微生物学报 *Acta Microbiologica Sinica*
55(10):1356 – 1363; 4 October 2015
ISSN 0001 – 6209; CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn
doi:10.13343/j.cnki.wsxb.20150036

Phenotypic analysis of *Phytophthora parasitica* by using high throughput phenotypic microarray

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Abstract: [**Objective**] We studied the phenotypic characterization of *Phytophthora parasitica* Dastur var. *nicotianae*. [**Methods**] Phenotypic characterization of the pathogen was studied to provide information for disease management program by using BIOLOG phenotype MicroArray (PM). Using PM plates 1 to 10, 950 different phenotypic characterizations were tested. [**Results**] *P. parasitica* was able to metabolize 74% of tested carbon sources, 96% of nitrogen sources, 100% of sulfur sources, and 98% of phosphorus sources. Most informative utilization patterns for carbon sources of *P. parasitica* were organic acids and carbohydrates, and for nitrogen were various amino acids. The pathogen presented 285 different nitrogen pathways. It had wide range adaptabilities in osmolytes with up to 1% sodium chloride, up to 3% potassium chloride, up to 5% sodium sulfate, up to 20% ethylene glycol, up to 2% sodium formate, up to 5% urea, and up to 2% sodium lactate. It also exhibited active metabolism under pH values between 3.5 and 10, with optimal pH of around 7.0. The pathogen showed both decarboxylase and deaminase activities in the presence of various amino acids. [**Conclusion**] These phenotypic characterizations of *P. parasitica* provided the theoretical basis for the next study of the pathogen in physiology and metabolism, and provided potential new way for tobacco black shank management.

Keywords: tobacco black shank, Biolog phenotype microarray, metabolic fingerprint, phenotypic characterization
CLC number: Q933 Article ID: 0001-6209(2015)10-1356-08

Black shank, caused by the soil borne pathogen *Phytophthora parasitica* Dastur var. *nicotianae* Breda de Haan Tucker (*P. parasitica*) is one of the most destructive diseases of tobacco (*Nicotiana tabacum* L.) worldwide. Infection of roots, stems, and leaves can occur at any stage of plant growth, resulting in

Supported by the National Natural Science Foundation of China (31360448), by the Guizhou Science Technology Foundation ([2011]2337) and by the Guizhou Tobacco Company Program (201305, 2013036, 2014036)

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Received: 21 January 2015/Revised: 15 February 2015

root necrosis, wilting, chlorosis, stem lesions, stunting, and plant death[1]. The disease also is present in most tobacco-growing regions of the world and can result in yield losses on all tobacco types[2-3]. Losses can reach 100% if disease management practices are not used[4], and common annual losses approach 85% in China[5].

Isolates of *P. parasitica* vary widely in aggressiveness[6]. Four races (i.e., 0, 1, 2, and 3) of *P. parasitica* have been described[7]. Races 0 and 1 have been reported in the United States, South Africa, India and major tobacco growing areas of China. A recent study suggests that race 1 is the major race in Guizhou Province of China[8], where more than 30% of the total Chinese tobacco crop is produced. The pathogen survives in infected plants and infested soil, with dissemination through use of infected plants, latently infected planting material, and contaminated irrigation water. Integrated black shank disease management program has been conducted for many years; it is still a serious problem for tobacco production in China[5]. Up until now, little is known about the phenotypic characters of isolates of *P. parasitica*, especially for the dominant strain of race 1 in China. A better understanding of the phenotypes of the pathogen will be much valuable to assess and develop management practices to decrease the impact of the disease below economic thresholds.

