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Comparative fitness of *Alternaria* species causing leaf blotch and fruit spot of apple in Australia

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Abstract The reason for the high prevalence of the *Alternaria arborescens* species group compared to other species groups associated with leaf blotch of apple in Australia is not well understood. In order to determine if *A. arborescens* has a biological fitness advantage over the other species groups, this study compared the mycelial growth rate, fecundity and competitive spore production attributes of three isolates of each of four *Alternaria* species groups and examined the relationship between saprophytic and pathogenic fitness traits. Overall, this study revealed that the fitness attributes of the *Alternaria* isolates are significantly different among and within each of the species groups and suggests a strong relationship exists between high aggressive isolates and fast mycelial growth rate. A possible trade-off between fecundity and mycelial growth rate and contribution of mycelial growth rate in host invasion processes and factors that contribute to prevalence of the *Alternaria* species groups associated with leaf blotch and fruit spot of apple in Australia are discussed.

Keywords Apple · *Alternaria* · Leaf blotch · Fruit spot · Fitness

Introduction

Alternaria mali (syn. *A. alternata* apple pathotype) causes leaf blotch and fruit spot of apple (*Malus x domestica* Borkh.) worldwide (Bulajic et al. 1996; Filajdic and Sutton 1991; Gagkaeva and Levitin 2000; Li et al. 2013; Ozgonen and Karaca 2006; Sawamura 1972). In Italy (Rotondo et al. 2012) and Australia (Harteveld et al. 2013), Alternaria leaf blotch and fruit spot were associated with multiple *Alternaria* species groups. In Australia, these included the *A. arborescens, A. tenuissima/ A. mali, A. alternata/ A. tenuissima* intermediate and *A. longipes* species groups (Harteveld et al. 2013). In this study the *A. tenuissima* refers to the *A. tenuissima/ A. mali* species group and *A. alternata* refers to the *A. alternata/ A. tenuissima* intermediate species group as described by (Harteveld et al. 2013). Among the four Australian *Alternaria* species groups, *A. arborescens* is the dominant species obtained from leaf blotch symptoms, whereas, the *A. tenuissima* and *A. alternata* species groups are more associated with fruit spot in Queensland and New South Wales (Harteveld et al. 2013). All species groups were obtained from leaf symptoms, but it is unclear why *A. arborescens* was more commonly isolated from the apple leaves.

Biological fitness of plant pathogens is described as the relative ability to persist in an environment for an extended period of time (Nelson 1979) and constitutes pathogenic and saprophytic fitness. These combined attributes lead to increased reproduction and survival through mycelial growth and mitotic or meiotic sporulation, infection efficiency, pathogenicity, and aggressiveness of fungal plant pathogens (Leach et al. 2001; Pringle and Taylor 2002). Saprophytic fitness attributes such as mycelial growth and fecundity were used to compare fitness of *Alternaria* isolates (Karaoglanidis et al. 2011). These fitness characters have not been investigated or used to distinguish Australian *Alternaria* species groups affecting apple. It is unknown if the distribution and prevalence of the species groups on apple can be attributed to their saprophytic fitness attributes.

This study tests the hypothesis that *A. arborescens* has a competitive advantage over the *A. longipes, A. alternata* and

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A. tenuissima species groups based on saprophytic fitness attributes. Specifically, this study aimed to compare the saprophytic fitness attributes of the *Alternaria* species groups as a way to examine the reason for the preferred prevalence of the species groups in Australia. In addition, this study aimed to determine the relationship between pathogenic fitness and saprophytic fitness attributes of the four *Alternaria* species groups associated with Alternaria leaf blotch and fruit spot in Australia. A better understanding of the biological fitness of Australian *Alternaria* species groups affecting apple leaves and fruits will aid in the estimation of the prevalence of each species and assist in the development of targeted disease control strategies.

