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Phenotypic Analysis of *Alternaria alternata*, the Causal Agent of Tobacco Brown Spot

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ABSTRACT

Tobacco brown spot caused by *Alternaria alternata* is a devastating disease of tobacco worldwide. Phenotypic characterization of the pathogen was investigated to provide some basic information for biology and pathology by using BIOLOG Phenotype Microarray (PM). Using PM plates 1-10, 950 different growth conditions were tested. Results exhibited that the pathogen was able to metabolize 24.74% of tested carbon sources, 85.26% of nitrogen sources, 97.14% of sulfur sources and 89.83% of phosphorus sources. Most informative utilization patterns for carbon sources of *A. alternata* were carbohydrates and for nitrogen were various amino acids. The pathogen presented 274 different nitrogen pathways. It had wide range adaptabilities in osmolytes with up to 10% sodium chloride, up to 6% potassium chloride, up to 5% sodium sulfate, up to 20% ethylene glycol, up to 6% sodium formate, up to 6% urea, up to 12% sodium lactate, up to 200 mM sodium phosphate, up to 100 mM ammonium sulfate, up to 100 mM sodium nitrate and up to 20 mM sodium nitrite. It also exhibited active metabolism in the range of pH values between 3.5 and 10, with optimal pH of around 6.0. The pathogen showed active decarboxylase activity, whereas no deaminase activity in the presence of various amino acids.

Key words: Biolog phenotype microarray, metabolic fingerprint, tobacco brown spot

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is an annual, leafy and solanaceous plant grown commercially for its leaves. China is the biggest single tobacco market in the world and accounts for more than 39.6% of the total global tobacco production (Wang *et al*., 2013) and 40% of the global tobacco consumption (Wang *et al*., 2014). During tobacco production period, the major destructive foliar disease tobacco brown spot, occurs every field where tobacco growing throughout the country. Tobacco leaves infected by this disease normally become incomplete, uneven baking leaf color and leaf thickness which results in poor quality of tobacco leaves and low value of industrial use (Jenning *et al*., 2002; Yakimova *et al*., 2009). Losses can reach more than 60% if disease management practices are not utilized. In the last five years, it has been a major serious problem for tobacco production in China (Tong *et al*., 2012). This disease is caused by the notorious fungal pathogen *Alternaria alternata* (Fr.) Keissl (Main, 1969; Dobhal and Monga, 1991). It normally happens from the lower leaves of a plant and gradually spreads to the upper leaves during leaf harvest period (Stavely and Slana, 1970). The typical symptom of brown spot on leaf is a brown necrotic centre surrounded by a yellow or yellowish-green halo (Slavov *et al*., 2004).

During the early part of the growing season, *A. alternata* grows extensively over the leaf surface and only seldom penetrates tobacco leaf for invasion. Whereas at harvest period, the pathogen directly penetrates the cuticle after formation of an appressorium or infects leaves from stomata

(Huang *et al*., 1996); later on, necrotic lesions are formed after host cells have collapsed due to fungal action (Main, 1969; Cheng *et al*., 2011). To the best of our knowledge, the mechanism of infection differences in two stages is still unclear. There might be some different chemicals on tobacco leaf interrelated with the invasion for the different growing stages and these chemicals could influence the metabolic activity of *A. alternata*. Up until now, little is known about the metabolic characters of the isolate of *A. alternata*. A better understanding of the phenotypes of the pathogen will be much valuable to develop management practices to decrease the impacts of the disease.

Recently, a high throughput Phenotypic Microarray (PM)/ Omnilog system, was developed by the company Biolog (Hayward, CA) to assay nearly 1000 metabolic phenotypes (Bochner *et al*., 2001). In the 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. 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.

To address these questions, we aim to elucidate phenotype analysis of *A. alternata*, a species that is emerging as a model for the studies of fungal biology and pathology (Velez *et al*., 2007; Jia *et al*., 2010; Llorens *et al*., 2013). The outcome would provide useful information to know detail biology of *A. alternata* and helpful knowledge for brown spot management.

MATERIALS AND METHODS

Fungal strain and culture conditions: One isolate of *A. alternata*, with wild-type sensitivity and pathogenicity to tobacco, was selected as the modal species for analysis. The isolate was grown and maintained on AEA agar (yeast power 5 g L^{-1}, $NaNO_3$ 6 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, KCl 0.5 g L^{-1}, $MgSO_4$ 0.25 g L^{-1}, glycerol 20 mL L^{-1} and 16.0 g L^{-1} agar) (Li *et al*., 2005; Jin *et al*., 2007), in a controlled climate cabinet at 25°C in darkness. After 7 days' incubation, conidia produced on the Petri dishes were washed with distilled water, 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 conidia suspension was diluted to a final concentration of 1×10^5 spores mL^{-1}.

