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Morphological, physiological and pathogenic variability of small-spore *Alternaria* sp. causing leaf blight of Solanaceous plants in Algeria

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Due to premature defoliation, early blight epidemics can cause major yield losses. Large-spore *Alternaria* species such as *A. solani* and *A. tomatophila* have long been recognized as important pathogens responsible for such blight disease in the family *Solanaceae* and thus represent a serious risk for crop production. Small-spore *Alternaria* species have also been frequently isolated from plant samples with typical blight symptoms but their incidence as primary pathogens is often controversial. In order to study the diversity of small-spore *Alternaria* species, 32 isolates were selected from a larger collection of 130 isolates from infected leaves, fruits and stems of tomato from various growing regions of North-West Algeria. Morphological characterization under standard conditions and polymerase chain reaction (PCR) analyses using specific primers to amplify a part of the ITS regions and the 5.8S gene were conducted to confirm their identification as members of the *alternata* section. They were then examined according to morphological characteristics of conidia and sporulation patterns on potato carrot agar (PCA) and were segregated into three morphological species: *A. alternata*, *A. tenuissima* and *A. arborescens.* Colony type, substrate colour, margin, zonation, pigmentation, colony diameter and conidia production were studied on potato sucrose agar (PSA). Physiological parameters and nutritional requirements of the isolates were also assessed and a data matrix based on cluster analysis and Euclidean distance was constructed. Results of pathogenicity test on tomato showed obvious diversity among the isolates and they could be separated into two groups based on their virulence. The dendrogram based on the influence of cultural, nutritional and physiological characters suggests moderate heterogeneity within the populations of *A. alternata* and *A. tenuissima*. The small-spore species formed five clusters that fundamentally paralleled the morphological groupings. However, the results provided no evidence for geographical and pathogenicity clustering of isolates.

Key words: Epidemiology, *Solanaceae*, small-spore *Alternaria* species, pathogenic variability, cultural characters, physiological diversity.

INTRODUCTION

Tomato *(Lycopersicon esculentum L)* often called as "queen of kitchen" is one of the oldest known and important vegetable crops grown in the world. It is commonly used for culinary purposes and consumed in

diverse ways, including raw, as an ingredient in many dishes, sauces, salads, and drinks. In Algeria, it represents a surface of 12173 Ha which covers 57.36% of the global cultivated area. Its production is ca 10 MQx of which 3.8 MQx is for industrial use (that is equivalent to 95% of the total industrial culture production) (Snoussi, 2009). Due to its high adaptability, early blight has the potential to become a serious threat for tomato crops in northwestern Algeria, because of the favorable environment; it affects foliage as well as tomato fruit reducing yield both qualitatively and quantitatively. Epidemics occur when the weather is warm and dry with short periods of high moisture and moderate temperatures (18 to 30°C). The fungal pathogens responsible for the disease belong to the genus *Alternaria* and infect the plant through conidia which are either wind-blown or splashed onto plant surfaces (Andersen and Frisvad, 2004; Leiminger et al., 2010). Both large-spore forming species (*A. solani* and *A. tomatophila*) and small-spore *Alternaria* spp. have been isolated from diseased tomato (Simmons, 2000).

The *Alternaria* genus includes nearly 275 species (Simmons, 2007) with saprophytic and plant-pathogenic lifestyles that may affect crops in the field or cause harvest and post-harvest decay of plant products (Logrieco et al., 2009). *Alternaria* taxonomy has long been mainly based on conidia morphology and sporulation pattern. Nishimura and Kohmoto (1983), using a statistical analysis of the size of conidia concluded that *Alternaria* isolates producing small spores belong to what they called a "collective species" *alternata*. From then, several authors (Kusaba and Tsuge, 1995; Johnson et al., 2000; Tsuge, 2003) have accepted these findings. To facilitate segregation and identification, Simmons and Roberts (1993) introduced the 3-dimensional sporulation pattern as a means of scoring small-spore isolates from Asian pears into groups. They described six major groups with characteristic sporulation patterns of which three will be treated in this paper. More recently based on phylogenetic studies, the *Alternaria* genus was separated into 24 sections and small-spore forming *Alternaria* were grouped into the *alternata* section that comprises almost 60 *Alternaria* species (Woudenberg et al., 2013; Lawrence et al., 2013). The molecular variation within the *alternata* section is low and these species were thus mainly differentiated based on phenotypic variation.

Phenotypic variations within fungal populations can generally be detected using morphological, cultural and pathogenic criteria. Understanding pathogen population structure and mechanisms by which such variations arise within a population is of paramount importance for devising a successful disease management strategy.

Most important environmental factors controlling the growth and sporulation of fungi are temperature, hydrogenion concentration and both carbon and nitrogen sources. A small variation in these factors may induce clear differences in their morphological characters, growth and sporulation (Bilgrami and Verma, 1978). Concerning pathogenic variability, it has been well established that *A. solani* and *A. tomatophila* could be considered as primary pathogens responsible for early blight of tomato (Simmons, 2000; Lourenço et al., 2009), while tomato isolates belonging to the *alternata* section have often been considered as successful invaders of preformed necrotic lesions with poor capability to infect green foliage in the field (Spits et al., 2005). Despite this, it is also well recognized that pathogenic populations (pathotypes) with narrow host range exist within the *alternata* section. *A. arborescens* responsible for the tomato stem canker constitutes a typical example (Grogan et al., 1975, Mesbah et al., 2000).

The objective of this study was to evaluate the importance of small–spore *Alternaria* species and to analyze the phenotypic variability within these populations isolated from tomato with early blight symptoms. We have therefore collected several isolates from diseased tomato in Algeria, analyzed their morphological characteristics based on growth and sporulation patterns and cultural behaviour on different media. We have also studied their virulence on tomato and showed that they can be divided into two groups based on their relative aggressiveness.

To the best of our knowledge, this constitutes the first report of leaf blight in solanaceous crops caused by small-spore *Alternaria* that is belonging to the *alternata* section, in the northwestern Algeria.

MATERIALS AND METHODS

Isolation

Samples of fresh infected leaf, stem and fruit of tomato were collected from 65 fields of commercial farmers located in the main vegetable producing cities in northwestern of Algeria including: Oran, Mascara, Mostaganem, Ain témouchent Relizane, Tlemecen and Sidi Bel-Abbès.

Identification of isolates

Isolation of *Alternaria* strains was done from small pieces of infected tissues after surface disinfection using 3% sodium hypochlorite (NaOCl) for 5 min. Tissue samples were then rinsed with sterile water and placed onto potato sucrose agar (PSA) medium and incubated at 25°C for 7 days. Cultures that were contaminated with bacteria were transferred on PSA amended with streptomycin (50 µg ml^{-1}). Pure cultures were obtained for each of the isolates using the single spore technique according to Hansen (1926).

Single spore colony was transferred to the same PSA medium. The

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Table 1. Scale for evaluation of the mean mycelia growth and conidial production of *Alternaria* isolates.