Materials and methods

Sources of isolates

A total of 12 isolates, consisting of three isolates of each species group *A. arborescens*, *A. tenuissima*, *A. alternata* and *A. longipes*, were obtained from apple in Australia (Table 1) (Harteveld et al. 2013; Horlock 2006). The isolates were recovered from storage at −80 °C in 15 % glycerol solutions. Cultures were maintained on ½-strength PDA plates as described in Harteveld et al. (2013).

Mycelial growth rate and fecundity of *Alternaria* species groups

Mycelial growth rate of each isolate was determined using 5 mm diameter mycelial plugs taken from the margin of 2-week-old cultures. Mycelial plugs were placed on 90 mm diameter ½-strength PDA plates in three replicates and incubated at 25 °C±1 °C in alternating 12 h/12 h light/dark cycle for 7 days. Colony diameter was measured daily for 7 days, thereafter, the growth rate was calculated using the formula: $[\sum(d_{i+t}-d_i)/t]/n$; where di=colony diameter (mm) at ith day, $di+t$=colony diameter at $i+i$th day, t=days after incubation, and n=the number of observations. In order to measure the fecundity of each isolate, after 7 days of incubation, each plate was flooded with 4 mL of sterile distilled water and the conidia were obtained by scraping the colony with a sterile scalpel blade to dislodge the conidia. The spore concentration from each plate was determined in triplicates using a haemocytometer. The experiment was repeated once.

Competitive spore production of *Alternaria* isolates

In order to determine competitive fitness of spore production between isolates of different species groups, conidia suspensions of two isolates of different species groups were mixed. Each isolate per species was mixed in three combinations with different isolates, for a total of 18 combinations. Mixed conidia suspensions were prepared by diluting conidia suspensions to the same concentration and mixing appropriate volumes to produce final conidia suspensions containing 1:1 ratios of the two isolates. All suspensions had a final volume of 2 mL and contained a final concentration of 10^5 conidia/mL. PDA plates were inoculated with mixed conidia suspensions and incubated at 25 °C in alternating 12 h/12 h light/dark cycle. After 7 days of incubation, a conidia suspension was obtained from the resultant colony and the single spore procedure as described by Harteveld et al. (2013) was used to

Table 1 Isolates used in the study of four *Alternaria* species groups of different aggressiveness obtained from leaves and fruit of apple in Australia

Identifier	*Alternaria* species group[a]	Accession number[b]	Substrate	Location[c]	Aggressiveness
arb-1	*A. arborescens*	46349	Fruit	NSW	Unknown
arb-2	*A. arborescens*	46567	Leaf	Qld	Unknown
arb-3[d]	*A. arborescens*	48600	Leaf	Qld	Moderate
ten-1[d]	*A. tenuissima*	46361	Leaf	NSW	High
ten-2[d]	*A. tenuissima*	46395	Leaf	NSW	Moderate
ten-3[d]	*A. tenuissima*	48081	Fruit	Qld	Low
alt-1	*A. alternata*	46390	Leaf	NSW	Unknown
alt-2[d]	*A. alternata*	46545	Leaf	Qld	High
alt-3[d]	*A. alternata*	46550	Fruit	Qld	High
lon-1[d]	*A. longipes*	46356	Fruit	Qld	High
lon-2[d]	*A. longipes*	46455	Leaf	WA	Moderate
lon-3[d]	*A. longipes*	47966	Leaf	Qld	Low

[a] *Alternaria* species group of the isolates (Harteveld et al. 2013)

[b] Accession numbers represent the BRIP codes of the isolates as coded by the Queensland Plant Pathology Herbarium, Brisbane, Australia

[c] Australian states: New South Wales (NSW), Queensland (Qld) and Western Australia (WA)

[d] Isolates included in multivariate analysis of saprophytic and pathogenic fitness traits

obtain a total of 10 randomly picked germinating spores. Each spore was transferred to a fresh ½-strength PDA plate. The plates were incubated at 25 °C in the dark for 7 days, thereafter, the plates were grouped into different species based on cultural and morphological characteristics and the identity of isolates of selected plates in each species group was confirmed using DNA sequencing of the endoPG and Alta1 regions as described by Harteveld et al. (2013) and aligned with the sequences of the used isolates for identification. The frequency of each species group obtained from the single spore isolation of the mixed cultures was recorded. Reproducibility of the results was examined in a repeated experiment using a subset of isolate combinations.

