VARIATION IN AGGRESSIVENESS AND AFLP AMONG *Alternaria solani* ISOLATES FROM INDONESIA

Variasi Keganasan dan AFLP Antarisolat Alternaria solani *Asal Indonesia*

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ABSTRACT

Alternaria solani is a necrotroph fungus that causes three-phased diseases in tomato. Management of the pathogen by using resistant cultivars requires knowledge on the aggressiveness and genetic diversity of the fungus. The aims of this study were to isolate *A. solani* from major tomato and potato producing areas in Indonesia and to study their aggressiveness and genetic variability. Twenty two *A. solani* isolates were recovered from early blighted tomato and potato in Central and West Java. *A. alternata* was also isolated from tomato leaves in West Java and North Sumatra, indicating that early blight in Indonesia may be caused by more than one *Alternaria* species. Resistance tests of four tomato genotypes to selected *A. solani* isolates revealed that local isolates were more aggressive in inciting early blight and stem lesion than an imported isolate from USA. This implies that introduced breeding materials must be tested to local isolates to obtain effective resistance genes. Cluster analysis based on amplified fragment length polymorphism (AFLP) obtained from *Eco*RI+AG and *Mse*I+C primer amplification separated 28 local and Taiwan isolates from the US isolate, which was coincided with aggressiveness separation between the local isolates and the US isolate. Three clusters of AFLP genotypes which did not associate with geographic origin were observed among tropical isolates. The low genetic diversity among the Indonesian isolates suggests clonal population structure with wide distribution. Successful local tomato breeding requires the availability of local *A. solani* collection with well-characterized aggressiveness level and molecular diversity to obtain effective resistance genes.

[Keywords: aggressiveness, AFLP, early blight, stem lesion, tomato]

ABSTRAK

Alternaria solani *adalah jamur nekrotof penyebab tiga gejala penyakit pada tanaman tomat. Pengelolaan patogen ini dengan menggunakan varietas tahan memerlukan informasi keragaman genetik* A. solani. *Penelitian bertujuan untuk mengisolasi* A. solani *dari daerah produksi utama tomat dan kentang di Indonesia dan mempelajari keragaman keganasan dan genetiknya. Sebanyak 22 isolat* A. solani *diisolasi dari daun-daun tomat dan kentang bergejala hawar dini di Jawa Tengah dan Jawa Barat.* A. alternata *juga diperoleh dari daun tomat bergejala hawar dini di Jawa Barat dan Sumatera Utara, sehingga di Indonesia penyakit ini mungkin disebabkan oleh lebih dari satu spesies* Alternaria. *Uji ketahanan empat genotipe tomat terhadap isolat* A. solani *terpilih di rumah kaca menunjukkan bahwa isolat lokal lebih agresif dalam menyebabkan penyakit hawar dini dan lesio batang dibandingkan dengan isolat asal Amerika Serikat. Implikasinya adalah galur tomat introduksi harus diuji terhadap isolat lokal untuk mendapatkan gen ketahanan yang efektif. Pengelompokan genotipe marka* amplified fragment length polymorphism *(AFLP) hasil amplifikasi DNA menggunakan kombinasi primer* Eco*RI+AG dan* Mse*I+C memisahkan 28 isolat* A. solani *asal Indonesia dan Taiwan dari isolat asal Amerika Serikat, yang sesuai dengan pemisahan derajat keganasan antara isolat lokal dan isolat Amerika Serikat. Tiga gerombol genotipe marka AFLP yang tidak berkaitan dengan asal geografis isolat terbentuk di antara isolat Indonesia dan Taiwan. Keragaman genetik yang rendah antarisolat* A. solani *Indonesia menunjukkan adanya struktur populasi klonal yang tersebar luas. Keberhasilan program pemuliaan lokal ketahanan tomat terhadap* A. solani *memerlukan ketersediaan koleksi isolat lokal yang terkarakterisasi keganasan dan genetiknya dengan baik agar diperoleh gen ketahanan yang efektif.*

[*Kata kunci: keganasan, AFLP, hawar dini, lesio batang, tomat*]

INTRODUCTION

The necrotroph fungus *Alternaria solani* (Ellis & Martin) Sorauer incites early blight of foliage, collar rot of seedlings or stem lesion of adult plants of tomato (*Solanum lycopersicum*) and lesion on tomato fruit (Chaerani and Voorrips 2006). On potato (*Solanum tuberosum*) the fungus also infects tubers during storage in addition to causes early blight disease (Odilbekov et al. 2016). Early blight is the

most common and destructive phase of the disease syndromes because it can cause premature defoliation which weakens plants and exposes fruits to sunscald injury (Chaerani et al. 2007). Early blight symptoms on foliage are characterized by dark brown to black lesions with concentric rings, which produce a "target spot" effect (Sherf and MacNab 1986). Necrotic lesions first affect older leaves and spread upward as plants become mature.

In Indonesia early blight epidemics was reported for the first time in the early 1900s to severely devastated potato fields in the highlands of West Java and later spread to potato fields at lower elevations causing considerable yield losses (Chaerani 2006). The disease soon spread to potato fields in the highlands of North Sumatra in 1920s. At present early blight is one of the common tomato and potato diseases in Indonesia, especially on tomato grown in lower altitudes, where late blight is less thriving (Chaerani 2006).

A. solani is difficult to control since it has a short life cycle, is primarily air borne, has long survival ability in plant debris and soil, and has a wide solanaceaous host range (Chaerani and Voorrips 2006). Host resistance is the most economically and environmentally sound approach over fungicidal spray to restrict early blight incidence in the field, but tomato and potato cultivars with high level of early blight resistance and desirable horticultural traits have not been identified (Gardner and Panthee 2012; Odilbekov 2015; Adhikari et al. 2017).