Phenotypic characterization: The metabolic abilities of *A. alternata* were tested by using the Phenotype Microarray (PM) system (Biolog, Hayward, CA, USA) according to the published procedure (Bochner *et al*., 2001; Zhou *et al*., 2003; Von Eiff *et al*., 2006). 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, nitrogen phosphorus, sulfur, as well as for biosynthetic pathways and plates 9-10 which test for osmotic/ion and pH effects, were utilized in this study. Conidial suspension of *A. alternata* 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 mM D-glucose, 5 mM potassium phosphate (pH 6.0) and 2 mM sodium sulfate was used for plates 3, 5, 6, 7 and 8. FF-IF plus 100 mM D-glucose was used for plate 4. FF-IF plus yeast nitrogen base and 100 mM D-glucose was used for plates 9 and 10. Plates were incubated in the OmniLog at 28°C for 7 days with readings taken every 15 min. 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 experiments were conducted twice.

RESULTS

Phenotypic characterization: Isolate of *A. alternata* tested in our study presented typical phenotypic fingerprint. The pathogen was able to metabolize 24.74% of tested carbon sources (26/95 in plate PM1 and 21/95 in plate PM2), 85.26% of nitrogen sources (67/95 in plate PM3, 95/95 in plate PM6, 80/95 in plate PM7 and 82/95 in plate PM8), 97.14% of sulfur sources (34/35 in plate PM4, Wells F02-H12) and 89.83% of phosphorus sources (53/59 in plate PM4, Wells A02-E12) (Fig. 1). The pathogen presented apparent growth in the negative control without any phosphorus source (plate PM4, Well A1) or any sulfur compound (plate PM4, Well F01). The phosphorus compounds that were effectively utilized by *A. alternata* included D-2-phospho-glyceric acid, 2-deoxy-D-glucose 6-phosphate, D-mannose-1-phosphate, O-phospho-D-tyrosine and O-phospho-L-tyrosine (Table 1, Fig. 1). Meanwhile, nearly all S-containing compounds (35/35 tested, plate PM4, Wells F1-H12) tested could be assimilated by the pathogen, except for D, L-Ethionine (plate PM4, Well G06) (Fig. 1), while three sulfur compounds were effectively utilized by the pathogen, including dithiophosphate (plate PM4, Well F06), L-cysteine (plate PM4, Well F07) and butane sulfonic acid (plate PM4, Well H09). Simultaneously, the pathogen presented 94 different biosynthetic pathways (94/94 tested, plate PM5, Wells A3-H12).

Using data from PM1 and PM2 (carbon sources), isolates of *A. alternata* from tobacco were able to use 103 different carbon sources (Fig. 1), about 34 compounds significantly