Relationship between saprophytic and pathogenic fitness traits

Any significant association between the saprophytic fitness traits measured as in vitro mycelial growth rate and fecundity and pathogenic fitness trait measured as aggressiveness levels was examined using nine *Alternaria* isolates (Table 1). Aggressiveness of the isolates, as the leaf area with leaf blotch was determined in detached leaf assays. Each isolate was grown on PDA as described above and spore suspensions were obtained by flooding the culture plates with sterile water and filtered through cheesecloth to remove mycelia. The spore suspension was adjusted to 10^5 spores/ml for the inoculations. The first five fully expanded leaves of young potted plants of the cultivar Royal Gala were detached, rinsed with deionised water and air-dried. One 0.5 cm^2 square sterile filter paper disk was soaked in spore suspension and placed on the abaxial side of each leaf, on one side of the midrib of the leaf blade. A control inoculation was performed by soaking the filter paper in sterile water. The inoculated leaf was placed in a plastic seal bag with wet cotton wool to provide continuous high relative humidity (>90 %) and incubated at 25 °C in darkness. After 5 days, leaf blotch development on the leaves was recorded. Aggressiveness was scored as low, moderate and high, covering 0–25, 25–50 % and 50–100 % of the leaf blade, respectively. The experiment was repeated once.

Data analysis

Statistical analyses were performed using GenStat (14th Edition, VSN International, Hertfordshire, UK). Variations in mycelial growth rate, fecundity and aggressiveness among the *Alternaria* species groups, the isolates and their interactions were analyzed using analysis of variance. The isolate was nested within the species group. Fecundity data was logarithm (log_{10}) transformed to stabilize variance. Reproducibility between experiments was confirmed with no significant difference between experiments. The mycelial growth rate and fecundity were expressed as the mean of three replicates for each isolate.

The relationships between the saprophytic traits: fecundity (spores/cm^2) and the mycelial growth rate (mm/day) and the pathogenic trait (aggressiveness) of the *Alternaria* isolates was explored using principal component analysis procedure in XLSTAT software version 2013.4 (Addinsoft, Paris, France). Aggressiveness levels were coded as values of 1, 2 and 3 for low, moderate and high aggressiveness of the nine *Alternaria* isolates, respectively. XLSTAT was used to calculate the Pearson's correlation coefficients for the associations between the fitness traits, the eigenvectors of the components and to compute a correlation biplot of the observations and variables using the two most significant components.

Results

Mycelial growth rate and fecundity of *Alternaria* species groups

There was no significant difference between the repeated experiments of the mycelial growth rate and fecundity. Irrespective of the species groups, there was significant ($P < 0.001$) variation among the *Alternaria* isolates within each species group in both saprophytic fitness traits, whereas, the traits did not vary significantly among the species groups. The mean and range of mycelial growth rate of *A. arborescens* isolates were the lowest at 6.4 mm/day (range 2.0–8.3 mm/day) compared to isolates of *A. alternata* with mean, 8.2 mm/day (range 5.0–10.5 mm/day) and *A. tenuissima*, with mean 8.4 mm/day (7.4–9.3 mm/day), whereas, the mean 8.4 mm/day was highest in *A. longipes* with range 6.2–10.3 mm/day (data not shown). Among the isolates, *A. longipes* isolate lon-1 and *A. alternata* isolate alt-3 were the fastest growing isolates, with an average growth rate of 9.3 mm/day. *A. arborescens* isolate arb-3 was the slowest growing isolate with an average growth rate of 4.1 mm/day (Fig. 1a).