Recently, a high throughput phenotypic microarray (PM)/Omnilog system, was developed by the company Biolog (Hayward, CA) to assay nearly 1000 metabolic phenotypes[9-10]. In this system, microbial cells in defined medium containing a reducible tetrazolium dye are inoculated into ten 96-well plates, where the plates contain various substrates to assay for: the utilization of carbon, nitrogen, sulfur, phosphorus source; biosynthetic pathways; and varying effects of osmotic, ionic, and pH environments. Cell growth and respiration lead to the reduction of the tetrazolium dye and result in blue color. The intensity of the color, which is proportional to microbial growth, is recorded every 15 min by a CCD camera and analyzed by Omnilog software, yielding a quantitative analysis of all collected data. The software plots the kinetic data of color formation in arbitrary units against time for each well and also assigns artificial color to each plot.

In order to provide insights that will support development of novel control strategies, we aim to elucidate phenotype analysis of *P. parasitica*, a species that is emerging as a model for the studies of oomycete biology and pathology[11-13]. The outcome would provide useful information to know detail biology of *P. parasitica* and helpful knowledge about black shank management.

1 Materials and Methods

1.1 Fungal strain and culture conditions

One isolate of *P. parasitica* identified as race 1[8], with wild-type sensitivity and pathogenicity to tobacco, was selected as the modal species for analysis. The isolate was grown and maintained on lima bean agar medium (LBA, 60.0 g/L lima beans boiled for one hour, 16.0 g/L agar), in a controlled climate cabinet at 25 ℃ in darkness. For zoospore production, agar plugs were removed from the edge of an actively growing culture and placed in Petri dishes containing 8 mL of 10% V8 liquid medium (10% V8, 100 mL Campbell's V8 juice to 900 mL sterile water, 0.2 g $CaCO_3$). Following a 96 h incubation period at 25 ℃ in light, the suspension was filtered through a double-layer of sterile cheesecloth (Grade #40: 24 × 20 threads per inch) to remove mycelia fragments and the resulting sporangia suspension was maintained at 4 ℃ for 30 min and then at 25 ℃ for 2 h in the dark to release the zoospores[14]. For long-term storage, 5 mm agar plugs from the leading edge of individual colonies were transferred into several sterile 1.5 mL microcentrifuge tubes containing 1 ml of sterile distilled water, and tubes were stored in the dark at 15 ℃[15-16].

1.2 Phenotypic characterization

The metabolic abilities of *P. parasitica* were tested by using the PM system (Biolog, Hayward,

CA) according to the published procedure[9, 17-18] using turbidity measurements. Using PM system, 950 different growth conditions were tested, including 190 different carbon sources, 95 nitrogen sources, 59 phosphorus sources, 35 sulfur sources, 94 biosynthetic pathways, 285 nitrogen pathways, and 192 tolerances to different osmolytes and pH conditions. All materials, media, and reagents for the PM system were purchased from Biolog. Plates 1 - 8, which test for catabolic pathways for carbon (PM 1 - 2), nitrogen (PM 3, 6 - 8), phosphorus (PM 4), sulfur (PM 4), biosynthetic pathways (PM 5), as well as for biosynthetic pathways, and plates 9 - 10, which test for osmotic/ion (PM 9) and pH effects (PM 10), were utilized in this study. Zoospore suspension of *P. parasitica* was prepared as mentioned above and suspended in appropriate medium containing sterile FF-IF; 100 μL of a dilution of an 62% transmittance suspension of cells were added to each well of the PM plates. FF-IF was used for PM plates 1 and 2. FF-IF plus 100 mmol/L D-glucose, 5 mmol/L potassium phosphate (pH6.0), and 2 mmol/L sodium sulfate was used for plates 3, 5, 6, 7 and 8. FF-IF plus 100 mmol/L D-glucose was used for plate 4. FF-IF plus yeast nitrogen base, and 100 mmol/L D-glucose was used for plates 9 and 10. Plates were incubated in the OmniLog at 28 ℃ for 7 days with readings taken every 15 minutes. Incubation and recording of phenotypic data were performed in the OmniLog station by capturing digital images of the microarrays and storing turbidity values in a computer file displayed as a kinetic graph. Data analysis was conducted using Kinetic and Parametric software (Biolog). Phenotypes were determined based on the area under the kinetic curve of dye formation. The experiment was conducted twice.