Effectiveness of host resistance to *A. solani* is greatly influenced by the genetic variation of the pathogen (Odilbekov et al. 2016). Previous studies revealed considerable variability among isolates with respect to morphology, physiology, biochemistry, aggressiveness and genetics (Bonde 1929; Henning and Alexander 1959; Petrunak and Christ 1992; Weir et al. 1998; Castro et al. 2000; Martinez et al. 2004; van der Waals et al. 2004; Varma et al. 2006; Lourenço et al. 2009; Lourenço Jr et al. 2011; Weber and Halterman 2012; Meng et al. 2015; Kumar et al. 2016; Odilbekov et al. 2016). Great variability in *A. solani* is unexpected, since the sexual stage of this pathogen is not known (Meng et al. 2015). The occurrence of differential aggressiveness among isolates has led to the conclusion for the presence of physiological races (Bonde 1929). However, the race concept in *A. solani* has not been widely accepted due to lack of characterized differential varieties (Chaerani and Voorrips 2006; Gannibal et al. 2014; Adhikari et al. 2017)

Genetic variation within *A. solani* populations has been assessed using various molecular markers which included random amplified polymorphic

DNA (RAPD) (Weir et al. 1998; Varma et al. 2006; Kumar et al. 2008; Lourenço Jr et al. 2011; Weber and Halterman 2012; Leiminger et al. 2013), random amplified microsatellites (RAMS) (van der Waals et al. 2004), amplified fragment length polymorphism (AFLP) (Martinez et al. 2004; Lourenço Jr et al. 2011; Odilbekov et al. 2016), and simple sequence repeats (SSRs) (Meng et al. 2015), as well as sequence analyses of *A. solani* genome portion (Lourenço et al. 2009; Weber and Halterman 2012). Among different types of molecular markers, AFLP marker has become one of the major methods of choice for studies of genetic diversity because it does not require prior knowledge of genomic sequence and generates a large number of polymorphic loci in a single experiment (Martinez et al. 2004; Odilbekov et al. 2016). The highly polymorphic nature of AFLP marker makes it especially useful for differentiating clonal lineages of fungi that reproduce asexually (McDonald 1997).

AFLP DNA analysis combines the power of restriction fragment length polymorphism (RFLP) with the flexibility of PCR-based technology (Vos et al. 1995). The technique is based on a multistep process which includes DNA digestion with a rare and frequent cutter, fragment ligation to adaptors, pre-selective PCR amplification, selective PCR amplification, and fragment separation. The use of two selective primers under stringent PCR conditions gives the AFLP technique reproducible results unlike RAPDs which use one arbitrary decamer primers.

Previous AFLP analyses of *A. solani* isolates from tomato and potato revealed great diversity among exotic and local isolates in Cuba (Martinez et al. 2004), whereas clonal population with high genetic variability was observed among Brazilian isolates (Rodrigues et al. 2008; Lourenço Jr et al. 2011). Exotic isolates from China, Turkey, Greece and USA tended to group separately from isolates from Cuba and Brazil. Both studies found genetic differentiation between isolates from tomato and potato.

The combination of molecular markers with pathogenicity assays represents a useful tool in pathogen population genetics (Bouajila et al. 2007). However, little information is available on the association of *A. solani* genotypic diversity with aggressiveness. Van der Waals et al. (2004) found a relatively high variability in RAMS profiles among potato *A. solani* isolates from South Africa, but failed to obtain apparent correlation with isolate aggressiveness.

Despite its widespread distribution in Indonesia, intensive collection and genetic studies of the pathogen

has not been pursued before. The knowledge of genetic diversity of this pathogen is useful for improvement of early blight disease management using resistant cultivars and for selection of resistance genes in breeding program. The objective of this research was thus to reveal the variability among *A. solani* collected from major tomato and potato producing areas in Indonesia based on aggressiveness of selected isolates and AFLP analysis.

MATERIALS AND METHODS

Collection, Isolation and Identification of Fungal Cultures

Tomato and potato leaves with typical early blight lesions collected from main tomato and potato producing areas in 10 localities of West and Central Java, and North Sumatra were transported in a cooler box or packed in paper bags containing silica gels and brought to the laboratories of Indonesian Ornamental Crops Research Institute (IOCRI) and Indonesian Vegetables Research Institute (IVEGRI) for isolation. *A. solani* was isolated by using either monospore isolation or diseased tissue plating technique on 1.5% water agar (WA). Purification was done by transferring hyphal tips to potato dextrose agar (200 g potato, 20 g dextrose, 15 g agar, and dH$_2$O to 1 liter). Cultures were maintained on V8 juice agar medium (200 ml V8 juice, 3 g CaCO$_3$, 20 g agar, and dH$_2$O to 1 liter) (Ribeiro 1978) at 21–22°C in a 12-h diurnal period of fluorescent light. For each isolate, single-spore cultures were established on V8 juice agar medium. Species identification was performed morphologically based on colony appearance and conidial characteristics (Ellis and Gibson 1975).

Aggressiveness Test of Selected Isolates

Inoculation experiments were done at Plant Research International (PRI) glasshouse. Three *A. solani* isolates selected to represent different geographic localities in West Java (Cianjur, Sukabumi and Lembang) and one isolate from USA contributed by Dr. E. Cobb (Department of Plant Pathology, Cornell University, Ithaca, New York, USA) were compared for their aggressiveness on four tomato genotypes. *A. solani* sporulation requires special conditions which include mycelial wounding, exposure to florescent light and partial desiccation (Barksdale 1969; Rodrigues et al. 2010a). Sporulation was induced by following Barksdale (1969) procedure.