Fecundity of the isolates within and between the species groups was significantly different ($P<0.001$) (Fig. 1b). Fecundity of the isolates within the *A. arborescens* species group varied from 0.3×10^3 to 7.2×10^5 spores/cm^2, whereas for *A. tenuissima* the fecundity ranged from 0.5×10^3 to 3.7×10^4 spores/cm^2, in *A. alternata* from 0.5×10^3 to 9.0×10^4 spores/cm^2 and in *A. longipes* from 5.3×10^3 to 7.4×10^4 spores/cm^2 (data not shown). Among all isolates, *A. arborescens* isolate arb-3 showed the highest average fecundity (3.4×10^5 spores/cm^2), followed by arb-1 (0.8×10^5 spores/cm^2), while isolate arb-2 had the lowest (0.7×10^3 spores/cm^2) average fecundity, followed by *A. tenuissima* isolate ten-1 with 0.3×10^4 spores/cm^2 (Fig. 1b).

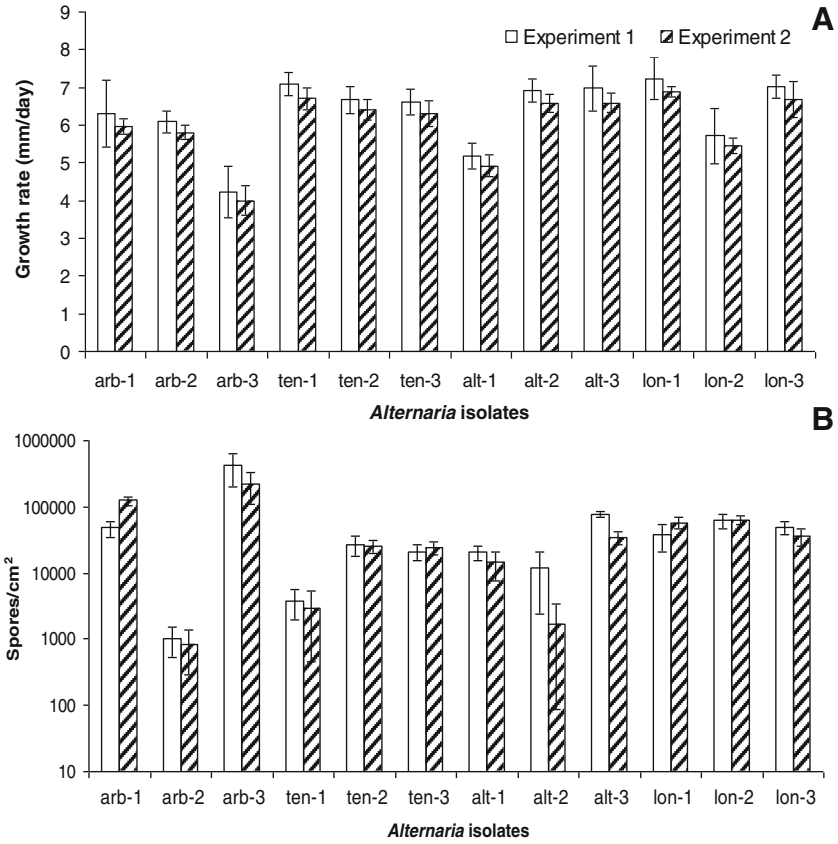

Fig. 1 Average growth rate (**a**) and fecundity (**b**) of two experiments of three *Alternaria* isolates of four *Alternaria* species groups associated with leaf blotch and fruit spot of apple. Bars indicate the standard deviation

Competitive spore production of *Alternaria* isolates

Amount of spores produced by each isolate within each *Alternaria* species groups in competition with isolates of different species group varied significantly (Fig. 2). The ratio of spores produced is dependent on the corresponding isolate rather than the species group. Overall, *A. arborescens* isolates were consistently and significantly ($P<0.05$) the most competitive, with >60 % of spores recovered when in mixed culture with other isolates of other species, except with *A. tenuissima* isolates ten-1 and ten-2 where about 40 % of the spores produced in the mixture were *A. arborescens* (Fig. 2). Only *A. tenuissima* isolate ten-3 was recovered when mixed with either *A. alternata* isolate alt-1 or *A. longipes* isolate lon-2 and 100 % of *A. longipes* isolate lon-3 was recovered in a mixture with *A. alternata* isolate alt-2 or *A. arborescens* isolate arb-2 (Fig. 2).