2 Results

2.1 Phenotypic characterization

The pathogen was able to metabolize 74% of tested carbon sources, 96% of nitrogen sources, 100% of sulfur sources, and 98% of phosphorus sources (Figure. 1). It was very efficient in utilizing unusual phosphorus compounds (58/59 tested, plate PM4, Wells A1-E12). The phosphorus compound that was poorly utilized by *P. parasitica* was phosphono acetic acid. Meanwhile, nearly all S-containing compounds (35/35 tested, plate PM4, Wells F1-H12) tested could be assimilated by the pathogen. The pathogen presented 94 different biosynthetic pathways (94/94 tested, plate PM5, Wells A3-H12).

Using data from PM1 and PM2 (carbon sources), isolate of *P. parasitica* from tobacco was able to use 140 different carbon sources; more than 100 compounds significantly supported the growth of the pathogen (Table 1). In comparison, fifty compounds significantly inhibited the growth of the pathogen (Table 2). Using the PM3 plate, the isolate of *P. parasitica* was tested for their ability to grow on 95 different nitrogen sources (Figure. 1). The first striking result was the apparent growth in the negative control without any nitrogen source (PM3, well A1). More than forty compounds supported the growth of the pathogen (Table 3). In comparison, four out of 95 nitrogen sources supported growth at the level of the negative control, indicating that *P. parasitica* cannot metabolize these compounds; these were nitrite (PM 3, well A03), nitrate (PM 3, well A04), D-serine (PM 3, well C08), and hydroxylamine (PM 3, well D04).

Using data from PM6 to PM8 (nitrogen pathways), *P. parasitica* presented 285 different nitrogen pathways, indicating that various combinations of different amino acids supported the growth of the pathogen (Figure 1). Plates PM9 and PM10 were used to test growth under various stress conditions. *P. parasitica* showed active metabolism with up to 1% sodium chloride, up to 3% potassium chloride, up to 5% sodium sulfate, up to 20% ethylene glycol, up to 2% sodium formate, up to 5% urea, up to 2% sodium lactate, up to 200 mmol/L sodium phosphate (pH7.0), up to 50 mmol/L ammonium sulfate (pH8.0), and up to 100 mmol/L sodium nitrate. The pH range where *P. parasitica* exhibited active growth was between 3.5 and 10, with