Mean mycelial growth (mm/day)	Conidial production (×10^5 spores.mm^{-2})	Rating
0 to 3.0	0 to 10	0
3.1 to 6.0	10.1 to 20.0	1
6.1 to ≤	20.1 to ≤	2

130 pure isolates were stored at 4°C on PSA slants for further study. Identification of small spore *Alternaria* was performed according to a previously established protocol (Simmons, 2007).

DNA isolation and molecular characterization

DNA isolation from the studied *Alternaria* isolates was carried out according to the microwave miniprep procedure described by Goodwin and Lee (1993). The PCR amplification was carried out in a Biorad thermocycler using the following scheme: initial denaturation at 95°C for 3 min, then 30 cycles consisting of 95°C for 30 s, 55°C for 50 sand 72°C for 1 min, and a final elongation at 72°C for 10 min. Amplifications were carried out using primers AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3') and AAR3 (5'-ATGGATGCTAGACCTTTGCTGAT-3') able to amplify a part of the ITS regions and the 5.8S gene specific to *A. alternata*-related species (Konstantinova et al., 2002). Amplification products were resolved on 1.2% agarose gels in 0.5×TAE buffer (20 mMTris-acetate pH 8, 0.5 mM EDTA) followed by ethidium bromide staining.

Cultural, nutritional and physiological characters of fungal strains

Thirty-two isolates were studied for their cultural and morphological variations on six culture media, potato carrot agar (PCA) (Simmons, 2007), malt extract agar (Messiaen et al., 1991), potato sucrose agar (PSA) (Samson et al., 2002), Czapek dox agar (Salam et al., 2006), Mathur (Mathur et al., 1950) and Sabouraud (Dongyou, 2010) selected for their ability to support *A. alternata* growth. Each figure was inoculated by transferring 5-mm mycelia disc from seven days old culture of each isolate and was incubated at 25±1°C for growth.The different colony characters like pigmentation, sporulation, and zonation were recorded in PSA medium by visual observation after 7 days. For morphological conidia chain examinations, the unsealed inoculated PCA figures were incubated under a daily natural light/dark cycle and maintained at a moderate temperature (22°C); cultures were observed after 5 days at 50× magnification with a stereomicroscope. Further examination was done at ×400 magnification using a compound microscope. Each spore suspension was mounted in lactophenol on a microscope slide and measured using a micrometer (septation and size). Thirty records per isolate were made for the purpose.

Effect of carbon and nitrogen sources

Czapekdox was used as a basal medium to study the effect of nutrients such as carbon and nitrogen compounds. Ten carbon sources were tested at 3% (v/v): sucrose, glucose, fructose, lactose, maltose, starch, cellulose, glycerol, mannitol and citric acid. Eight nitrogen sources were also tested at 0.2% (v/v): potassium nitrate, sodium nitrate, ammonium sulfate, asparagine, valine, leucine, arginine and peptone as described by Attrassi et al. (2007).

Effect of pH

To test the influence of pH on fungal growth, isolates were cultured in PSA initially adjusted to different pH ranging from 4.0 to 10.0 using dilute acid or alkali (Vijayalakshmi et al., 2012).

Effect of temperature

Petri dishes containing PSA medium were inoculated with 5-mm mycelial discs from ten-day-old culture of different isolates. The inoculated figures were incubated at different temperature: 5, 10, 15, 20, 25, 30 and 35°C (Hubballi et al., 2010).

Fungal growth and sporulation

Measurements on radial colony diameter were recorded 2^{th}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} day after inoculation. Growth rate per day was calculated by the formula (Sofi et al., 2012):

χ = *Growth at day N (mm) - Growth at day N-2 (mm)/2*

Sporulation was determined by harvesting the conidia from the surface of ten-day-old colonies by flooding each figure with 10 ml of sterilized distilled water and scratching the agar surface with the help of rubber spatula. The resulting suspension was filtered through muslin cloth and concentration of the spores was measured with the help of haemacytometer. Total number of spores on the colony and number of spores per unit area were calculated. In this study, all assays were replicated three times.

All the 32 isolates were categorized into groups depending on rates attributed on the mean mycelia growth and conidial production using the scale described by Attrassi et al. (2007) and modified as follow in Table 1.

Bioassays for pathogenicity of fungal isolates

In order to confirm the identification of the disease and its causal agent, the pathogenicity test was performed using a detached leaf technique as described by Reni et al. (2007). Detached healthy leaflets of tomato cultivar Saint Pierre from leaf N^o 5 were cleaned with sterile distilled water, dried and transferred to Petri dishes with moisten filter papers. Leaflets were inoculated by two different methods. For the first method, conidial suspensions of 7 days old culture grown on PSA medium were prepared as previously described. Spore density was adjusted using a heamacytometer technique to 10^5 cfu/ml (Brame and Flood, 1983). A 20µl drop of the suspension was then applied to the surface of the leaf. A control experiment was carried out simultaneously using sterile distilled water. For the second method, leaflets were inoculated with 5-mm plugs of 7-day-old cultures of the isolate. The inoculum was placed in the middle of each leaf. Leaves for control were mock inoculated with a plug of sterile PDA (Loladze et al., 2005; Park et al., 2008).

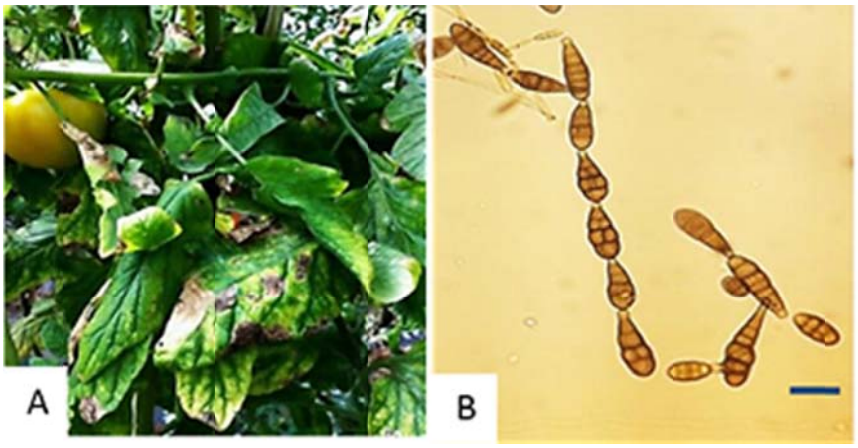

Figure 1. **A)** Leaves of tomato plants showing typical symptoms of early blight disease. **B)** Light micrographs of conidia of strains isolated from diseased tomato organs. Bars= 15 µm.

Table 2. Collected samples from northwestern Algeria regions from different fields of tomato production.