Relationship between saprophytic and pathogenic fitness traits

The isolates ten-3 and lon-3 showed a low aggressiveness on the inoculated apple leaves. Isolates arb-3, ten-2 and lon-2 showed a moderate and ten-1, lon-1, alt-2 and alt-3 showed high aggressiveness (Table 1). There was no disease development in the control inoculations and no significant variation ($P<0.001$) between the first and repeated experiment.

The PCA analysis showed a significant negative correlation between the two saprophytic attributes (fecundity and mycelial growth rate), but not between the pathogenic fitness trait aggressiveness and the saprophytic fitness traits (Table 2). However, a negative association was observed between aggressiveness and fecundity (Table 2). The PCA analysis showed that the first component explained 67 % of the association of the saprophytic fitness traits that showed a strong positive weight on mycelial growth rate and a strong negative weight on fecundity (Fig. 3). The second component that explained 31 % of the relationship showed a strong positive weight on the pathogenic fitness trait aggressiveness (Fig. 3). The correlation biplot of all the isolates showed that isolates with high aggressiveness and mycelial growth rate were strongly partitioned on the positive axes of the plot showing a strong host invasion process may correspond with high growth rate of the pathogen in the host tissue (Fig. 4). In contrast, isolates with low aggressiveness were grouped on the negative axes, while moderately aggressive isolates were separated based on their fecundity (Fig. 4). The moderately aggressive isolate arb-3 with very high fecundity was strongly

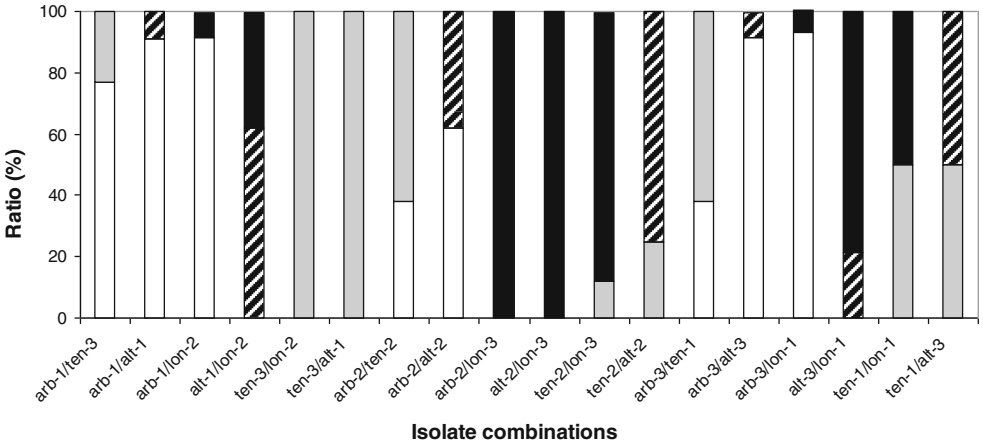

Fig. 2 Ratio of competitive spore production in 18 combinations of three different isolates of four *Alternaria* species groups. *Alternaria* species groups: arb: *A. arborescens* (□), alt: *A. alternata* (▨), ten: *A. tenuissima* (▩), lon: *A. longipes* (■)

drawn to the left (negative) on the *x*-axis or the PCA component 1, whereas, the moderately aggressive isolate ten-2 with low fecundity was partitioned to the negative of the *y*-axis (PCA component 2) and to the positive on the *x*-axis (PCA component 1) due to its high growth rate (Fig. 4).