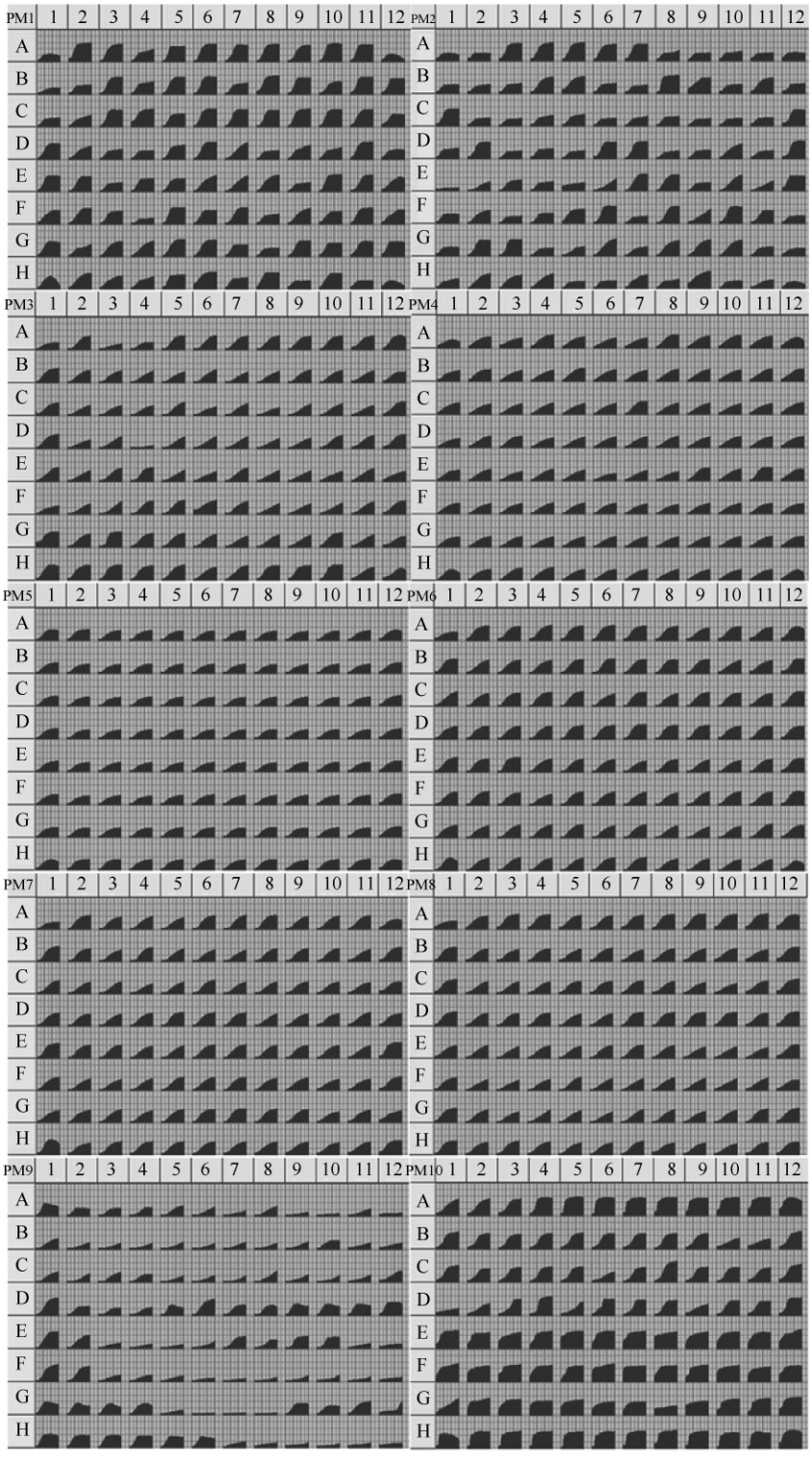

Figure 1. Data for Biolog phenotype MicroArray PM 1 – 10 plates of the pathogen *Phytophthora parasitica*. Utilization of the isolate of *P. parasitica* from tobacco was indicated by grey areas in the growth curve for each substrate.

an optimal pH of around 7.0. When combined with various amino acids at the pH of 4.5, *P. parasitica* showed active growth in all tests except for combining with the amino acids of L-isoleucine (PM 10, well B10), L-leucine (PM 10, well B11), L-tryptophan (PM 10, well C06), anthranilic acid (PM 10, well

D01), and 5-hydroxytryptophan (PM 10, well D09). In comparison, when combined with various amino acids at the pH of 9.5, the pathogen presented active growth in all tests. PM 10, wells B1-D12 and E1-G12, tested the decarboxylase and deaminase activities of the pathogen in the presence of amino acids at pH4.5 and pH9.5, respectively. *P. parasitica* showed both decarboxylase and deaminase activities in the presence of most of the amino acids, except for anthranilic acid (PM 10, well D1).