Zone	Season	Isolate	Sample
Oran	Spring and Summer	A26, A21	48
Mascara	Spring and Summer	A13, A15, A17, A18, A19, A20, A24	11
Mostaganem	Spring and Summer	A4, A6, A7, A8, A9, A10, A11, A16, A22, A23, A25, A27, A30	31
Ain témouchent	Summer	A1, A3, A12, A14, A28	10
Relizane	Summer	A31, A32	3
Telemcen	Summer	A2	11
SidiBel-Abbès	Summer	A5, A29	16

(20−23°C). All isolates were evaluated and each assay was replicated three times.

Disease assessment

The impact of the pathogens was determined by visual rating of disease symptoms 1 week after inoculation; the assessment was carried out by measuring the diseased area of the leaflets and compared to the control using a scale as follows: 0 = no symptoms, 1 = 0 to 30% of leaf area necrotic and chlorotic, 2 = 31 to 60% of leaf area necrotic and chlorotic, and 3 = 61 to 100% of leaf area necrotic and chlorotic (Van der Waals et al., 2004; Kheder et al., 2012).

Statistical analysis

Averages of colony diameter and number of conidia/ml for each treatment combination were evaluated for subsequent data analysis. Analysis of variance and separation of means to determine differences in growth rates and sporulation under different growth conditions was carried out. The data were statistically analyzed and the treatment means were compared. Agglomerative hierarchical clustering (Ward, 1963) of cultural, nutritional and physiological parameters were combined to construct a dendrogram using the STATISTICA 5.0 software. Euclidean distances were transformed according to the formula: 100 × distance between two points / maximal distance. This enabled the interpretation of results as 0–250%.

On the other hand, the analysis of the results from the virulence assays was performed on the basis of the means and standard deviation using nonhierarchical classification (Digby and Kempton, 1987). It is often found that nonhierarchical methods provide a more acceptable classification into fewer major groups. The groupings obtained are also more robust to any aberrant similarities between individual pairs of units (Digby and Kempton, 1987).

RESULTS AND DISCUSSION

Isolation of *Alternaria*

All isolates were collected from plants displaying similar symptoms characteristic of the early blight disease. They affected leaves, stems and fruits (Figure 1A).

Mycological analysis indicated that tomatoes in the seven main producer cities of vegetables in northwestern of Algeria were strongly invaded by small-spore *Alternaria* spp (section *alternata*). The cultural and morphological characters of 130 isolates were closely similar to those described by Simmons (2007) (Table 2). Conidiophores arising singly or in small groups produced spores in chains and conidiospores were large with longitudinal and transverse septa and a short beak typical for *A. alternata* and related species (Figure 1B). Their identification was further confirmed by molecular analysis using specific primers. As shown in Figure 2, the amplification of DNA from these small-spores isolates using the AAF2

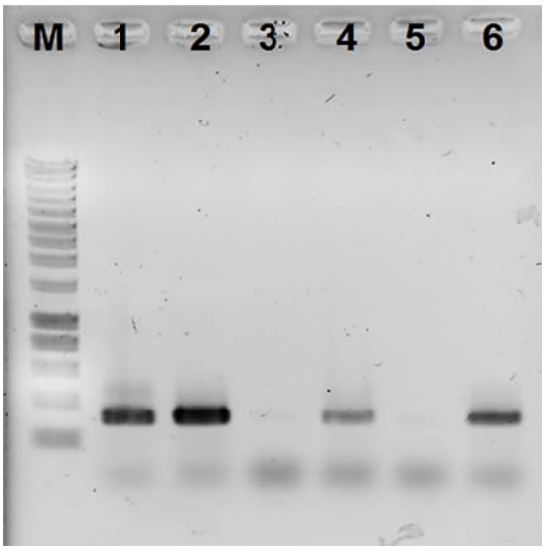

Figure 2. Sample gel showing amplification products from *Alternaria* genomic DNA using the AAR2/AAF3 primer pair. Template DNA was isolated from: *A. alternata* (lane 1), *A. solani* (lane 3), *A. tomatophila* (lane 5), *Alternaria* isolates from diseased tomato (lanes 2,4, and 6). Lane M, 100 bp molecular weight marker.

/AAR3 primer pair resulted in a specific 341-bp PCR product. At the same time, no amplification product was observed using the same primer pair and DNA from *A. tomatophila* and *A. solani*, two species often isolated from symptomatic tomato leaves, confirming the identity of the chosen isolates as members of the *alternata* section.

Cultural characteristics of the isolates on PSA medium

Thirty-two fungal isolates were randomly selected for further characterization of their colony morphology on potato sucrose agar (PSA). The results revealed a considerable variation in macroscopic characters of these isolates collected from different locations this morphological species were. Colony color varied from light to dark olivaceous with greenish or brownish tinge. Mostly, the colonies had velvety or cottony mycelia growth with slight variations and regular to irregular margin and without concentric zonation. All the isolates impregnated the media with a color mostly grey to brown with some variations that were clearly visible from the underside of figures. These observations are in agreement with Pusz (2009) who reported that the colonies of *A. alternata* isolated from *Amaranthus retroflexus* varied from light grey to dark grey. Similarly, Rai and Kumari (2009) observed loose, cottony, compact and dense colonies with light to dark black color for *A. alternata* infecting Periwinkle. The findings of Hubballi et al. (2010) showed variation in the pigmentation of 15 *A. alternata* isolates producing black, brownish black, greenish black, brown and yellow pigmentation.

Variation with respect to topography of the colony type was also observed. Two isolates (A21 and A26) had cottony sub-aerial mycelia growth while, 13 isolates (A4, A6, A7, A8, A9, A10, A11, A16, A22, A23, A25, A27 and A30) had medium cottony growth. Five isolates (A1, A3, A12, A14 and A28) had cottony central growth. Seven isolates (A13, A15, A17, A18, A19, A20 and A24) produced velvety, oppressed mycelium whereas, two isolates (A5 and A29) grown with velvety mycelium and furrowed margin. One isolate (A2) had velvety mycelium with oppressed margin.

All the isolates produced spores on PSA (Figure 5B). High sporulation was observed for one isolate (A12), while for most of the isolates moderate sporulation was recorded. Poor sporulation was observed only for one isolate (A7). Variation in growth rates on PSA was also observed (Figure 3). The fastest mean growth rate was obtained for isolate A31 (mean 14.90 ± 0.614 mm/day) and the slowest in the (A2) isolate (mean 8 ± 0.487 mm/day). Isolates in the present study depicted periodic changes in their growth rates. Four isolates grew very fast in the initial 2–4 days of observation (A12, A13, A16 and A17) but decreased afterwards while most of the isolates had the highest growth rate at 6 days and one isolate (A10) grew faster at the end of the experiment. Almost similar observations were performed by Pusz (2009) and Hubballi et al. (2010) when measuring growth rates of *A. alternata* and *A. mali* isolates, respectively.