Discussion

This study showed that fitness attributes of isolates among and within each of the Australian *Alternaria* species groups that are associated with leaf blotch and fruit spot of apple are highly variable. However, the fecundity of *A. arborescens* isolates when in competition with other *Alternaria* species groups was in most cases higher than isolates of other species. This could contribute to its dominance as the most frequently isolated species group associated with leaf blotch of apple in Australia (Harteveld et al. 2013). Analysis of the relationship between the saprophytic and pathogenic fitness traits revealed that highly aggressive isolates with fast mycelial growth rate were strongly partitioned together on the positive axes of the correlation biplot showing. This suggests that high growth rate of the *Alternaria* isolates may contribute to a strong host invasion process of the host tissue. The analysis of the relationships between the fitness traits indicate a significant trade-off between fecundity and mycelial growth, indicating that isolates with lower growth rate in the species groups may be translated to higher fecundity.

Table 2 Pearson correlation coefficients of pathogenic and saprophytic fitness traits of isolates of four *Alternaria* species groups associated with Alternaria leaf blotch and fruit spot of apple in Australia

Fitness trait	Aggressiveness	Fecundity	Mycelial growth rate
Aggressiveness	1		
Fecundity	−0.125	1	
Mycelial growth rate	0.314	−0.917*	1

*Significant with P=0.05

Generally, the high variability observed in the characteristics of isolates in each species group may be due to a lack of distinct taxonomic species delineation of the isolates. Recent studies have shown that the *Alternaria* species group concept used to define the *Alternaria* isolates in this study, may not sufficiently resolve the identity of the isolates and therefore isolates in some of the species groups could be considered as belonging to one species, *A. alternata* (Andrew et al. 2009; Lawrence et al. 2013). Nevertheless, irrespective of the species groups, we clearly show that significant variation in saprophytic characters exist among the *Alternaria* isolates associated with leaf blotch and fruit spot of apple in Australia. Possible recombination between subpopulations has been suggested as a source of variation in the *Alternaria* species groups (Stewart et al. 2013).

PCA analysis of the saprophytic fitness attributes of each isolate showed that a significant negative correlation exist between the two saprophytic attributes (fecundity and mycelial growth rate). Therefore, a large number of isolates is required for morphological or cultural characterization of the species. This is important when assessing chemical or other control methods that are focused on reducing either mycelial growth or spore production of a pathogen (Badawy and Rabea 2013; de Oliveira et al. 2012). A somewhat consistent trend among isolates of *A. arborescens* is slower mycelial growth rate and smaller colony sizes in culture in comparison with other small-spored *Alternaria* species (Harteveld et al. 2013; Pryor and Michailides 2002; Rotondo et al. 2012). This indicates that this feature is a consistent characteristic of isolates in the *A. arborescens* species group.

The variability among the isolates observed in this study suggests that other additional mechanisms such as metabolites/types or quantity of toxins produced may be

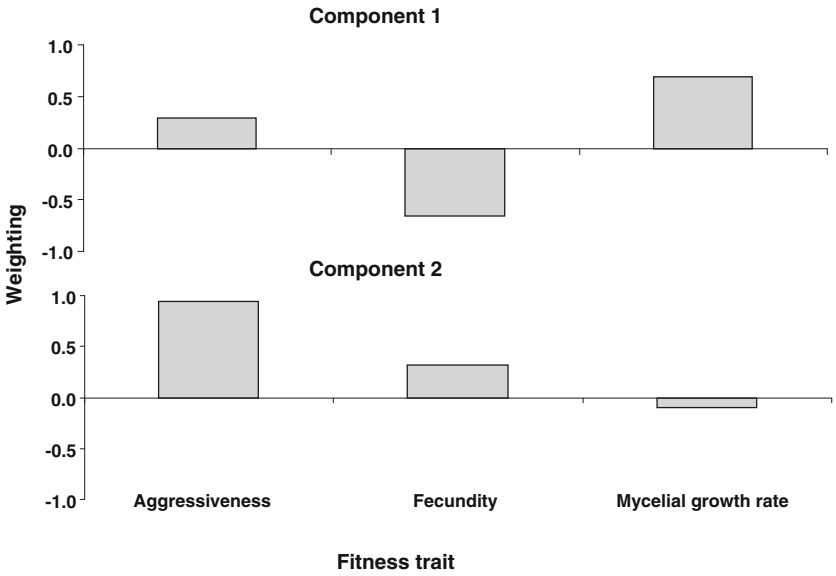

Fig. 3 Weighting (eigenvectors) of the first two principal components explaining 67 % and 31 % of the variance, respectively, of the fitness of nine *Alternaria* isolates including aggressiveness, fecundity and mycelial growth rate

