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Multiple associations of *Alternaria* species on high density apple (*Malus* × *domestica*) plantation system in Jammu and Kashmir, India

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ABSTRACT

The study was carried out during 2021–2022 at Sher-e Kashmir University of Agricultural Sciences and Technology, Shalimar, Srinagar, Jammu and Kashmir to evalaute symptomatological development of Alternaria leaf blotch and spot of apple (*Malus* × *domestica* Borkh.) cultivars. The target of concern was to discern the specific *Alternaria* spp. intricately linked to manifestation of *Alternaria* fruit and leaf disease in apple trees under high density plantation system. Similarly, morpho-cultural characteristics of seven isolates were studied. Further, protein coding plasma membrane ATPase gene was used and the amplicon sequences were uploaded to *GenBank* and the accession numbers acquired after submissions for the ATPase were OQ355499, OQ222078, OQ333060 - OQ333064. The ATPase gene delimited all the isolates into three clades in which Clade-I contain sequences of *Alternaria tenuissima.* Clade-II consists sequences of *Alternaria alternata* while as Clade-III contains sequences of *Alternaria arborescens*. According to morphocultural, pathogenicity and molecular identification, the pathogens, viz. *Alternaria alternata, Alternaria tenuissima*, and *Alternaria arborescens* were found associated with apple leaf disease in India.

Keywords: ATPase, BLASTn, Clade, Morphocultural, Pathogenicity

The domesticated apple (*Malus* × *domestica* Borkh.) traces its origins to the Tien Shan Mountains of Kazakistan and southeastern Europe (Gastier 2000). Despite its historical and agricultural importance, apple cultivation faces numerous challenges worldwide due to its vulnerability to various diseases and pests (Way *et al.* 1991). In Kashmir, important diseases affecting apple trees consist of apple scab, Alternaria leaf blotch, powdery mildew, Marssonina leaf blotch, crown rot, and root rot. Apple scab is the most important foliar disease among these, followed by Alternaria leaf blotch.

Alternaria leaf blotch was first time reported in the USA by Roberts (1924). Since then, it has spread around the world and caused notable financial losses due to premature defoliation. Reports from North Carolina indicate defoliation rates ranging from 60–80% (Filajdic and Sutton 1991). In contrast, Alternaria fruit spot was first reported in Japan during 1950, Italy in 2003 and California in 2023. The disease was first documented from Himachal Pradesh and Kashmir in India (Gupta and Agarwala 1968, Puttoo 1987). Despite being considered of minor significance initially, the disease has evolved into a major threat in the UT of Jammu and Kashmir in recent years (Shahzad *et al.* 2002).

Alternaria mali is identified as the main pathogen causing alternaria leaf blotch, with the potential involvement of other *Alternaria* spp. being hypothesized (Simmons 1999). The identification of these species is complex and varies across regions. In different studies, the disease has been attributed to *A. mali* as reported by Filajdic and Sutton (1991) and Sawamura (2014). Some studies classify it as the *A. alternata* apple pathotype (Johnson *et al.* 2001, Abe *et al.* 2010, Ding *et al.* 2024, Ullah *et al.* 2024) or *Alternaria alternata* f. sp. *mali* (Yoon *et al.* 1989, Chauhan and Gupta 2019). In Italy, Australia, France and California, USA leaf and fruit symptoms have been associated with three *Alternaria* spp. groups *A. tenuissima, A. alternata*, and *A. arborescens* (Rotondo *et al.* 2012, Harteveld *et al.* 2013, Fontaine *et al.* 2021, Elfar *et al.* 2023), respectively. The implementation of high-density orchard systems induces alterations in microclimatic conditions, potentially elevating the risk and susceptibility to fungal diseases. Hence the current study was carried to ascertain the association of different *Alternaria* spp. with fruit spot and leaf blotch

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of apple in Kashmir valley under high density plantation systems.

MATERIALS AND METHODS

The study was carried out during 2021–2022 at Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar (34º9′22″N and 74º52′55″E; 1685 m amsl), Srinagar, Jammu and Kashmir.