Sporulation patterns of the isolates on PCA medium

All the 130 *Alternaria* isolates collected from plants in the family *Solanaceae* in northwestern Algeria were characterized for their sporulation patterns and conidial characteristics on potato carrot agar (PCA) at 7 days. All isolates could be grouped into three types that corresponded to type 3, 4 and 5 sporulation patterns as defined by Simmons and Roberts (1993). Figure 4 shows the morphological characteristics of conidia and sporulation patterns of representative isolates of each group. Type 4 sporulation pattern (Figure 4A) appeared as low bushy clumps of well-branched chains. Conidia varied from obpyriform to ovate to obclavate, yellowish-brown to brown, with 1-8 transverse and 0-3 longitudinal or oblique septa. Spore body size was 6.5-59.8 × 4.2-16.5µm. This typical *A. alternata* accounted for 33. 85% and the selected isolates from this morphological species were A1, A2, A10, A11, A12, A13, A15, A16, A19, A21, A24, A26, A28, A30 and A32. Type 5 sporulation pattern (Figure 4B) corresponded to moderately long to long chains of more than 9 conidia, branching of chains usually was minor (1 to 2 conidia) or lacking. Conidia produced were obclavate or ellipsoidal, brown to golden brown, some conidia with minutely verrucose walls. Mature conidia with 4-9 transverse septa and 0-4 longitudinal or oblique septa were

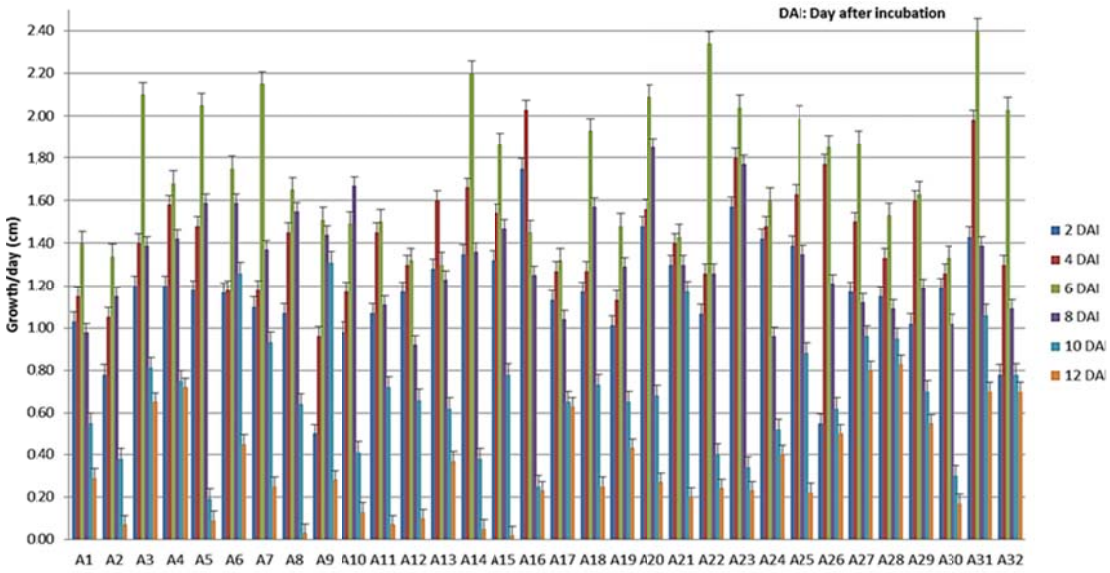

Figure 3. Variability in the growth rates of *Alternaria* isolates on PSA medium.

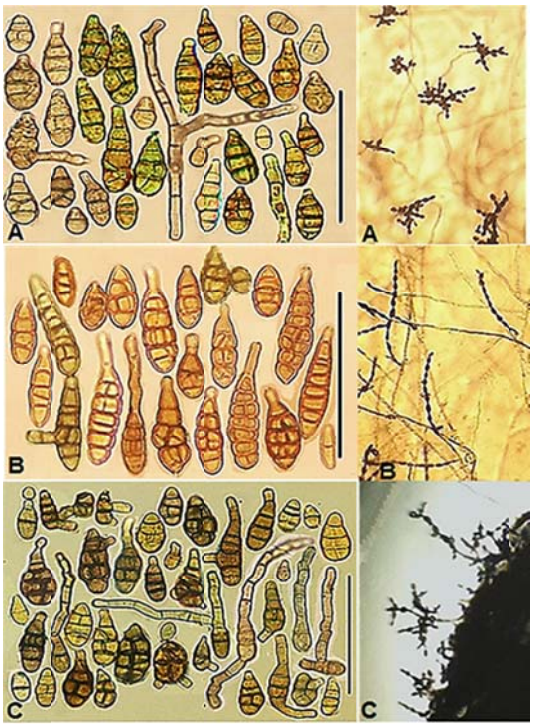

Figure 4. (A) *Alternaria alternata* conidia and sporulation pattern (PCA 7d) from isolate A15. **(B)** *Alternaria tenuissima* conidia and sporulation pattern (PCA 7d) from isolate A8. **(C)** *Alternaria arborescens* conidia and sporulation pattern (PCA 7d) from isolate A23. Bars=50 µm.

observed. Conidium body size was 9.8-60.20 × 8.6-15.5 µm. This typical *A. tenuissima* accounted for 64.61% and the chosen isolates were A3, A4, A5, A6, A7, A8, A9, A14, A17, A18, A20, A22, A25, A27, A29 and A31. Type 3 sporulation pattern (Figure 4C) appeared as conidial chains of 2-6 units long and typically produce branches