involved in causing the differences between the isolates (Takai 1980; Tunali et al. 2012). In other countries, *A. mali* has been shown to produce the host specific AM-toxin (Johnson et al. 2000; Kohmoto et al. 1976) and the involvement of specific metabolites produced by each species group or isolates has been proposed as a factor that contributes to pathogenic variation among the species groups (Andersen et al. 2002; Greco et al. 2012; Logrieco et al. 2009). At present, it is not known if the Australian isolates of the *Alternaria* species groups associated with leaf blotch and fruit spot of apple produce any metabolites/toxins that may influence their prevalence and/or pathogenicity. Further study is needed to identify any metabolites/toxins produced by the Australian *Alternaria* isolates and establishes their involvement in the biological fitness for leaf blotch and fruit spot of apple.

Alternaria species are sensitive to their environment (Rotem 1994) and their fitness can be affected by culture media (Simmons 2007), light regulations (Ansari et al. 1989; Roberts et al. 2012; Singh 2000) and climatic factors such as temperature and relative humidity (Rotem 1994). In this study, we performed the experiments under controlled conditions, and assessed the saprophytic fitness traits using in vitro assays. Since, the saprophytic behavior of the isolates may be influenced by their pathogenic behavior, future studies should assess the biological fitness traits of *Alternaria* isolates affecting apple using *in planta* assays (Moya-Elizondo et al. 2011).

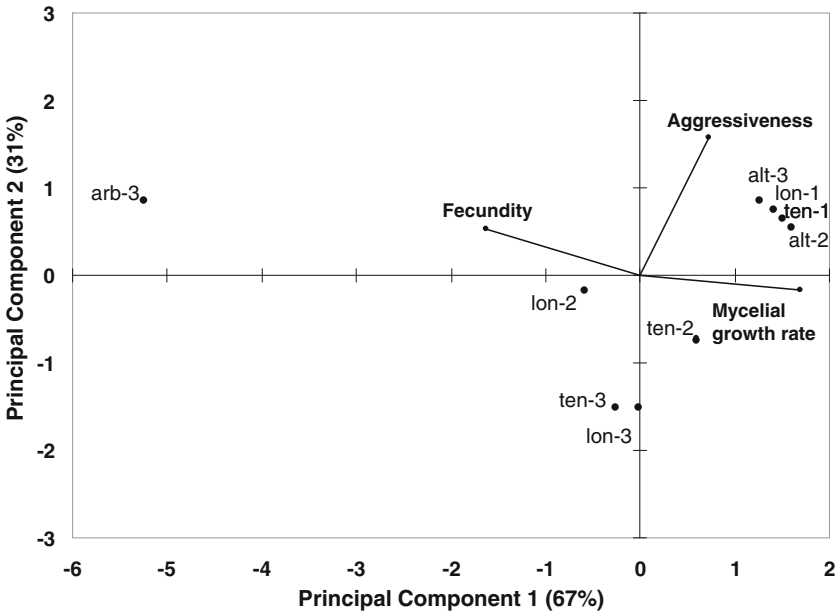

Fig. 4 Correlation biplot of the first two principal components explaining 98 % of the variance, showing the fitness variation of nine *Alternaria* isolates based on pathogenic (aggressiveness) and saprophytic (mycelial growth rate and fecundity) fitness traits. *Alternaria* species groups: arb: *A. arborescens*, alt: *A. alternata*, ten: *A. tenuissima*, lon: *A. longipes*

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