Aerial mycelia were scraped off from 7 to 10-day old cultures using a scalpel and the plates were returned to the same incubation conditions with the bottom of the Petri dishes facing down and leaning on inverted lids. After 48 h spores were removed using an L-shaped glass rod in the presence of 5 ml sterile dH$_2$O. Seeds of three introduced moderately resistant tomato breeding lines (FT94-978; 99-213 obtained from Prof. M. Mutschler, Department of Plant Breeding, Cornell University, Ithaca, New York, USA; NC-EBR6 obtained from Dr. R. Gardner, North Carolina University, Raleigh, USA; and HRC90.145 obtained from Dr. V. Poysa, Agriculture and Agri-Food Canada, Harrow Research Center, Harrow, Ontario, Canada) and a susceptible tomato genotype (cv. Moneymaker) were grown for three weeks on peat soil in boxes of 34 cm × 29.5 cm × 128.4 cm. Each box contained eight rows of four plants, with the four genotypes planted in two alternating rows. Boxes were placed on a bench lined with a wet mat. Leaves were sprayed with *A. solani* spores at a rate of 10^3 ml^{-1} tap water until run-off using a glass atomizer. Immediately after spraying, the boxes were covered with a transparent lid and placed on a bench lined with a wet mat. The bench was covered with a transparent plastic forming a tunnel (Figure 1a and c).

Intermittent misting for 15 min at 45-min intervals to maintain high humidity inside the tunnel was supplied from a humidifier (Figure 1b). After 24 h the lids were removed and each side of the plastic tunnel was opened. During the day the humidifier was turned off for 8 h to allow the plant surface to dry. The tunnel was closed during the night. The glasshouse received periodic misting for 16 h to maintain the relative humidity from 40% to 72% during the day and 85% to 100% during the night. The temperatures in the glasshouse were maintained at 20–27°C during the day and 16–24°C during the night. Treatments were replicated three times. A same experimental set up was performed to investigate the aggressiveness of the four isolates in inciting stem lesion severity. Spores (10^3 ml^{-1} in tap water) were sprayed onto plant stem base until run-off.

Early blight severity on each leaf was recorded four times at 3, 7, 10 and 14 days after inoculation (DAI) on a scale of 0 to 5, where 0 = no visible lesions on leaf; 1 = up to 10% leaf area affected; 2 = 11 to 25%; 3 = 26 to 50%; 4 = 51 to 75%; and 5 = more than 75% leaf area affected or leaf abscised (Vakalounakis 1983). Leaves that were not completely unfurled during the inoculation were not assessed. The disease scales were converted into percentage of early blight index (PEBI) for each plant using the following formula (Pandey et al. 2003): PEBI = (sum of all ratings × 100)/(no. of leaves sampled × maximum disease scale). PEBI was used to calculate the area under the disease progress

curve (AUDPC) using the formula: AUDPC = Σ {([R_{i+1} + R_i]/2 × (t_{i+1} − t_i)}, where R_i is the PEBI at the ith observation, t_i is time (days after planting) at the ith observation, and n is the total number of observations (Nash and Gardner 1988). Stem lesion severity was recorded twice at 10 and 14 DAI using a modified scale from Barksdale and Stoner (1977), where 0 = no lesions, 1 = lesion size 1 to 2 mm, 2 = lesions between 2 mm and 1 cm, and 3 = lesion size equal to or more than 1 cm. Stem lesion index and AUDPC were calculated using the same formulas as described. AUDPC values were analyzed as 4×4 factorial experiments in a randomized completely block design.

DNA Isolation

Genomic DNA was extracted using DNA extraction kit (Puregene-Biozym BV) from mycelia which were scraped off from 7 to 10-day old cultures grown on V8-juice agar medium in 9-cm diameter Petri plates. DNA concentration was estimated by 1% agarose gel electrophoresis and compared to that of lambda DNA.

AFLP Analysis

AFLP analysis was performed at the PRI following the procedure described in Vos et al. (1995). Briefly, genomic DNA (50 ng) from each isolate was digested with the six-base restriction enzyme *Eco*RI and the four-base restriction enzyme *Mse*I, then the digested fragments were ligated with *Eco*RI–biotine and *Mse*I–biotine adaptors in the presence of T4 DNA ligase. The resulting primary template was diluted and pre-amplified with *Eco*RI primer (E00) and *Mse*I primer (M00). The pre-amplification product was diluted and used as the secondary template for selective amplification with

*Eco*RI+AG and *Mse*I+C primers which contained two and one selective bases, respectively, at their 3' end after the core sequences. The *Eco*RI primer was radio labeled with [32]P (Invitrogen).

AFLP fragments were then separated on a 6% denaturing polyacrylamide gel run with a constant power of 70 W for 2 h 45 min using OWL sequencing gel system. DNA fragment sizes were estimated by comparing migration distance to that of a 30–330-bp AFLP ladder which was also radio labeled with [32]P. The gels were placed into a cassette and exposed to Kodak X-OMAT AR film overnight. AFLP fragments between 50 and 330 bp were scored manually by eye for the presence (1) or absence (0) for each isolate. Only bands that could be scored unambiguously were recorded.

Four *A. solani* isolates obtained from Ir. Gunarto (East-West Seed Indonesia), two from Dr. L.L. Black (AVRDC, Taiwan), and the US isolate were included in the AFLP analyses. In the case where isolates showed cultural variation, each variant was also run in the gel analysis to check whether morphological variation can be differentiated at molecular level. Two *A. alternata* isolates obtained in the surveys (see Results and Discussion) were also included as comparison in the analysis. Several genetic studies showed that AFLPs were able to distinguish fungal species within the same genus regardless of the primer combination used (Casasnovas et al. 2013; Oviedo et al. 2013).