Table 1. Substrates in PM 1 and 2 MicroPlates significantly supported the growth of *Phytophthora parasitica*

#Cond.	Substrates	#Cond.	Substrates	#Cond.	Substrates
1-A02	L-Arabinose	1-E04	D-Fructose-6-Phosphate	2-A05	g-Cyclodextrin
1-A03	N-Acetyl-D-Glucosamine	1-E05	Tween 80	2-A06	Dextrin
1-A05	Succinic Acid	1-E06	a-Hydroxyglutaric Acid-g-Lactone	2-A07	Gelatin
1-A06	D-Galactose	1-E07	a-Hydroxybutyric Acid	2-B04	Amygdalin
1-A07	L-Aspartic Acid	1-E08	b-Methyl-D-Glucoside	2-B05	D-Arabinose
1-A08	L-Proline	1-E10	Maltotriose	2-B08	Arbutin
1-A09	D-Alanine	1-E11	2′-Deoxyadenosine	2-B09	2-Deoxy-D-Ribose
1-A10	D-Trehalose	1-E12	Adenosine	2-B11	D-Fucose
1-A11	D-Mannose	1-F01	Gly-Asp	2-C01	Gentiobiose
1-B03	Glycerol	1-F02	Citric Acid	2-C12	Palatinose
1-B04	L-Fucose	1-F03	m-Inositol	2-D02	Salicin
1-B05	D-Glucuronic Acid	1-F05	Fumaric Acid	2-D06	D-Tagatose
1-B06	D-Gluconic Acid	1-F06	Bromosuccinic Acid	2-D07	Turanose
1-B08	D-Xylose	1-F07	Propionic Acid	2-D10	g-Amino-N-Butyric Acid
1-B09	D, L-Lactic acid	1-F09	Glycolic Acid	2-D12	Sodium butyrate
1-B10	Formic Acid	1-F10	Glyoxylic Acid	2-E07	4-Hydroxybenzoic Acid
1-B11	D-Mannitol	1-F11	D-Cellobiose	2-E08	b-Hydroxybutyric Acid
1-B12	L-Glutamic Acid	1-F12	Inosine	2-E10	2-Oxovaleric acid
1-C03	D, L-Malic Acid	1-G01	Gly-Glu	2-E12	5-Keto-D-Gluconic Acid
1-C04	D-Ribose	1-G03	L-Serine	2-F05	Oxalomalic Acid
1-C05	Tween 20	1-G04	L-Threonine	2-F06	Quinic Acid
1-C06	L-Rhamnose	1-G05	L-Alanine	2-F08	Sebacic Acid
1-C07	D-Fructose	1-G06	Ala-Gly	2-F10	Succinamic Acid
1-C08	Acetic Acid	1-G07	Acetoacetic Acid	2-F11	D-Tartaric Acid
1-C09	a-D-Glucose	1-G09	Mono-Methylsuccinate	2-G02	L-Alaninamide
1-C10	Maltose	1-G10	Methylpyruvate	2-G03	N-Acetyl-L-Glutamic Acid
1-C11	D-Melibiose	1-G11	D-Malic Acid	2-G06	L-Histidine
1-C12	Thymidine	1-G12	L-Malic Acid	2-G08	Hydroxy-L-Proline
1-D01	L-Asparagine	1-H01	Gly-Pro	2-G09	L-Isoleucine
1-D05	Tween 40	1-H02	p-Hydroxyphenyl Acetic Acid	2-G10	L-Leucine
1-D06	a-Ketoglutaric Acid	1-H05	D-Psicose	2-H02	L-Phenylalanine
1-D07	a-Ketobutyric Acid	1-H06	L-Lyxose	2-H03	L-Pyroglutamic Acid
1-D09	a-D-Lactose	1-H08	Pyruvic Acid	2-H04	L-Valine
1-D11	Sucrose	1-H10	D-Galacturonic Acid	2-H07	D, L-Octopamine
1-E01	L-Glutamine	2-A03	a-Cyclodextrin	2-H09	Dihydroxyacetone
1-E02	m-Tartaric Acid	2-A04	b-Cyclodextrin		

Table 2. Substrates in PM 1 and 2 MicroPlates not utilized by *Phytophthora parasitica*