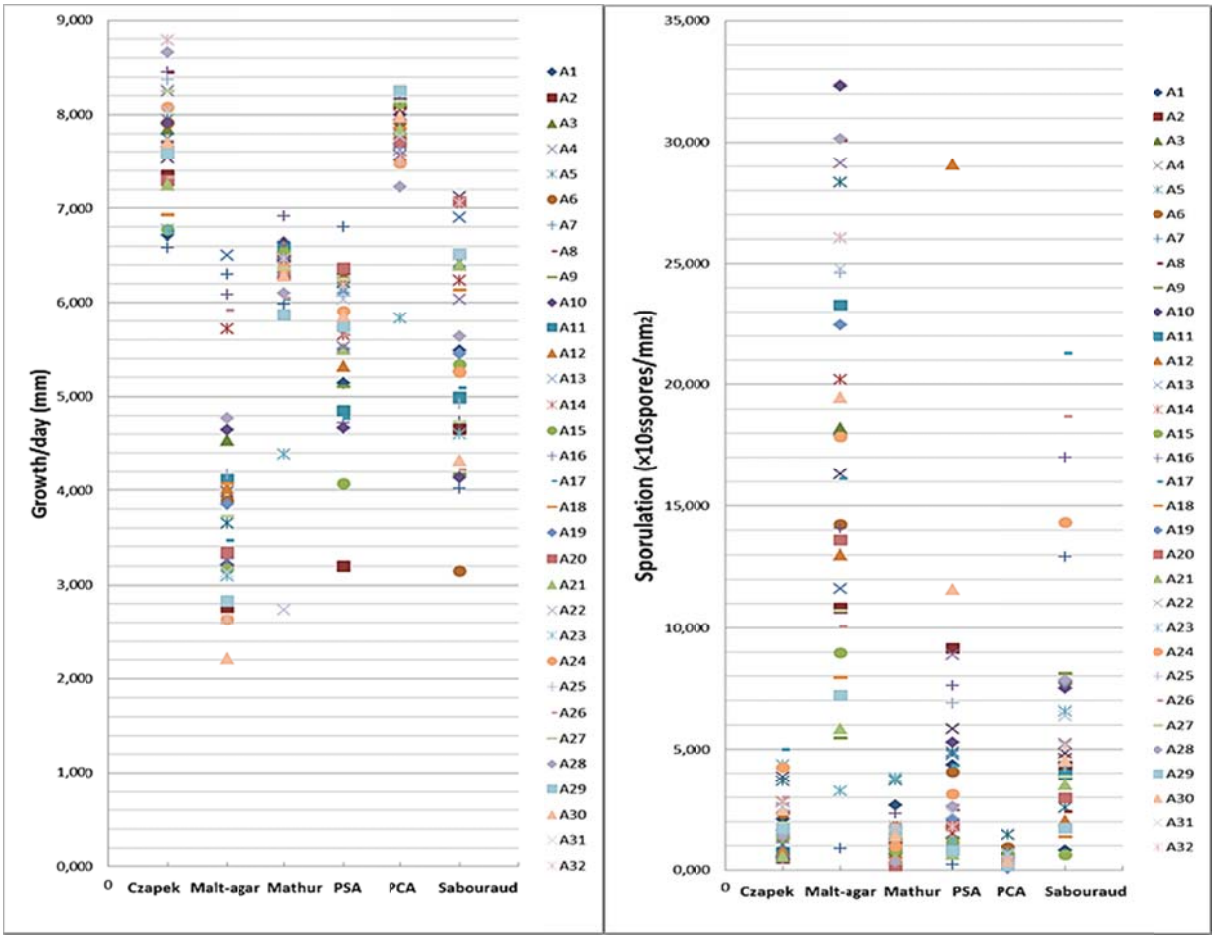

Figure 5. Variability in the growth (A) and sporulation (B) rates of small- spore *Alternaria* isolates on different medium.

(1 to 5 conidia) having a long defined primary conidiophore with few terminal and sub terminal branches. Conidia were ovate, obpyriform to ellipsoidal, mature conidia had 1-6 transverse septa and 0-3 longitudinal or oblique septa; spore body measured 9.6-38.4 × 3.2-12.8 µm. This group accounted for only 1.53% of the isolates and A23 was chosen for further studies. To compare, Xia and Tian-Yu (2008) reported that the conidia of 27 *A. alternata* isolates varied from 16.5-56.5 × 6.5-14.5 µm and conidia size variability among 53 isolates of *A. tenuissima* was 22.5-42 × 8.5-12.5 µm. Although morphological characteristics of conidia and conidiophores have often provided the major taxonomic criteria for delimitation of fungal species, these characters may be strongly affected by environmental factors. Thus in quantifying size dimensions, number of transverse or longitudinal septa, isolates often fit a number of different species and accurate assignment of small-spore *Alternaria* to one of the described species is often difficult based on this sole criteria. By contrast, 3D-sporulation patterns provide a much robust morphological criteria for the delineation of *Alternaria* species within the *alternata* section.

Response of the isolates to different culture conditions

Base medium

Growth and sporulation of the 32 selected isolates was first tested on different culture media. Significant vegetative growth was observed on all tested media (Figure 5). The relatively high growth rates recorded irrespective of the medium indicate that small-spore *Alternaria* isolates have the ability to utilize a wide range of carbon sources and other nutrients. For all the 32 isolates, Czapek Dox agar supported the maximum growth (mean: 7.667±0.591 mm/day) but poor sporulation (2.166±0.98210^5 spores /mm^2) maybe due to the presence of chloride ion. Growth rates on PCA medium were almost similar (mean: 7.763±0.425 mm/day) with the minimum fungal sporulation (mean: 0.450± 0.207 10^5 spores/mm^2). This medium, which is rich in nutrients, may probably favor mycelia growth with ultimate loss of sporulation (Nasraoui, 2006). By contrast, the slower mycelia growth rate was measured on malt extract agar (mean: 3.902±1.141 mm/day) which supported the maximum sporulation