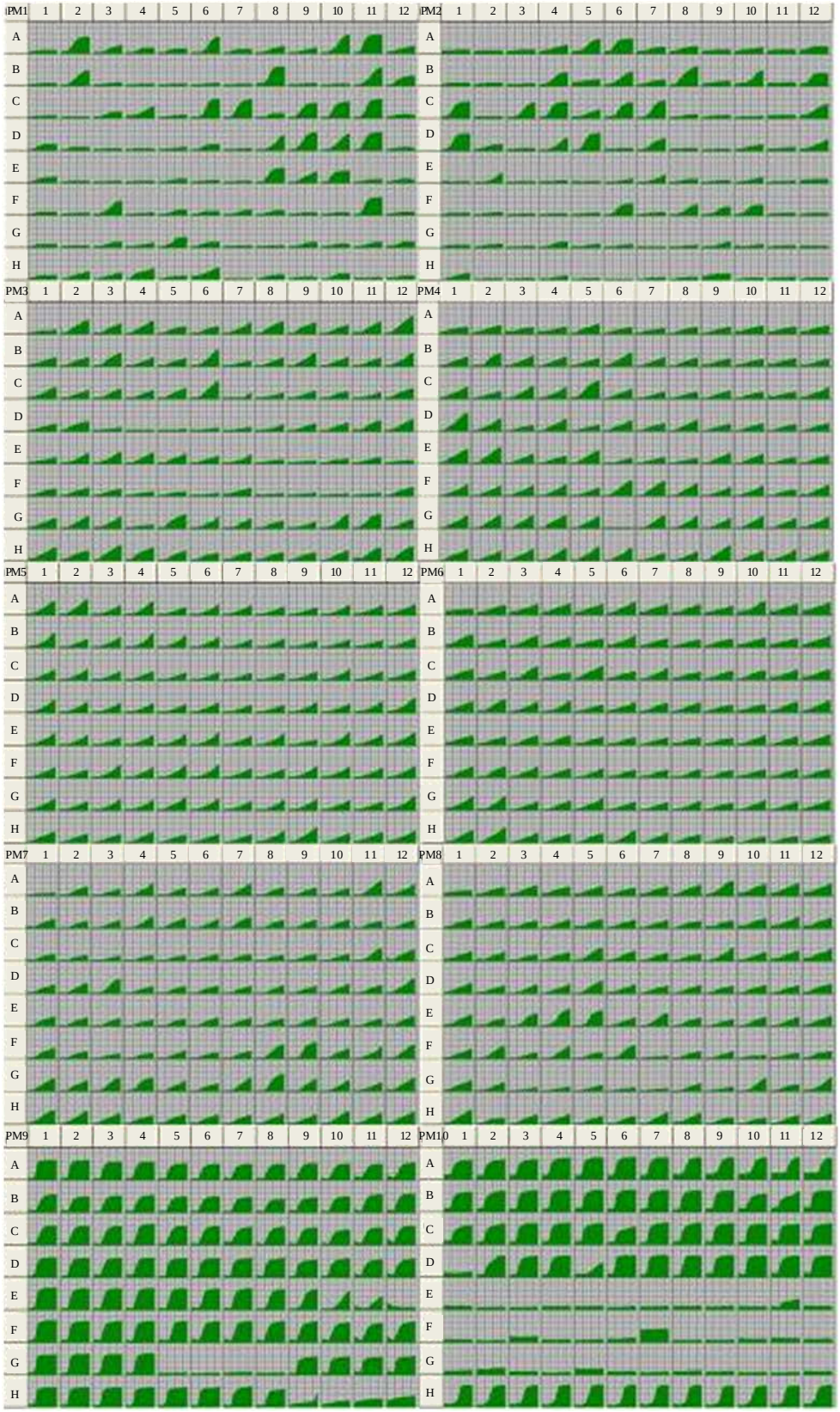

Fig. 1: Data for biolog phenotype microarray PM 1-10 plates of the pathogen *Alternaria alternata*, (Utilization of the isolates of *A. alternata* from tobacco was indicated by green areas in the growth curve for each substrate)

Table 1: Substrates in PM 1-10 Micro plates significantly supported the growth of *Alternaria alternata*