Morpho-cultural characterization of Alternaria *spp.*

Collecting, isolating, purifying, and maintaining the Alternaria *isolates*: Diseased apple leaf samples exhibiting symptoms of Alternaria leaf spot were collected from seven apple cultivars, viz. Fuji Zehn Aztec (FZY2), Royal delicious (RODLA1), Early Redone (EROC2), Decoste Robigen (DCRA1), Red delicious (RDZ1), Mitch Gala (MGA2), Vance Delicious (VDM9A2) in May 2021. The samples were taken to the laboratory for pathogen isolation on potato dextrose agar (PDA) media. After isolation, the pathogens were purified using single-spore technique with sustained cultures maintained on PDA at 23 ± 1°C.

Morpho-cultural characterization: For morpho-cultural characterization, the single-spore isolates were analyzed as per the systematics given by Pryor and Michailides (2002) and Hong *et al.* (2006). Fungal mycelia were inoculated to PDA petriplates in triplicates, incubated at 23 ± 1°C for seven days, and assessed for colony texture, colour, margin, and diameter. The descriptions of colony type given by Nobles (1948) and Ridgway's (1912) colour standards were used to classify isolates. The morphological characteristics were observed after 10 days of incubation. Temporary mounts were prepared from freshly sub-cultured sporulating cultures on the slides. The measurements taken using a compound microscope at 40x magnification. Standard references of *Alternaria* spp. (Simmons 2007) were used for identification, and 20 observations per isolate were recorded.

Pathogenicity bioassays: Pathogenicity bioassays were conducted using conidial suspensions prepared from 10-day-old colonies on PDA. Employing a sterilized razor blade, spores dislodged from the colonies were passed across a double layered sterilized muslin cloth and collected in a suspension. A hemocytometer was used to adjust the spore concentrations to 10^5 conidia/mL. Inoculation was performed on both wounded and unwounded detached apple leaves of seven cultivars taken for isolation following a modified variant of the procedure by Sofi *et al.* (2013). The pathogenicity of all *Alternaria* isolates was tested to assess their pathogenicity.

Molecular identification: The study's investigation of molecular variability involved isolation of DNA, amplification in PCR, sequencing, and phylogenetic analysis to understand the genetic diversity of *Alternaria* spp. isolated from diseased apple leaves. DNA was isolated from representative samples by CTAB method, a technique established by Murray and Thompson in 1980.

PCR amplification was conducted using specific primer pairs (ATPDF1/ATPDR1) as outlined by Lawrence *et al.* (2013). A 25 µl reaction volume was used for the PCR reactions, which contained 12.5 µl of GoTaq Mix, 1 µl of each primer (10 µl total), and 1 µl of template DNA. This method was based on protocols by Zhu and Xiao (2015). The reaction mixture underwent a brief spin in a microfuge. The amplification process was performed using a T-Gradient PCR, which involved a 4 min heating phase at 94°C, 35 cycles of denaturation at 94°C for 30 sec, annealing of 35 sec at 53°C, extension at 72°C for 1 min, and a final extension for 5 min at 72°C. The amplified products were separated on a 1% agarose gel via electrophoresis, which allowed the visualization of DNA bands. The bands were visualized using an Alfa Imager gel documentation system, and the results were documented photographically.

Sequencing of DNA and phylogenetic analysis: Post-PCR, the amplified products were submitted for DNA sequencing. Sequences from all forward and reverse primers were obtained and aligned using BioEdit software, version 7.09. This alignment process ensured that the sequences were accurate and high-quality, enabling creation of consensus sequences for each isolate, as per the methodology by Hall (1999). The chromatograms of these sequences were carefully trimmed and aligned at both ends to ensure uniformity. These consensus sequences were then analyzed using BLASTn against sequences available in the National Center for Biotechnology Information (NCBI) database. This step was crucial for identifying the species of *Alternaria* isolates by comparing them with known sequences in the database. Additionally, five sequences of *Alternaria* spp. from the NCBI database were used for comparative analysis. To explore the phylogenetic connections among various *Alternaria* isolates, multiple sequence alignments were carried out using CLUSTALW within the MEGA software, version 11.0. The phylogenetic tree was constructed using Kimura-2-parameter substitution model, and the reliability of the tree was tested using a bootstrap test with 1000 replicates, adhering to Kimura's methodology from 1980. This analysis revealed distinct clustering of the isolates into different clades, indicating genetic variability among the isolates. The acquired sequences were uploaded to Gene Bank and given accession numbers.