Phylogenetic Analysis

The resulting binary data describing the presence or the absence of AFLP fragments at all scored loci were used to generate a similarity matrix based on simple matching coefficient (Sneath and Sokal 1973). The level of relatedness among the isolates was determined
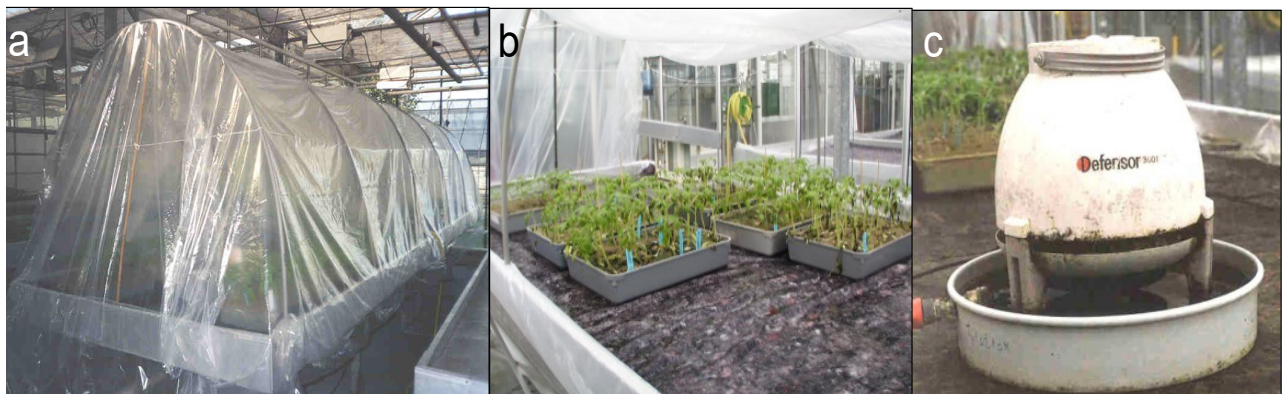

Figure 1. *Alternaria solani* inoculation experiment on tomato plants in glasshouse. Closed (a) and open (b) plastic tunnel where plants were incubated on a bench lined with wet mat. Periodic misting during the night was supplied from a humidifier (c).

using the sequential agglomerative hierarcical nested (SAHN) cluster analysis and a dendrogram was constructed using the unweighted pair-group method of arithmetic averages (UPGMA) module available in the software. All calculations were done with the aid of the software NTSYSpc (v2.01, Exeter Software, Setauket, NY, USA).

RESULTS AND DISCUSSION

Fungal Isolation

Twenty two *A. solani* isolates were successfully recovered from infected tomato and potato leaves collected from nine localities surveyed in West and Central Java (Table 1). Tomato leaf samples collected

Table 1. Hosts and origins of *Alternaria solani* and *A. alternata* isolates used in this study

Isolate code	Name	Species	Host	Locality	Source
1	Cgn 01	*A. solani*	Tomato	Cugenang, Cianjur, West Java	This study
30	As 99041	*A. solani*	Tomato	Cugenang, Cianjur, W. Java	This study
4	Pct 03	*A. solani*	Tomato	Pacet, Cianjur, W. Java	This study
5	Pct 04	*A. solani*	Tomato	Pacet, Cianjur, W. Java	This study
6	Pct 08	*A. solani*	Tomato	Pacet, Cianjur, W. Java	This study
7	Pct 09	*A. solani*	Tomato	Pacet, Cianjur, W. Java	This study
9	Pct 11	*A. solani*	Tomato	Pacet, Cianjur, W. Java	This study
32	As 20001	*A. solani*	Tomato	Songgom, Cianjur, W. Java	This study
10	Smi 101	*A. solani*	Tomato	Agrabinta, Sukabumi, W. Java	This study
12	Smi 106	*A. solani*	Tomato	Agrabinta, Sukabumi, W. Java	This study
13	Smi 111	*A. solani*	Tomato	Agrabinta, Sukabumi, W. Java	This study
16	Smi 205	*A. solani*	Tomato	Agrabinta, Sukabumi, W. Java	This study
17	Smi 209	*A. solani*	Tomato	Agrabinta, Sukabumi, W. Java	This study
31	As 99049	*A. solani*	Tomato	Selabintana, Sukabumi, W. Java	This study
23	As 99006	*A. solani*	Tomato	Margahayu, Lembang, W. Java	This study
25	As 99014	*A. solani*	Tomato	Margahayu, Lembang, W. Java	This study
26	As 99024	*A. solani*	Potato	Margahayu, Lembang, W. Java	This study
27	As 99025	*A. solani*	Tomato	Margahayu, Lembang, W. Java	This study
28	As 99029	*A. solani*	Tomato	Margahayu, Lembang, W. Java	This study
19	L.2	*A. solani*	Tomato	Leles, Garut, W. Java	This study
20	L.6	*A. solani*	Tomato	Cisurupan, Garut, W. Java	This study
33	As 20008	*A. solani*	Tomato	Demplongan, Getasan, Central Java	This study
35	As-08	*A. solani*	Tomato	Lembang, W. Java	Ir. Gunarto[1]
36	As-23	*A. solani*	Tomato	Lembang, W. Java	Ir. Gunarto[1]
90	As Lembang	*A. solani*	Tomato	Lembang, W. Java	Ir. Gunarto[1]
88	As-33	*A. solani*	Tomato	Wanayasa, Purwakarta, W. Java	Ir. Gunarto[1]
104	A.sol-1	*A. solani*	Tomato	Taiwan	Dr. Lowell L. Black[2]
105	A-sol-2	*A. solani*	Tomato	Taiwan	Dr. Lowell L. Black[2]
34	34	*A. solani*	Tomato	USA	Dr. E. Cobb[3]
29	As 99032	*A. alternata*	Tomato	Margahayu, Lembang, W. Java	This study
91	Ajj 01	*A. alternata*	Tomato	Brastagi, North Sumatra	This study