Well	Substrate	Well	Substrate	Well	Substrate
PM1					
A02	L-arabinose	B11	D-Mannitol	D09	a-D-lactose
A06	D-galactose	C06	L-Rhamnose	D10	Lactulose
A10	D-trehalose	C07	D-Fructose	D11	Sucrose
A11	D-mannose	C09	a-D-Glucose	E08	b-methyl-D-glucoside
B02	D-sorbitol	C10	Maltose	E10	Maltotriose
B08	D-xylose	C11	D-Melibiose	F11	D-cellobiose
PM2					
A05	g-cyclodextrin	B12	3-0-β-D-Galactopyranosyl-D-arabinose	C12	Palatinose
A06	Dextrin	C01	Gentiobiose	D01	D-Raffinose
B04	Amygdalin	C03	D-Lactitol	D05	Stachyose
B06	D-arabitol	C04	D-Melezitose	F6	Quinic acid
B08	Arbutin	C06	a-Methyl-D-glucoside		
B10	i-erythritol	C07	b-Methyl-D-galactoside		
PM3					
A12	L-glutamic acid	G10	D,L-a-Amino-Caprylic acid	H11	Gly-Met
B06	L-lysine	G11	d-Amino-N-Valeric acid	H12	Met-Ala
C06	D-glutamic acid	H03	Ala-Glu		
PM4					
B06	D-2-phospho-glyceric acid	E01	O-Phospho-D-tyrosine	F07	L-Cysteine
C05	2-deoxy-d-glucose 6-phosphate	E02	O-Phospho-L-tyrosine	H09	Butane aulfonic acid
D01	D-mannose-1-phosphate	F06	Dithiophosphate		
PM5					
A04	L-arginine	D12	2’-Deoxy cytidine	G12	Myo-inositol
B01	L-glutamine	E12	2'-Deoxy uridine	H09	Tween 20
B04	L-isoleucine				
PM6					
A06	Ala-Glu	B01	Ala-Ser	C05	Arg-Tyr
A10	Ala-Lys	C03	Arg-Ser	H02	Ile-Tyr
PM7					
A11	Lys-Lys	G01	Trp-Phe	H01	Tyr-Trp
D03	Phe-Trp	G03	Trp-Trp	H02	Tyr-Tyr
D12	Pro-Tyr	G04	Trp-Tyr	H10	Val-Tyr
F08	Trp-Asp	G07	Tyr-Glu	H12	γ-Glu-Gly
F09	Trp-Glu	G08	Tyr-Gly		
F12	Trp-Lys	G12	Tyr-Phe		
PM8					
A09	Asp-Gln	D05	Pro-Trp	F06	β-Ala-Phe
A10	Asp-Gly	E03	Trp-Val	G10	Phe-β-Ala
A11	Glu-Ala	E04	Tyr-Ile	G12	D-Ala-Gly-Gly
A12	Gly-Asn	E05	Tyr-Val	H01	Gly-Gly-Ala
C05	Phe-Asp	E07	Val-Gln	H07	Val-Tyr-Val
C09	Phe-Tyr	F02	β-Ala-Ala		
C12	Pro-Asn	F04	β-Ala-His		
PM9					
A01	1% NaCl	C06	6% NaCl+glutathione	E11	6% Urea
A02	2% NaCl	C07	6% NaCl+glycerol	F01	1% Sodium lactate
A03	3% NaCl	C08	6% NaCl+trehalose	F02	2% Sodium lactate
A04	4% NaCl	C09	6% NaCl+trimethylamine-N-oxide	F03	3% Sodium lactate
A05	5% NaCl	C10	6% NaCl+trimethylamine	F04	4% Sodium lactate
A06	5.5% NaCl	C11	6% NaCl+octopine	F05	5% Sodium lactate
A07	6% NaCl	C12	6% NaCl+trigonelline	F06	6% Sodium lactate
A08	6.5% NaCl	D01	3% Potassium chloride	F07	7% Sodium lactate
A09	7% NaCl	D02	4% Potassium chloride	F08	8% Sodium lactate
A10	8% NaCl	D03	5% Potassium chloride	F09	9% Sodium lactate
A11	9% NaCl	D04	6% Potassium chloride	F10	10% Sodium lactate
A12	10% NaCl	D05	2% Sodium sulfate	F11	11% Sodium lactate
B01	6% NaCl	D06	3% Sodium sulfate	F12	12% Sodium lactate
B02	6% NaCl+betaine	D07	4% Sodium sulfate	G01	20 mM Sodium phosphate pH 7
B03	6% NaCl+N-N dimethyl glycine	D08	5% Sodium sulfate	G02	50 mM Sodium phosphate pH 7
B04	6% NaCl+Sarcosine	D09	5% Ethylene glycol	G03	100 mM Sodium phosphate pH 7
B05	6% NaCl+dimethyl sulphonyl propionate	D10	10% Ethylene glycol	G04	200 mM Sodium phosphate pH 7
B06	6% NaCl+MOPS	D11	15% Ethylene glycol	G09	10 mM Ammonium sulfate pH 8
B07	6% NaCl+ectoine	D12	20% Ethylene glycol	G10	20 mM Ammonium sulfate pH 8
B08	6% NaCl+choline	E01	1% Sodium formate	G11	50 mM Ammonium sulfate pH 8
B09	6% NaCl+phosphorylcholine	E02	2% Sodium formate	G12	100 mM Ammonium sulfate pH 8
B10	6% NaCl+creatine	E03	3% Sodium formate	H01	10 mM Sodium nitrate
B11	6% NaCl+creatinine	E04	4% Sodium formate	H02	20 mM Sodium nitrate