RESULTS AND DISCUSSION

The main purpose of our study was to identify different small spored *Alternaria* spp. linked with Alternaria leaf blotch of apple. The trees and leaves were tagged for recording disease development or appearance of symptoms on each cultivar. During the course of investigation, symptomatological studies revealed that in case of Alternaria leaf blotch, the disease was first noticed during second week of May as small, circular, 0.5–2.0 mm size and light brown in colour. More lesions were observed followed by coalescing of 2–5 spots form blotch (6.0–10.0 mm) in the fourth week of May. In the last week of June, the infected leaves became yellow and undergo went premature defoliation. While in case of Alternaria leaf spot, the symptoms appeared in third week of May as circular brown lesions having purple

margins 2.0 to 3.0 mm in size. In the last week of June, the lesions were silvery grey in colour having maximum sizes of 9.0–10.0 mm followed by yellowing and premature defoliation of leaves. More or less similar observations have been recorded by other workers: Horlock (2006), Rotondo *et al.* (2012), Wenneker *et al.* (2018), Fontaine *et al.* (2021).

Morpho-cultural characteristics of Alternaria *isolates*: Morpho-cultural characteristics of seven isolates were examined, focusing on colony colour, colony type, colony diameter, colour and size of hyphae, conidiophores, and conidia. The isolates varied in cultural and morphological characteristics. The study found it challenging to differentiate small-spored *Alternaria* owing to high resemblance in their morphological characteristics.

Isolates RDZ1 and VDM9A2, identified as *A. alternata*, produced colonies ranging from velvety to woolly, appressed, light olivaceous green with slightly irregular to regular white rim. Their conidia were muriform, obpyriform, ovoid and ellipsoid, with dimensions averaging 25.64–29.71 × 09.14–13.02 µm [Table 1, Table 2, Fig. 1 (e, s and g, n, u)].

Isolates FZY2, RODLA1, and EROC2, identified as *A. tenuissima*, formed colonies with a range of velvety, felty olivaceous grey to green shades with slightly regular to regular dull white rim and produced conidia that were muriform, obclavate, and ovoid-ellipsoid, with dimensions averaging 29.34–38.02 × 09.36–10.98 µm [Table 1, Table 2, Fig. 1 (e, s and g, n, u)].

Isolates DCRA1 and MGA2, identified as *A. arborescens*, exhibited colonies type as velvety, appressed, colours range from light to dark olivaceous green to black and produced obpyriform-ovoid conidia with dimensions averaging 16.19–22.38 × 09.32–10.76 µm [Table 1, Table 2, Fig. 1 (d, k, r and f, m ,t)].

In the present study, the colour of colony varied between light to dark olivaceous green and greyish to black with white cottony center. These findings were in corroboration with studies carried by Harteveld *et al.* (2013) and Sofi *et al.* (2013). Similar results were observed by Serdani *et al.* (2002), Toome Heller *et al.* (2018), Wenneker *et al.* (2018), Ali *et al.* (2021) who observed dark olivaceous green colour and dark olivaceous black colonies in *A. arborescens*. Subsequently, Kou *et al.* (2014) and Zhu and Xiao (2015) reported light olivaceous green colour colonies in *A. tenuissima,* while Rotondo *et al.* (2012) and Harteveld *et al.* (2013) found grey and grey to green colonies in *A.tenuissima*, respectively. The studies in respect of conidial shapes were recorded under *in vitro* conditions revealed round, ovoid, ellipsoid, obpyriform and obclavate shapes. Similar observations were seen by Wee *et al.* (2016) in *A. tenuissima* as obclavate conidia and Riquelme *et al.* (2021) observed ellipsoid, obclavate, ovoid, obpyriform shapes of conidia in case of *A. alternata*, *A. arborescens* and *A. tenuissima*. Similarly, ovoid type of conidia were observed in *A. arborescens* by Wenneker *et al.* (2018) and Elfar *et al.* (2018).