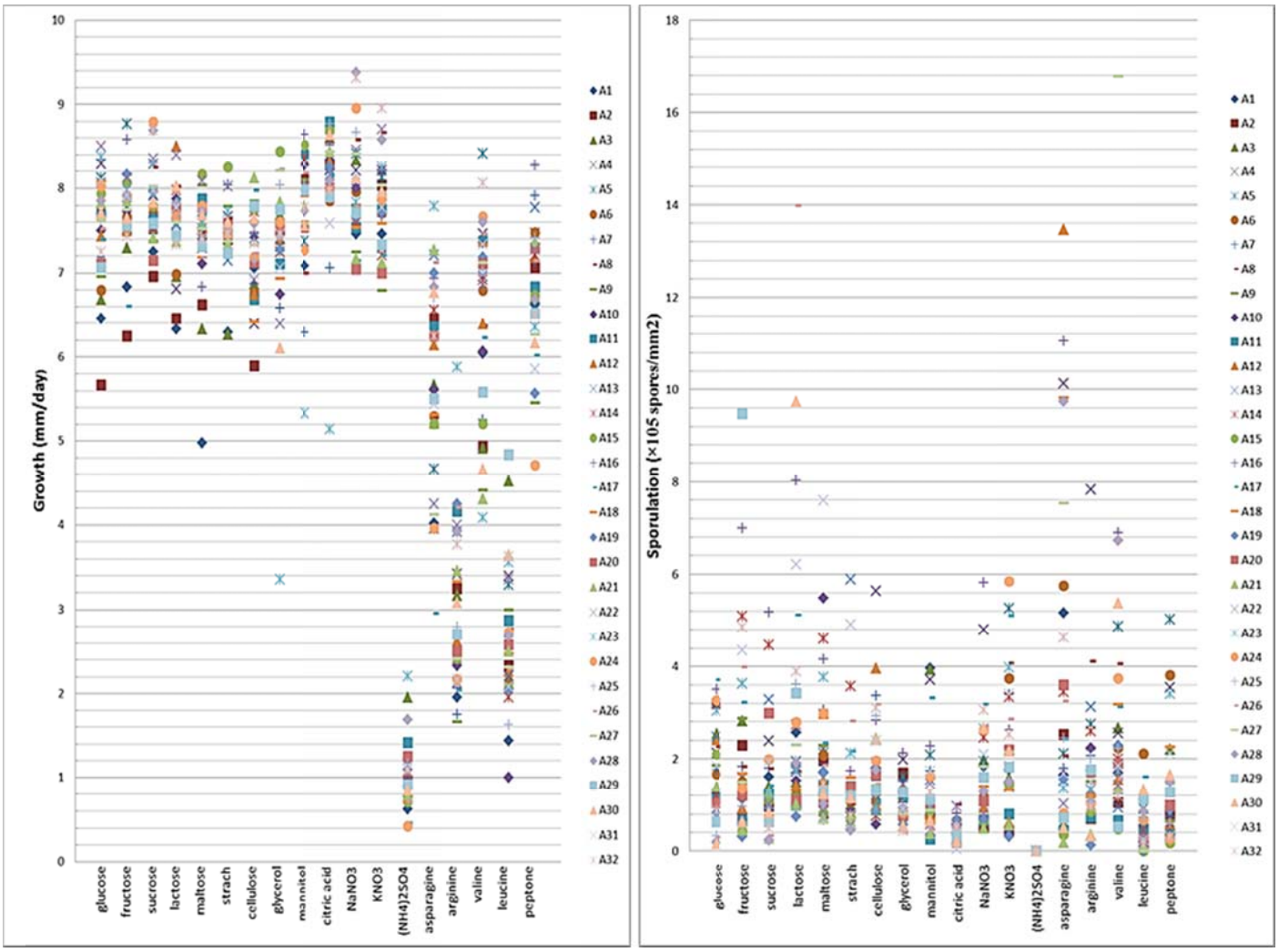

Figure 6. Variability in the growth (A) and sporulation (B) rates of small –spore *Alternaria* isolates on different carbon and nitrogen sources.

(mean: 16.721±6.915 10^5 spores/mm^2), albeit with large variation between isolates. Poor sporulation (mean: 1.334 ±0.581 10^5 spores/mm^2) and moderate mycelia growth (mean: 6.212±1.072 mm/day) was observed on Mathur medium. Sabouraud medium and PSA both supported moderate mycelia growth (mean: 5.501±1.072 mm/day and 5.639±0.766 mm/day, respectively) and sporulation (6.365±3.618 10^5 spores/mm2 and 4.348 ± 3.06410^5 spores/mm^2, respectively), and were the preferred media for culturing small-spore *Alternaria*. The influence of media base revealed a negative correlation between growth and sporulation of the isolates. The composition of the culture media thus constitutes an important physiological parameter that significantly affects the mycelia growth rate and conidial production of the isolates.

Carbon and nitrogen sources

Effects of ten carbon sources and eight nitrogen sources (Figure 6) were then tested using Czapek dox as basal medium. All the ten carbon sources were found suitable to sustain mycelia growth. Although the type of carbon source had low effect on the recorded growth rates, sporulation was much more affected by this parameter and varied from 0.431±0.215 10^5 spores/mm^2 on citric acid to 2.89±2.6910^5 spores/mm^2 on lactose. Contrarily to what was recorded for carbon sources, a stronger effect of the nitrogen source on growth parameters was observed. The mycelia growth of the isolates on different nitrogen sources was found to be highest on two inorganic nitrogen sources, sodium nitrate (7.977±0.584 mm/day) and potassium nitrate (7.827±0.503 mm/day) with moderate (1.778±1.242 spores/mm^2 and 2.297 ± 1.462 10^5 spores/mm^2, respectively) sporulation. The lowest fugal growth was recorded on ammonium sulfate containing medium (1.054±0.386 mm/day) with no sporulation. These observations are in agreement with those of Attrassi et al. (2007) studying *A. alternata*

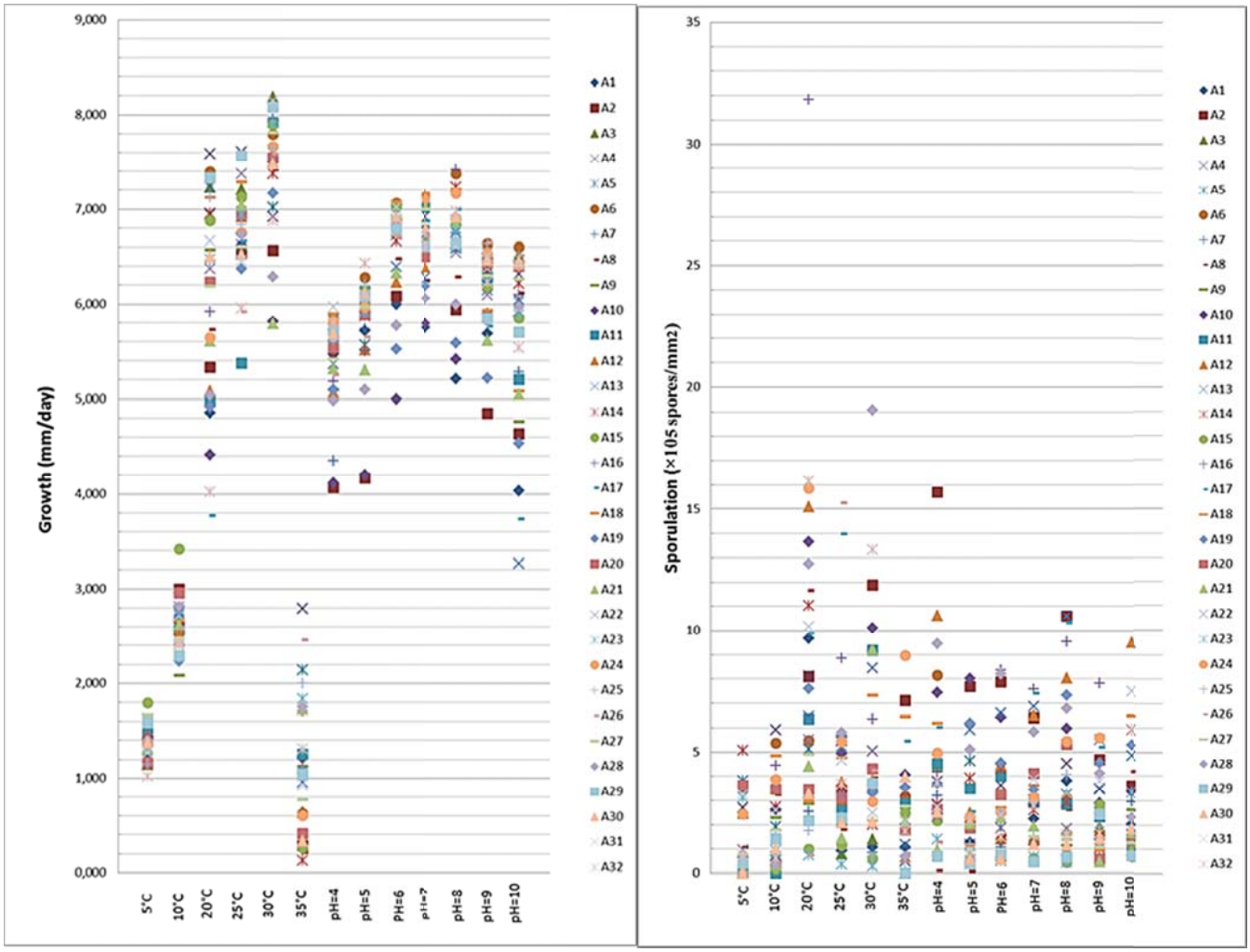

Figure 7. Variability in the growth (A) and sporulation (B) rates of small-spore *Alternaria isolates* on different pH and temperatures.