Table 1: Continue

Well	Substrate	Well	Substrate	Well	Substrate
B12	6% NaCl+L-Carnitine	E05	5% sodium formate	H03	40 mM Sodium nitrate
C01	6% NaCl+KCl	E06	6% sodium formate	H04	60 mM Sodium nitrate
C02	6% NaCl+L-proline	E07	2% Urea	H05	80 mM Sodium nitrate
C03	6% NaCl+N-acetyl-L-glutamine	E08	3% Urea	H06	100 mM Sodium nitrate
C04	6% NaCl+β-glutamic acid	E09	4% Urea	H07	10 mM Sodium nitrite
C05	6% NaCl+β–amino-N-butyric acid	E10	5% Urea	H08	20 mM Sodium nitrite
PM10					
A01	pH 3.5	B09	pH 4.5+L-histidine	D06	pH 4.5+b-Hydroxy glutamate
A02	pH 4	B10	pH 4.5+L-isoleucine	D07	pH 4.5+g-Hydroxy glutamate
A03	pH 4.5	B11	pH 4.5+L-leucine	D08	pH 4.5+5-Hydroxy lysine
A04	pH 5	B12	pH 4.5+L-lysine	D09	pH 4.5+5-Hydroxytryptophan
A05	pH 5.5	C01	pH 4.5+L-methionine	D10	pH 4.5+D,L Diamino-pimelic acid
A06	pH 6	C02	pH 4.5+L-phenylalanine	D11	pH 4.5+Trimethylamine-N-oxide
A07	pH 7	C03	pH 4.5+L-proline	D12	pH 4.5+Urea
A08	pH 8	C04	pH 4.5+L-serine	E11	pH 9.5+L-Leucine
A09	pH 8.5	C05	pH 4.5+L-threonine	H01	X-Caprylate
A10	pH 9	C06	pH 4.5+L-tryptophan	H02	X-alpha-D-glucoside
A11	pH 9.5	C07	pH 4.5+L-tyrosine	H03	X-beta-D-glucoside
A12	pH 10	C08	pH 4.5+L-valine	H04	X-alpha-D-galactoside
B01	pH 4.5	C09	pH 4.5+hydroxy-L-Proline	H05	X-beta-D-galactoside
B02	pH 4.5+L-alanine	C10	pH 4.5+L-ornithine	H06	X-alpha-D-glucuronide
B03	pH 4.5+L-arginine	C11	pH 4.5+L-homoarginine	H07	X-beta-D-glucuronide
B04	pH 4.5+L-asparagine	C12	pH 4.5+L-homoserine	H08	X-beta-D-glucosaminide
B05	pH 4.5+L-aspartic Acid	D02	pH 4.5+L-norleucine	H09	X-beta-D-galactosaminide
B06	pH 4.5+L-glutamic Acid	D03	pH 4.5+L-norvaline	H10	X-alpha-D-mannoside
B07	pH 4.5+L-glutamine	D04	pH 4.5+a- amino-N-butyric acid	H11	X-PO_4
B08	pH 4.5+glycine	D05	pH 4.5+a-amino malonate	H12	X-SO_4

supported the growth of the pathogen (Table 1), including L-arabinose, D-galactose, D-trehalose, D-mannose, D-sorbitol, D-xylose, D-mannitol, L-rhamnose, D-fructose, a-D-glucose, maltose, D-melibiose, a-D-lactose, lactulose, sucrose, b-methyl-D-glucoside, maltotriose, D-cellobiose, g-cyclodextrin, dextrin, amygdalin, D-arabitol, arbutin, i-erythritol, 3-0-β-D-galactopyranosyl-D-arabinose, gentiobiose, D-lactitol, D-melezitose, a-methyl-D-glucoside, b-methyl-D-galactoside, palatinose, D-raffinose, stachyose and quinic acid. In comparison, around 85 compounds significantly inhibited the growth of the pathogen (Fig. 1). Using the PM3 plate, the isolate of *A. alternata* was tested for their ability to grow on 95 different nitrogen sources (amino acids) (Fig. 1). More than 65 compounds supported the growth of the pathogen (Table 1), typical compounds included L-glutamic acid, L-lysine, D-glutamic acid, D,L-a-amino-caprylic acid, d-amino-N-valeric acid, Ala-Glu, Gly-Met and Met-Ala. In comparison, twenty-four out of 95 nitrogen sources supported growth at the level of the negative control, indicating that *A. alternata* cannot metabolize these compounds (Fig. 1).