Pathogenicity tests: The pathogenicity tests categorized isolates into three groups based on lesion colour and incubation period. Group 1 produced light brown lesions with an incubation period of 3–4 days, Group 2 produced dark brown lesions with a 4-days incubation period, and

Table 1 Cultural characters of different isolates of *Alternaria* spp. related with apple cultivars under high density orchard system

Isolate	Colony characteristics*				
	Type	Colour	Margin	Underside colour	Diameter of colony** (mm)
FZY2	Velvety, felty, slightly furrowed with greyish raised centre	Olivaceous grey	Regular with light grey with dull white rim	Dark black centre with white rim	80.36
RODLA1	Velvety, felty with sparse aerial mycelium	Black olivaceous green	Slightly irregular, olivaceous black with white rim	Dark black with white rim	75.23
EROC2	Velvety, felty with cottony aerial mycelium	Light olivaceous green	Regular, green and dull white margin	Dark black centre with white rim	81.37
DCRA1	Velvety and appressed, furrowed	Light olivaceous green with greyish white patch in the centre	Slightly irregular, light green with dull white margin	Dark black centre with white margin	49.45
RDZ1	Velvety, appressed, furrowed	Olivaceous green with cottony grey growth	Regular, light green with extreme white rim	Dark brown centre with white rim	74.12
MGA2	Velvety, appressed, furrowed	Dark black with white centre	Regular, black with light brown rim	Smoky light black	85.43
VDM9A2	Woolly, appressed, furrowed	Olivaceous green with greyish surface	Slightly irregular, olivaceous green with white margin	Dark brown with white rim	72.65

* Mean of 3 replicates; ** Seven days after inoculation.

Table 2 Conidial morphology of different isolates of *Alternaria* spp. associated with apple cultivars under high density orchard system

Isolates	Conidia*				
	Colour	Size (Length×Width) (µm)		Septation (T×L)#µm	Shape
		Range	Mean		
DCRA1	Dark brown	12.41–32.56 × 6.12–15.74	22.38 × 09.32	1–5 × 0–3	Obpyriform-ovoid
RDZ1	Dark brown	20.35–40.75 × 8.14–18.28	29.71 × 13.02	1–5 × 0–3	Obpyriform, ovoid-ellipsoid
MGA2	Dark brown	12.21–24.42 x 11.21–20.28	16.19 × 10.76	1–4 × 0–3	Obpyriform-ovoid
RODLA1	Golden brown	18.51–50.12 × 8.14–12.21	33.18 × 09.36	1–4 × 0–2	Muriform, obclavate-ellipsoid
EROC2	Dark brown	18.35–68.63 × 8.14–12.21	38.02 × 10.98	1–7 × 0–2	Muriform, obclavate-ellipsoid
FZY2	Light brown	20.12–44.77 × 7.71–13.21	29.34 × 10.56	1–6 × 0–2	Muriform, obclavate, ovoid-ellipsoid
VDM9A2	Dark brown	12.58–34.34 × 8.14–14.24	25.64 × 09.14	1–5 × 0–2	Muriform, obpyriform

*Mean of 20 observations in each isolate**.** T, Transverse; L, Longitudinal; L, Length; W, Width.