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[2]AVRDC, Taiwan
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in Lembang (West Java) and North Sumatra yielded *A. alternata* isolates. Both *Alternaria* species could presence in the same field and even on the same leaf, and therefore the necrotic spots caused by *A. solani* can be confused with those caused by *A. alternata* (Tymon 2014; Odilbekov et al. 2016). Generally, lesions caused by *A. alternata* are smaller, darker, sunken and can be numerous in number compared to those caused by *A. solani* (Tymon 2014). Our results, where surveys were mostly done on tomato fields, showed that *A. solani* was more commonly detected than *A. alternata*. On the contrary, Tymon (2014) detected *A. alternaria* more often than *A. solani* in the potato fields and concluded that the former species is the dominant causal agent of early blight potato in the Pacific Northwest of USA. *A. alternata* was also identified among early blighted potato foliage in Germany, the Netherlands and Poland (Latorse et al. 2010).

Both species showed similar cultural morphology on V8 juice agar medium, i.e. grey olive to black colonies with cottony and velvety mycelia, but *A. solani* could clearly be distinguished from *A. alternata* by the formation of large, dark, obclavate and muriform conidia with beaks (Figure 2d and e) which were born singly on simple conidiophores. On the contrary, *A. alternata* produced beakless, small conidia which were born in chains on conidiophores and readily sporulated in culture in complete darkness without special treatment.

Recently, molecular identification of the causal agent of early blight disease has been possible through the use of species-specific primers (Edin 2012; Chowdappa et al. 2014). These primers allowed large field assays for rapid and sensitive detection of *A. solani* simply by PCR amplification of DNA samples from early blighted leaves without the need to isolate the fungus.

Three types of *A. solani* culture on V8 juice agar medium were observed: abundant sporulating type which was characterized by compact mycelia with distinct radial zonation in the medium (Figure 2a), non-sporulating type which produced white, sterile and massive aerial mycelia (Figure 2b), and intermediate type (Figure 2c). Mixed culture types which appeared as growth sectoring could presence in the same Petri dish. Only the sporulating type section was transferred to a fresh medium when subculturing it because sporulating ability may be lost during repeated subcultures (Bonde 1929).

We observed cultural variability among monospore cultures of parental isolates (isolate 16, 17, and 19) as was reported by Bonde (1929) and later by Henning and Alexander (1959). In spite of that, AFLP analysis
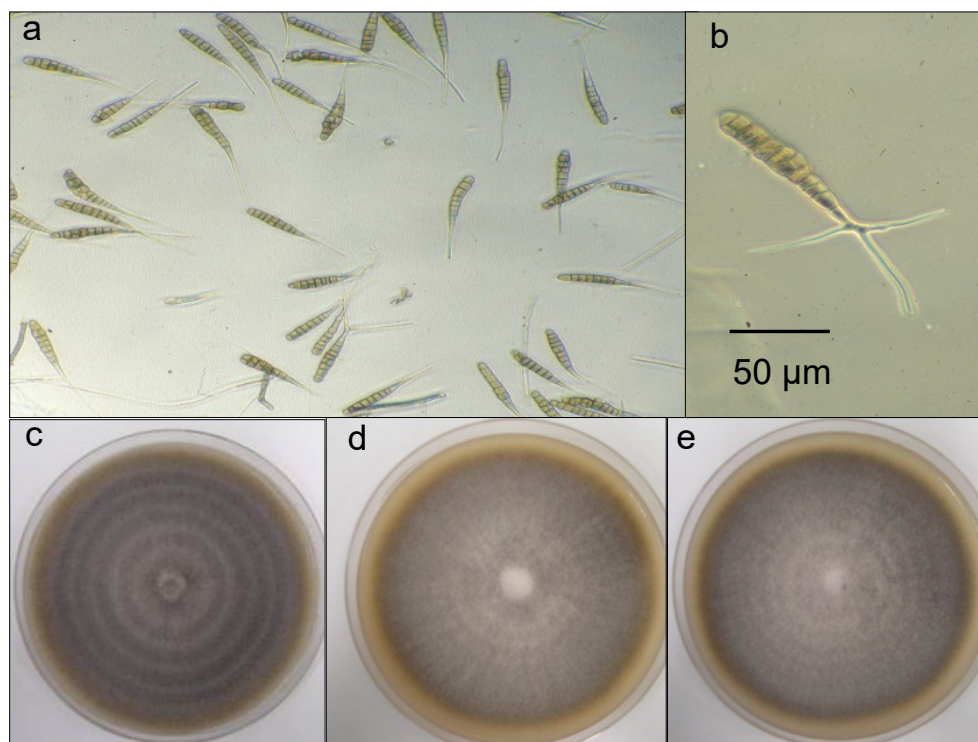

Figure 2. Morphological performance of *Alternaria solani*. Conidia (a) and an enlarged image of single conidium (b). Characteristic of *Alternaria solani* culture on V8 juice agar medium showing radial zonation with abundant (c), mycelial or non-sporulating (d), and intermediate spore production type (e).

revealed identical banding patterns among descendants of each of those parental isolates (Figure 3), indicating that such anonymous genome portions were not informative for differentiating cultural variation. A similar result was reported by Rotondo et al. (2012) among single-spore isolates of small-spores *Alternaria* from apple, where morphological variation was not in accordance with AFLP groupings.

