties skaičiuoklė pateikė $C_{18}H_{22}O_5$ formulę, paremtą jonu, masei esant *m/z* 319.1603.

Reikšminiai žodžiai: sėjamasis avinžirnis, *Ascochyta rabiei*, fitotoksinas, solanapironas, LC/MS metodas.