isolated from pears and Snapbeans, respectively, while Ramjegathesh and Ebenezar (2012) found that ammonium sulfate was the optimum nitrogen source of *A. alternata* isolated from leaf onion. While many fungi assimilate well the inorganic nitrogen sources like nitrates, they can also use a wide range of amino-acids which evoke different fungal responses and usually fungi grow well on asparagine unlike on leucine which supports low fungi growth (Nasraoui, 2006; Brzonkalik et al., 2011). In line with this, our results show that growth and sporulation were very poor on leucine and arginine as organic nitrogen sources while valine and asparagine yielded moderate growth (6.453±1.181 and 5.741±1.184 mm/day, respectively) and sporulation (2.862±3.019 and 3.552 ± 3.64810^5 spores/mm^2, respectively).

pH and temperature

The impact of the medium pH on mycelial growth and sporulation (Figure 7) of the 32 isolates was studied in the range of pH 4 - 10. In accordance with previous observations showing that generally fungi grew best at neutral or slightly acidic pH (Madan and Thind, 1998), the optimum pH for growth of our isolates was between 6 and 8 (ca. 6.6 mm/day). The pH values below 6 and above 9 led to a decreased mycelia growth and minimum growth rate was recorded at pH 10 (5.678±0.883 mm/day). Similar trend has already been reported by Ramjegathesh and Ebenezar (2012) and alkaline media are generally not recognized as favorable for fungal growth and sporulation. By contrast, pH 4 allowed the maximum mean of sporulation (4.058±3.309 10^5 spores/mm^2) followed by pH 8 (3.918±3.190 10^5 spores/mm^2). To compare, optimal pH values for sporu-lation varying between 4.8 and 6.3 have been reported for different *Alternaria* species (Mathur and Sarboy, 1977; Gemawat and Ghosh, 1980). In the present study, a single peak was recorded for optimum pH value for mycelia growth, which agrees with the behavior of fungi studied by Mehrotra (1964). However, two peaks were observed according to the mean sporulation of the isolates as already reported by Mathur et al. (1950) for *C. lindemuthianum*.

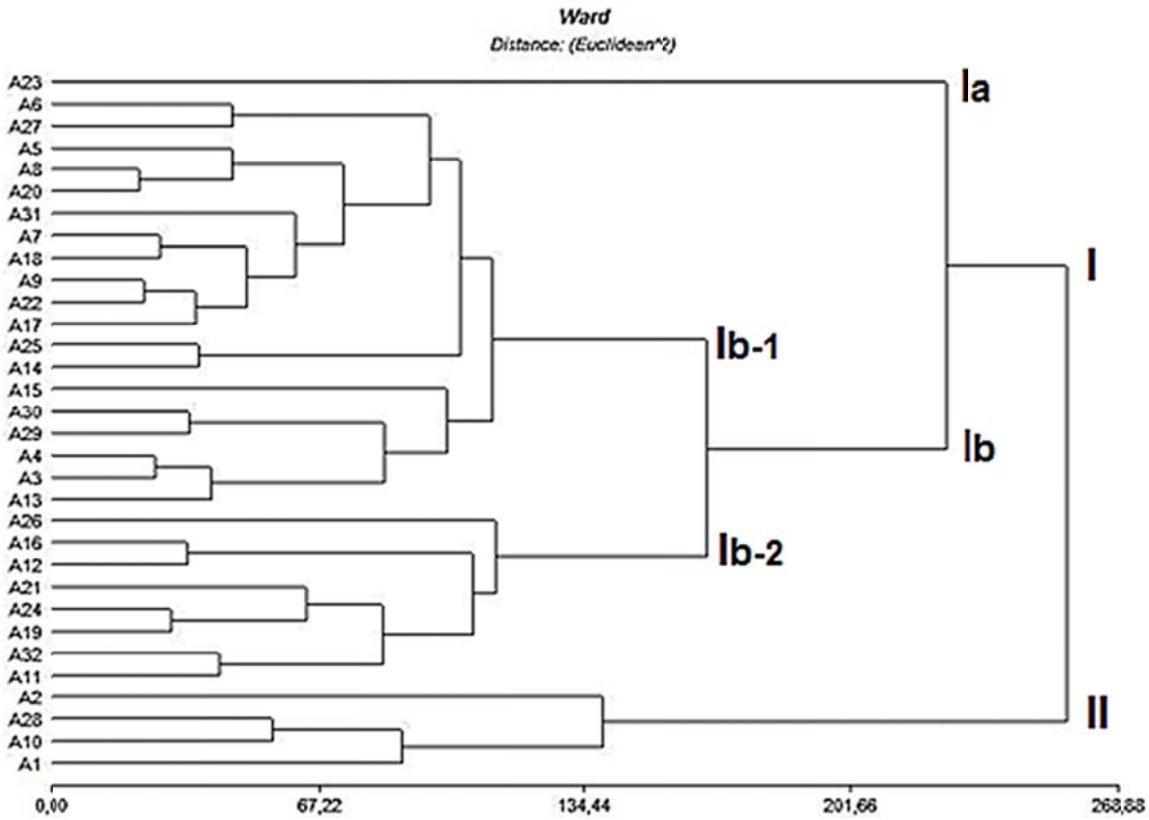

Figure 8. Dendrogram presenting the cluster analysis of morphological, cultural and physiological characteristics of 32 small-spored*Alternaria* isolates.

Temperature is often considered as the most important physical environmental factor for regulating the growth and reproduction of fungi. All the 32 isolates grew well at a temperature of 30°C (7.434±0.612 mm/day) followed by 25°C (6.751±0.457 mm/day). The lowest growth was observed at 35°C (1.108±0.678 mm/day). From this study, it is clear that temperatures ranging from 25 - 30°C are optimal for the growth of small-spore *Alternaria*. These results are in agreement with those of Hubballi et al. (2010), reporting that 27°C was the optimum temperature for the growth of *A. alternata*. The most suitable temperature for sporulation was 20°C (7.648±6.358 10^5 spores/mm^2) followed by 30 and 25°C. Poor sporulation was observed at 5°C (1.381±1.54610^5 spores/mm^2).