Aggressiveness Test

Necrotic spots with yellow halo on leaves were started to visible on 3 DPI. Necrotic spots incited by aggressive isolates on susceptible tomato genotypes enlarged and coalesced (Figure 4c) which often turned leaves into dark color leading to premature defoliation (Fig 4d), whereas necrotic spots caused by non-aggressive isolate failed to develop (Figure 4b). Dark, sunken necrotic spots developed on stems (Figure 4f) and progressed to girdle basal stems (Figure 4g).

No significant interaction among *A. solani* isolates and tomato lines was observed for both disease syndromes. *A. solani* isolates differed significantly in aggressiveness in inciting early blight and stem lesion diseases (P<0.05). Indonesian isolates were more aggressive than that of the US isolate in causing both disease syndromes (Table 2 and 3). The US isolate was even unable to incite stem lesion (Table 3). Although aggressiveness tests were done on representative isolates, the results showed that local isolates were more aggressive than that of the imported one. Repeated subculturing of the US isolate may account for its reduced aggressiveness as was encountered by Weber and Halterman (2012) in their experiments for *A. solani* reference isolate. Contrary to Gannibal et al. (2014) finding that isolates from tomato is more specialized and associated with tomato, our results showed that isolate 10 which was isolated from potato leaves in Sukabumi (West Java) was the most aggressive in causing tomato early blight and stem lesion.

Low to varying degrees of aggressiveness among *A. solani* were reported by several researchers. For example, Shahbazi et al. (2010) observed varying
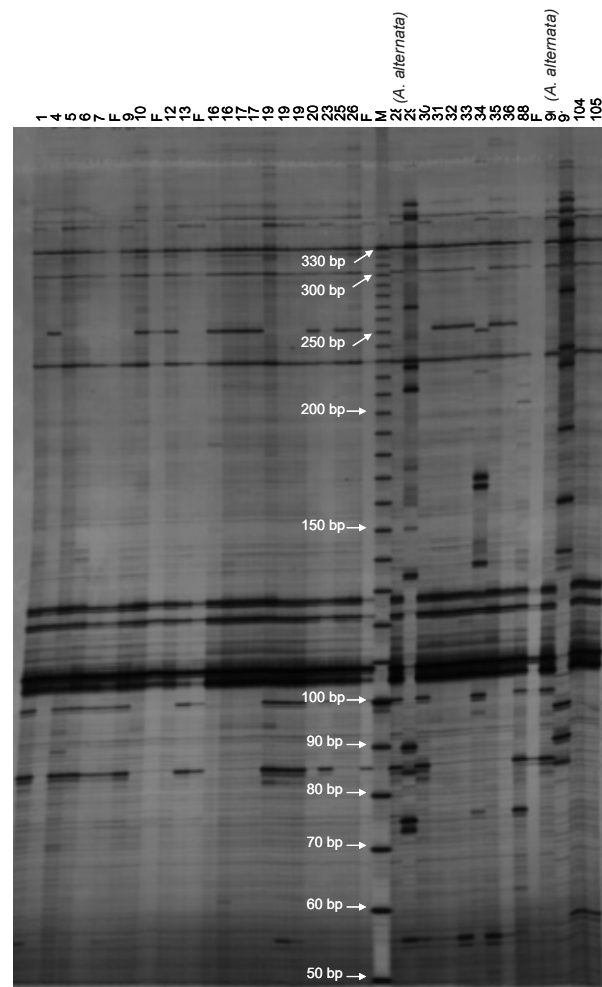

Figure 3. AFLP fingerprints of *Alternaria solani* isolates resulted from an AFLP analysis using primer combination *Eco*RI+AG and *Mse*I+C. Fail amplifications are marked as F. M is 10 bp DNA ladder. Isolate 16, 17 and 19 showed cultural variability among monospores isolates, and therefore each variant was PCR amplified and electrophoresed. Detail descriptions of the isolates used in this study are given in Table 1.

Figure 4. Early blight and stem lesion symptoms on resistant (b and f) and susceptible (c, d and g) tomato genotypes caused by aggressive *Alternaria solani* isolates. Plants sprayed with water were used as controls (a and e).

Table 2. Area under the disease progress curve (AUDPC) values of early blight severity observed at 14 days post-inoculation on four tomato genotypes incited by selected *Alternaria solani* isolates.

Isolate (host and origin)	Tomato genotypes[1]				Mean isolate
	HRC90.145	Moneymaker	NCEBR-6	FT94-978; 99-213	
9 (tomato, Cianjur, Indonesia)	140.2	113.7	71.4	32.6	89.5 a
10 (potato, Sukabumi, Indonesia)	160.7	119.7	78.5	37.7	99.1 a
26 (tomato, Lembang, Indonesia)	84.4	119.2	43.6	30.7	69.5 a
34 (tomato, USA)	0.0	0.0	0.0	0.0	0.0 b
Mean cultivar	96.4 a	88.1 a	48.4 ab	25.2 b	

[1]Averages of 8 plants × 3 replicates.
Values in a column or row followed by the same letters are not significantly different according to DMRT at P = 0.05. Data were arcsine [√(x/100)] transformed before statistical analysis.

Table 3. Area under the disease progress curve (AUDPC) values of stem lesion severity at 14 days post-inoculation on four tomato genotypes incited by selected *Alternaria solani* isolates.