#Cond.	Substrates	#Cond.	Substrates	#Cond.	Substrates
1-A12	Dulcitol	2-C04	D-Melezitose	2-E09	g-Hydroxybutyric Acid
1-B01	D-Serine	2-C06	a-methyl-D-Glucoside	2-E11	Itaconic Acid
1-F04	D-Threonine	2-C07	b-methyl-D-Galactoside	2-F03	Melibionic Acid
1-H09	L-Galactonic Acid-g-Lactone	2-C08	3-Methylglucose	2-F07	D-ribono-1,4-Lactone
1-H11	b-Phenylethylamine	2-C09	b-methyl-D-Glucuronic Acid	2-F12	L-Tartaric Acid
1-H12	2-Aminoethanol	2-C10	a-methyl-D-Mannoside	2-G04	L-Arginine
2-A02	Chondroitin sulfate C	2-C11	b-methyl-D-Xyloside	2-G05	Glycine
2-A08	Glycogen	2-D03	Sedoheptulosan	2-G07	L-Homoserine
2-A09	Inulin	2-D04	L-Sorbose	2-G11	L-Lysine
2-A10	Laminarin	2-D05	Stachyose	2-G12	L-Methionine
2-A11	Mannan	2-D08	Xylitol	2-H05	D,L-Carnitine
2-A12	Pectin	2-D09	N-acetyl-D-Glucosaminitol	2-H06	sec-Butylamine
2-B02	N-acetyl-Neuraminic Acid	2-D11	d-Amino Valeric Acid	2-H08	Putrescine
2-B07	L-Arabitol	2-E01	Capric Acid	2-H10	2,3-Butanediol
2-B10	i-Erythritol	2-E02	Caproic Acid	2-H11	2,3-Butanone
2-C02	L-Glucose	2-E04	D,L-Citramalic Acid	2-H12	3-Hydroxy 2-Butanone
2-C03	D-Lactitol	2-E05	D-Glucosamine		

Table 3. Substrates in PM 3 MicroPlates significantly supported the growth of *Phytophthora parasitica*

#Cond.	Substrates	#Cond.	Substrates	#Cond.	Substrates
3-A02	Ammonia	3-C05	D-Aspartic Acid	3-G05	Allantoin
3-A05	Urea	3-C07	D-Lysine	3-G06	Parabanic Acid
3-A06	Biuret	3-C12	L-Ornithine	3-G10	D,L-a-Amino-Caprylic Acid
3-A07	L-Alanine	3-D01	N-Acetyl-L-Glutamic Acid	3-H01	Ala-Asp
3-A08	L-Arginine	3-D11	Putrescine	3-H02	Ala-Gln
3-A09	L-Asparagine	3-D12	Agmatine	3-H03	Ala-Glu
3-A10	L-Aspartic Acid	3-E01	Histamine	3-H04	Ala-Gly
3-A11	L-Cysteine	3-E03	Tyramine	3-H05	Ala-His
3-A12	L-Glutamic Acid	3-E04	Acetamide	3-H06	Ala-Leu
3-B01	L-Glutamine	3-F05	Cytosine	3-H07	Ala-Thr
3-B02	Glycine	3-F06	Guanine	3-H08	Gly-Asn
3-B09	L-Proline	3-F12	Inosine	3-H09	Gly-Gln
3-B10	L-Serine	3-G01	Xanthine	3-H10	Gly-Glu
3-B11	L-Threonine	3-G02	Xanthosine	3-H11	Gly-Met
3-B12	L-Tryptophan	3-G03	Uric Acid	3-H12	Met-Ala
3-C01	L-Tyrosine	3-G04	Alloxan		

3 Discussion

While a large number of genetic and molecular biological studies have been conducted with *P. parasitica*, phenotypic diversity is still poorly explored in oomycetes. Direct high-throughput assessment of phenotypes using the PM system[9] has stirred much attention for molecular biological, genomic, and population studies of microorganisms[19-21]. Here, metabolic ability of the pathogen from host of tobacco was systematically analyzed using PMs and significant metabolic diversity was found.