Global analysis of the cultural variability of small-spore *Alternaria* isolates from tomato

Combined results of growth rates at different temperatures, colony morphology, and metabolite profiles were found to be useful in characterization and differentiation of small-spore *Alternaria* spp. when standardized conditions were applied and representative isolates employed for comparison (Andersen et al., 2005). We therefore performed a global analysis of the above-described phenotypical traits from our selected isolates collection. The matrix shown on Figure 8 consisted of 32 isolates and 65 characters. All the isolates were categorized into two main clusters (I and II). Cluster I contained one isolate of *A. arborescens* and 27 isolates of *A. tenuissima* and *A. alternata* species group respectively, cluster II accommo-dated only four isolates of *A. alternata* group. Cluster I was further subdivided into two groups accommo-dating the *A. arborescens* isolate A23 (Ia) and 16 A. tenuissima isolates as well as 11 isolates of *A. alternata species* (Ib), respectively. Similarly, cluster Ib was further subdivided into two subgroups. The Ib-1 subgroup accommodated the 19 isolates from our collection that had a typical A. *tenuissima* sporulation pattern and three isolates thathad the typical *A. alternata sporulation* pattern. The Ib-2 subgroup consisted of eight isolates that had the typical *A. alternata* sporulation pattern. Several workers have already reported cultural, morphological and pathogenic variability among isolates of small spore *Alternaria* spp. (Slavov et al., 2004; Tetarwal et al., 2008; Sofi et al., 2012) and Rotem (1994) claimed that the variation on the cultural characteristics (color, growth, and sporulation) allowed to identify almost as many races as the number of isolates tested. The observed diversity for the phenotypic characters within the isolates from tomato and assigned to the *A. alternata* and *A. tenuissima* morphological species might reflect various environmental conditions in regions

Figure 9. Tomato leaf blight disease development on detached leaves pathogenicity tests on *Lycopersicon esculentum L*; leaf inoculated with sterile growth medium and sterile distilled water (far left) remained free of symptoms 1 week after inoculation. Leaflets inoculated with mycelia plug (A) leaflets inoculated with conidial suspension (B).

where these isolates have been collected. However, no geographical clustering was obtained; all groups and sub groups contained isolates from different districts having almost similar agro-climatic conditions.

Pathogenicity variability among small-spore *Alternaria* isolates from tomato

In order to test whether the cultural characteristics of the 32 selected isolates could reflect differences in virulence, pathogenicity tests were performed by inoculating detached leaves either with conidial suspensions or with mycelial plugs. Similar results were obtained for all isolates with both methods. The small-spore *Alternaria* isolates differed in their ability to produce lesions from the point of inoculation on leaflets. Fifteen isolates were weakly pathogenic comprising eight isolates of the *A. alternata* species and seven for the *A. tenuissima species*. For these isolates, we observed that spores germinated profusely on the surface of tomato leaflets inoculated with conidial suspensions, but failed to cause disease. However, 17 of the isolates tested exhibited relatively strong ability to produce infection on leaflets of which seven were of the *A. alternata* species, nine from the *A. tenuissima* species and the *A. arborescens* isolate A23. Nonhierarchical grouping of results from virulence assays on detached leaves showed that isolates could be divided into two main groups (Table 3). Isolates in group 1 had average virulence ratings of either 0 or 1, while those in group 2 had average ratings of either 2 or 3. The highest incidence of disease on leaflets inoculated with conidial suspensions was observed in A22 isolate (86.51±5.60%) followed by A5 (76.90±3.94 %) while isolate A23 recorded 56.73 ±12.20%. The lowest disease incidence was noted with the A16 isolate (11.81±3.86 %). Disease incidence in leaflets inoculated with mycelia plug shows almost same rates with high ability to produce lesions for A22 (90.18±6.44 %), A5 (84.23±6.00 %) and A23 isolate (75.59±15.67%) and the lowest incidence (13.33±4.30%) for A16 isolate as it is represented in Figure 9.

The pathogenicity assays in this study showed a high degree of variation in virulence of the different isolates. Pathogen entry into the tissues may be an active process but opportunistic infections may also occur as a result of sunburns, or cracking of leaves by the wind. Given the abundance of aerial spores, both active and opportunistic infections could result in lesions being composed of genetic mixtures. Two types of small-spore *Alternaria* isolates have previously been isolated from diseased tomato: the saprophytic form, for which all *Lycopersicon esculentum* cultivars are resistant, but develops symptoms only on ripe tomato fruits (blackmold) (Cassol and St. Clair, 1994) and the pathotype *A. alternata* f. sp. *lycopersici*, also reported as *A. arborescens* (Simmons, 1999), which induces lethal dark brown cankers on stems, leaf necrosis and wilting in susceptible tomato cultivars by the action of its host-specific AAL-toxins (Grogan et al., 1975; Mesbah et al., 2000). Several *A.*

Table 3. Nonhierarchical grouping of *Alternaria* isolates from northwestern Algeria into two groups based on virulence on detached leaves of tomato.

Zone	Isolate	
	Group 1	**Group 2**
AinTemouchent	A1, A12	A3, A14, A28
Mascara	A13, A17, A18, A19	A15, A20, A24
Mostaganem	A4, A6, A8, A9, A16, A30	A7, A10, A11, A22, A23, A25, A27
Oran	A26	A21
Relizane	A31	A32
SidiBelabbes		A29, A5
Tlemcen	A2	

Group 1 is least virulent (average ratings of either 0 or 1) and group 2 is most virulent (average ratings of either 2 or 3).

alternata pathotypes are known as host specific-toxins (HST) producers, which are essential virulence factors and determine their host range (Kohmoto et al., 1995). Although we have not investigated the production of HST in this study, AAL-toxin might be synthesized by isolate A23. However, further studies are needed to investigate the other isolates from our collection of small spore *Alternaria* based on their secondary metabolites patterns to check whether they might also be considered as potential HST-producers.

Conclusion

Small-spore *Alternaria* have consistently been isolated from tomato organs showing symptoms of early blight in the northwestern region of Algeria. Based on their sporulation patterns, among the 32 studied isolates, only one corresponded to the tomato pathogenic *A. arborescens* species while all the other isolates were assigned to the *A. tenuissima and A. alternata* species. Members of the latter groups have often been considered as fungi with saprophytic or opportunistic lifestyles. Despite this, pathogenicity assays conducted on detached tomato leaves showed that some of the studied isolates, either from the *A. tenuissima* or the *A. alternata* species, were highly virulent resulting in almost complete browning of inoculated leaves in one week. Diversity within the *A. alternata* species, and to a lesser extent within the *A. tenuissima* species was also evidenced from their morphological characteristics and cultural behavior. Agglomerative hierarchical clustering of such parameters provided no correlation with virulence nor with geographic origin of the isolates, although the resulting dendrogram was coherent with the species grouping and confirmed that the identified morphotypes corresponded to different species.

Besides isolates belonging to the *alternata* section, *Alternaria* species belonging to the *porri* section (*A. solani* and *A. tomatophila*) are often considered as major cause for early blight on tomato. Future studies will therefore investigate the occurrence of this *Alternaria* lineage in northwestern Algeria and their possible interactions with isolates of the *alternata* section.

Conflict of Interests

The authors have not declared any conflict of interests.

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