Isolate (host and origin)	Tomato genotypes[1]				Mean isolate
	HRC90.145	Moneymaker	NCEBR-6	FT94-978; 99-213	
9 (tomato, Cianjur, Indonesia)	142.9	126.8	98.9	53.5	105.5 a
10 (potato, Sukabumi, Indonesia)	156.8	125.6	84.6	64.8	108.0 a
26 (tomato Lembang, Indonesia)	89.4	111.4	55.6	50.6	76.7 a
34 (tomato, USA)	0.0	0.0	0.0	0.0	0.0 b
Mean cultivar	97.3 a	90.9 a	59.8 ab	42.2 b	

[1]Averages of 8 plants × 3 replicates.
Values in a column or row followed by the same letters are not significantly different according to DMRT at P = 0.05. Data were arcsine [√(x/100)] transformation before statistical analysis.

levels of aggressiveness among *A. solani* from different geographic locations in Iran. In contrast, Kumar et al. (2008) obtained two aggressiveness groups among 11 *A. solani* isolates collected from different agroclimate zones in India and van der Waals et al. (2014) reported low to non-aggressive groups among 112 *A. solani* isolates from potato in South Africa which hampered characterization of the pathogen diversity. Weber and Halterman (2015) also found no significant aggressiveness variation on *A. solani* isolates from potato when tested *in vitro*.

Different susceptibilities to *A. solani* isolate were observed among tomato genotypes (P<0.05). The resistance degree of the tomato genotypes to early blight was consistent with the claimed resistance level, except for HRC90.145 which turned out to be susceptible to Indonesian isolates. HRC90.145 was previously rated as moderately resistant to *A. solani* (Poysa and Tu 1996). This implies that introduced breeding materials must be tested to local *A. solani* isolates to obtain an effective resistance in Indonesia. The resistance to stem lesion phase among the tomato genotypes tested was in accordance to the resistance to early blight. Previous classical genetic and genetic mapping studies found that resistance of tomato to both disease syndromes may be found in the same source, but may also segregated independently in other resistance sources (Chaerani and Voorrips 2006; Chaerani et al. 2007).

Varying levels of aggressiveness among *A. solani* isolates even among isolates originating from different germ tube tips from the same conidium were repeatedly reported (Bonde 1929; Neergaard 1945; Stall 1958), but consistent host-specific reactions of isolates which indicates the presence of physiological races could not be demonstrated (Castro et al. 2000). It is hardly possible to identify tomato and potato germplasm with race-specific resistance since results of genetic studies on resistance to *A. solani* derived from different donors point to the same conclusion that resistance to the pathogen is expressed quantitatively that is controlled by minor genes (Chaerani et al. 2007; Odilbekov 2015; Adhikari et al. 2017).

AFLP Polymorphism and Phylogenetic Analysis

By using two and one selective bases for *Eco*RI and *Mse*I primers, respectively, a total of 40 fragments were obtained. Nine polymorphic fragments in the size range of 165–325 bp were scored between *A. solani* and *A. alternata* (Figure 3), but only 5 polymorphic fragments were detected within *A. solani* isolates. Theoretically, the use of a two-nucleotide extension for the *Eco*RI primer along with a single nucleotide extension with the *Mse*I primer should generate more fragments and potentially more polymorphic markers (Kothera et al. 2003). However, low AFLP polymorphism was obtained in the current study, indicating low genetic variability among Indonesian *A. solani*. Similar studies by Lourenço *et al*. (2011) obtained higher AFLP polymorphism (>70 fragments) among Brazilian isolates although using one primer combination with the same number of selective nucleotides (*Eco*RI+AT and *Mse*I+A), whereas Martinez et al. (2004) obtained up to 452 polymorphic fragments among Cuban isolates when using three primer combinations (*Eco*RI+AT/+AC/+AG and *Mse*I+A).

A. solani were clearly separated from *A. alternata* at the similarity level of about 0.65 (Figure 5). Isolate subdivision with respect to temperate and tropical origin was observed at 0.71 coefficient similarity where the US isolate formed a distinct cluster. Within the tropical isolates, no subdivision based on geographic origin was observed. Three distinct genotypes were observed at 0.91 coefficient of similarity with one branch contained only isolate 88 obtained from Purwakarta (West Java). The other two branches contained 12 and 14 isolates, each with identical AFLP banding patterns.

Studies in USA, Cuba, Brazil, India and China using isozyme and molecular markers also found no location effect or no clear differentiation of *A. solani* collected from various regions in those countries (Petrunak and Christ 1992; Weir et al. 1998; Martinez et al. 2004; Varma et al. 2006; Kumar et al. 2008; Lourenço et al. 2011; Leiminger et al. 2013; Meng et al. 2015). Isolates from different agroclimatic conditions were found to be more genetically similar than isolates in close proximity to one another (Kumar et al. 2008). The observed low genetic diversity in our studied population suggests clonal population structure and is consistent with the asexual reproduction mode of the pathogen. The wide distribution of such population structure could be shaped by similar host adaptation processes on varieties with narrow genetic background. Most commercial tomato cultivars in Indonesia were developed from few high-yielding lines introduced from Taiwan (Chaerani 2006),
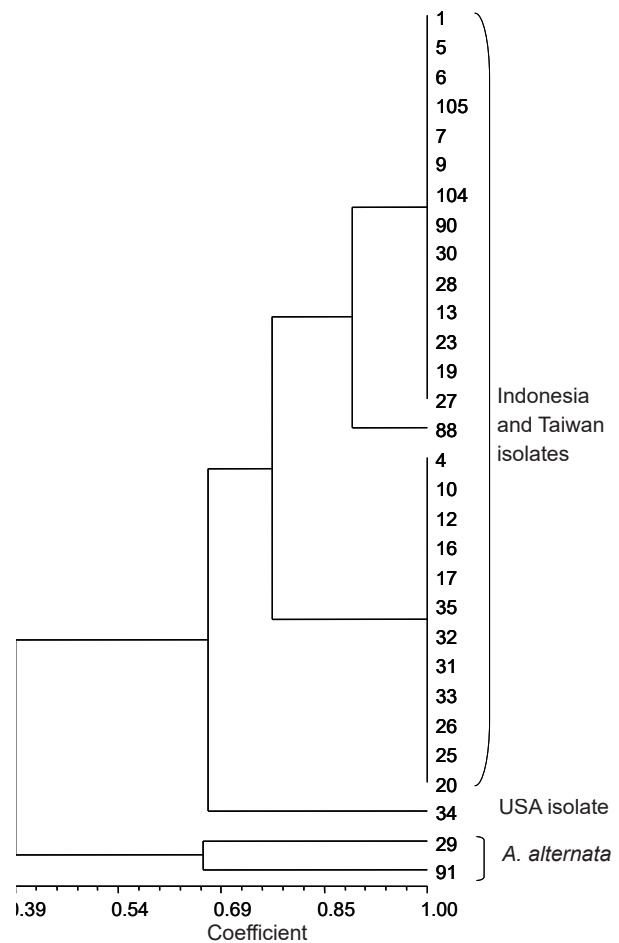