Using data from PM6 to PM8 (nitrogen pathways), *A. alternata* presented 274 different nitrogen pathways, indicating that various combinations of different amino acids supported the growth of the pathogen (Fig. 1). Plates PM9 and PM10 were used to test growth under various stress conditions. *A. alternata* showed active metabolism with up to 10% sodium chloride, up to 6% potassium chloride, up to 5% sodium sulfate, up to 20% ethylene glycol, up to 6% sodium formate, up to 6% urea, up to 12% sodium lactate, up to 200 mM sodium phosphate (pH 7.0), up to 100 mM ammonium sulfate (pH 8.0), up to 100 mM sodium nitrate and up to 20 mM sodium nitrite, however, it could not metabolize any sodium benzoate (pH 5.2) which ranged from 20-200 mM (plate PM 9, Well G05, G06, G07 and G08) in our analysis (Fig. 1). When combined with various osmolytes at the treatment of 6% sodium chloride, *A. alternata* presented active growth in all tests (plate PM9, Well B01 to B12 and C01 to C12). The pH range where *A. alternata* exhibited active growth was between 3.5 and 10, with an optimal pH of around 6.0. When combined with various amino acids at the pH of 4.5, *A. alternata* showed active growth in all tests except for combining with the amino acids of anthranilic acid (plate PM 10, well D01) and p-aminobenzoate (plate PM 10, well D05) (Fig. 1, PM10). In comparison, when combined with various amino acids at the pH of 9.5, the pathogen presented no 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 pH 4.5 and pH 9.5, respectively. *A. alternata* showed active decarboxylase activities in the presence of most of the amino acids, except for anthranilic acid (plate PM 10, Well D1) and p-aminobenzoate (plate PM 10, Well D05); in comparison, it exhibited no deaminase activities (Fig. 1, plate PM 10), except for L-leucine (plate PM 10, Well E11).

DISCUSSION

While a large number of genetic and molecular biological studies have been conducted with *A. alternata*, phenotypic diversity is still poorly explored. Direct high-throughput assessment of phenotypes using the Phenotype MicroArray (PM) system (Bochner *et al*., 2001) has stirred much attention for molecular biological, genomic and population studies of microorganisms (Velez *et al*., 2007). Here, metabolic ability

of the pathogen from host of flue-cured tobacco was systematically analyzed using PMs and significant metabolic diversity was found.

In this study, a pathogenic isolate of *A. alternata* from the host of flue-cure tobacco was analyzed by the high-throughput PM technique. A narrow range of carbon compounds could be utilized by the pathogen and most of the nitrogen, sulfur and phosphorus sources were metabolized. Since, carbon substrate is the most important nutrient in the life of most microorganisms, the narrow range of carbon utilization of *A. alternata* may indicate a poor adaptability of the pathogen in tobacco developing period. Early studies have proved that tobacco leaves are seldom infected by *A. alternata* during the early part of the growing season, whereas at harvest time all plant parts above ground become heavily infected (Ramm and Lucas, 1963; Stavely and Slana, 1970; Tong *et al*., 2012). There might be some carbon substrates produced on tobacco leaves only at harvest time to induce the infection of the pathogen. The narrow carbon substrates metabolized in our study may in some case explain the infection mechanism of *A. alternata* on tobacco. However, whether those carbon substrates are presence in the mature or near mature tobacco leaves or not is still unclear, more work is need to be conducted to confirm this hypothesis.

The most informative plates for *A. alternata* 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 just some carbohydrates and for nitrogen sources some amino acids. There were some carbohydrates reported in tobacco leaf that regulated leaf development (Miller *et al*., 2000), stimulated ethylene production (Philosoph-Hadas *et al*., 1985), metabolized just during leaf ageing progress (Crafts-Brandner, 1991) and that existed just on the leaf surface of tobacco (Menetrez *et al*., 1990). Such compounds might play a role in supporting the infection of tobacco leaves by *A. alternata*. Additionally, the pathogen had wide range adaptabilities in osmolytes and pH conditions which was found by using plates PM 9 and PM 10. The phenotypic diversity in osmolytes of the pathogen can be rationalized by considering the seasonal variation in osmolytes due to varying conditions of dryness and watering. Decarboxylase of the pathogen generates alkaline amines by the catabolism of amino acids which help to counteract an acidic pH (Maurer *et al*., 2005). A high pH on the other hand can be countered by deaminases which generate acids. *A. alternata* in our study showed active decarboxylase activities, whereas no deaminase activities. Hence, strong alkalic chemicals might have some potential ability to inhibit the metabolism of the pathogen and could be used for tobacco brown spot management. Consequently, phenotypic characters for utilization of those sources and wide range adaptabilities of *A. alternata* could have a high potential value in pathogen-tobacco interaction studies and survival of the pathogen in environment.

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 *A. alternata*, or decreasing the amount of these sources that could be utilized by the pathogen in the field may reduce the damage caused by brown spot. Meanwhile, changing the osmolytes and pH environment in tobacco leaves to make it unadaptable for the pathogen *A. alternata* may also lighten the happening of brown spot. These imagines could be proved in the next study in the near future.

CONCLUSION

In conclusion, phenomics study of *A. alternata* in our study increases our understanding of the pathogen.

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