In this study a pathogenic isolate of *P. parasitica* with wild-type sensitivity to fungicides was characterized by the high-throughput PM technique. A wide range of carbon compounds could be utilized and most of the nitrogen, sulfur, and phosphorus sources were also metabolized. These data indicate a great adaptability of *P. parasitica* in the natural environment, which is a notorious oomycete pathogen that is distributed worldwide in tropical and subtropical regions and has a fair ability to colonize both soils and plants. The most informative plates for *P. parasitica* were PM1/PM2 (carbon sources), PM3 (nitrogen sources), PM 9 (osmolytes conditions) and PM 10 (pH conditions). Most informative utilization patterns for carbon sources were organic acids and carbohydrates, and for nitrogen sources various amino acids. These compounds are commonly found in many plant exudates[22], and organic fertilizers[23]. Such compounds might play a role in supporting the infection

of *P. parasitica* on roots of solanaceous plants. Additionally, the pathogen had wide range adaptabilities in osmolytes and pH conditions, which was found by using plates PM 9 and PM 10. Decarboxylase of the pathogen generates alkaline amines by the catabolism of amino acids, which help to counteract an acidic pH. On the other hand, a high pH can be countered by deaminases, which generate acids [24]. This phenotypic diversity of the pathogen can be rationalized by considering the seasonal variation in soil pH and osmolytes due to varying conditions of dryness and watering. Consequently, phenotypic characters for utilization of those sources and wide range adaptabilities of the pathogen could have a high adaptive value in plant-microbe interaction and survival of the pathogen in soil.

Since the PM technique examines strains for many characters relating to how environmental stressors affected pathogen's activity, it becomes possible to determine whether it has a reasonable chance of being useful for disease management in agriculture. Enhancing the amount of carbon and nitrogen sources that could not be metabolized by *P. parasitica*, or decreasing the amount of these sources that could be utilized by the pathogen in the field may reduce the damage caused by black shank. Meanwhile, changing the osmolytes in soil to make it unadaptable for the pathogen *P. parasitica* may also lighten the happening of black shank. These imagines could be proved in the next study in the near future. In conclusion, phenomics study of *P. parasitica* in our study increases our understanding of the pathogen.

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烟草黑胫病菌的表型组学分析

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摘要: 【目的】为了阐明烟草黑胫病菌的代谢表型特征。【方法】采用 BIOLOG 细胞表型芯片技术系统地研究了烟草黑胫病菌的细胞表型; 采用 PM 1 – 10 代谢板, 对烟草黑胫病菌的 950 种代谢表型进行了测定。【结果】黑胫病菌能代谢 74% 的碳源、96% 的氮源、100% 的硫源和 98% 的磷源; 高效代谢的碳源为有机酸类和碳水化合物类, 高效代谢的氮源为氨基酸类; 病原菌具有 285 种不同的氮源代谢通路; 黑胫病菌具有广泛的适应性, 能在高达 1% 氯化钠、3% 氯化钾、5% 硫酸钠、20% 乙二醇、2% 甲酸钠、5% 尿素或 2% 乳酸钠中存活; 其适应 pH 值范围为 3.5 – 10, 最适 7.0; 在多种氨基酸的作用下, 烟草黑胫病菌均表现出脱羧酶和脱氨酶活性。【结论】这些代谢特征为烟草黑胫病菌进一步的生理和代谢研究提供了理论基础, 并对烟草黑胫病的防治提供了新的思路。

关键词: 烟草黑胫病, Biolog 表型芯片, 代谢指纹图谱, 表型特征

中图分类号: Q933 **文章编号:** 0001-6209(2015)10-1363-08

(本文责编: 张晓丽)

基金项目: 国家自然科学基金项目(31360448); 贵州省科技基金项目([2011] 2337); 贵州省烟草公司项目(201305, 2013036, 2014036)

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收稿日期: 2015-01-21; **修回日期:** 2015-02-15