Figure 5. Dendrogram showing genetic similarity among 29 isolates of *Alternaria solani* and two isolates of *A. alternata*, as revealed by UPGMA cluster analysis based on AFLP genetic fingerprints obtained from AFLP analysis using primer combination *Eco*RI+AG and *Mse*I+C. Detail descriptions of the isolates used in this study are given in Table 1.

whereas only one or two potato cultivars are cultivated in Indonesia (Balitsa 2014). Wide cultivation of host with such narrow genetic background explains the clustering of Taiwan isolates with the local isolates and the clear distinction of the USC isolate, which adapted to different tomato genetic background. Another possible explanation for the low genetic diversity in our *A. solani* isolates could be the lower number of isolates compared to previous studies and the fact that the isolates were mostly from tomato.

Separation between the US isolate from the three selected local isolates in AFLP groupings was in accordance to their aggressiveness levels, but AFLP groupings of local isolates separated isolate 9 from isolate 10 and 26, despite their non-significant differences in aggressiveness levels. A similar result was obtained by Weber and Halterman (2012) where

four representative *A. solani* with equal aggressiveness level to potato were placed in different RAPD groups. Several other genetic diversity studies of fungal pathogen also found no clear association of molecular groupings with degree of virulence or aggressiveness of the pathogen (Kothera et al. 2003; Atallah et al. 2011; Rotondo et al. 2012). This indicates that such anonymous genomic regions were not suitable for fine discriminations of virulence or aggressiveness.

Host subdivision could not be addressed in our study because of the low sample number from potato. Isolates obtained from contiguous tomato and potato fields such as in Margahayu, Lembang, could possibly originate from a tomato field infected with an adapted lineage to this host. Other studies in the USA, Brazil and Cuba found population subdivision according to the host of origin as detected by RAPD and/or AFLP markers (Weir et al. 1998; Martinez et al. 2004; Lourenço et al. 2011) as well as by sequence analyses of ITS region of rDNA and the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*), and *Alt a1* (Lourenço et al. 2009).

Re-identification of *Alternaria* species and *A. solani*-like from both hosts by morphological analysis, host plant association studies, and sequence analyses of the *Alt a1*, *gpd* and/or *calmodulin* genes confirmed that early blight of tomato and potato are caused by distinct *Alternaria* species (Simmons 2000; Rodrigues et al. 2010b; Latorse et al. 2010; Gannibal et al. 2014). Currently, there are four other *Alternaria* species beside *A. solani* that are associated with early blight disease: *A. alternata* was identified from potato in Germany, the Netherlands, Poland (Gannibal et al. 2014), and USA (Tymon 2014); *A. tomatophila* was identified from tomato in Russia (Gannibal et al. 2014) and Brazil (Rodrigues et al. 2010b); and *A. cretica* and *A. grandis* were known to cause early blight on tomato and potato, respectively, in Brazil (Rodrigues et al. 2010b). Our surveys found two isolates of *A. alternata* from early blighted tomato leaves, one of them was presence in the same area with *A. solani*. A thorough sampling and identification of early blight pathogen from tomato and potato in Indonesia could lead to a better understanding whether the disease is indeed caused by more than one *Alternaria* species.

Considering the almost continuous cropping systems, geographic proximity of tomato and potato fields in many producing areas, and polycyclic infection throughout the year in the absence of harsh environmental conditions in Indonesia, it is interesting to study the population dynamics of *A. solani* and changes in its epidemiology. Since host plant assays were performed on few isolates, tests on a wider *A.*

solani collection would provide a better understanding on the aggressiveness spectrum of the pathogen. The use of one primer combination was sufficient to separate the tropical isolates from that of the temperate isolate and to discriminate aggressiveness level among selected isolates, but was powerless in discriminating intra-variation among local isolates. Genetic analysis with more AFLP primer combinations and the use of SSR markers (Meng et al. 2015), would reveal more genetic variation. The information obtained from this study should be useful for selection of appropriate type of *A. solani* resistance and resistance genes in local tomato breeding programs.

CONCLUSION

Twenty two *A. solani* isolates were successfully recovered from early blighted tomato or potato leaves originated from nine localities in Central and West Java. Two isolates of *A. alternata* were also identified from tomato fields in West Java and North Sumatra. Host plant assays of selected *A. solani* isolates showed that local isolates were more aggressive than that of the temperate isolate from USA in inciting both early blight and stem lesion symptoms. AFLP analysis with *Eco*RI+AG and *Mse*I+C primer combination separated the US isolate from local and Taiwan isolates, which partly coincided with low aggressiveness of the former isolate. Overall, low genetic diversity among local and tropical *A. solani* isolates were observed, which suggests clonal population structure with wide distribution.

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