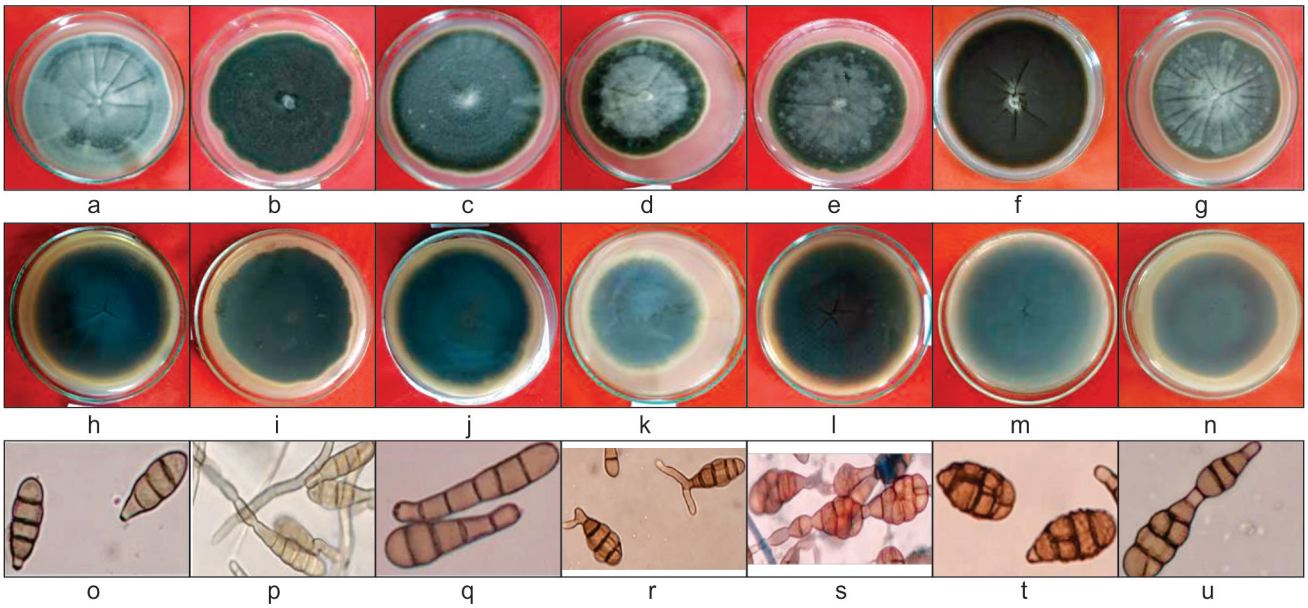

Fig. 1 Morphological characterization of *Alternaria* spp. isolated from different apple cultivars. a–g, Colony morphology on potato dextrose agar after 7 days; h–n, Underside colour of colonies produced by *Alternaria* spp.; and o–u, Conidial morphology of *Alternaria* spp.
a, h, and o, A. *tenuissima* isolate FZY2; b, i, and p, *A. tenuissima* isolate RODLA1; c, j, and q, *A. tenuissima* isolate EROC2; d, k, and r, *A. arborescens* isolate DCRA1; e, l, and s, *A. alternata* isolate RDZ1; f, m, and t, *A. arborescens isolate* MGA2; g, n, and u, *A. alternata* isolate VDM9A2.

Group 3 produced dark brown lesions with a yellow halo and an incubation period of 4.5 days (Table 3, Fig. 2). The colour of lesions varied between light to dark brown with a yellowish halo that suggest the presence of toxins secreted by different pathotypes of a pathogen. The study observed that only injured leaves developed symptoms, suggesting that *Alternaria* spp. are weak pathogens that need wounds to infect tissues.

These findings were in agreement with Pryor and Michailides (2002) and Rotondo *et al.* (2012) as they also reported substantial lesion development only on wounded leaves. Sofi *et al.* (2013) reported the variation in incubation period.

Phylogenetic analysis: The study focused on sequencing and phylogenetic analysis of the plasma

Table 3 Pathogenicity bioassay of *Alternaria* isolates using detached leaf method

Group	Isolates	*Pathogenicity Bioassay	
		**Incubation period (Days)	Colour of lesions
1	EROC2, FZY2	3.5	Light brown
	MGA2	3.0	
	RODLA1, RDZ1	4.0	
2	DCRA1	4.0	Dark brown
3	VDM9A2	4.5	Dark brown with yellow halo

*Mean of 3 replicates; ** Days after inoculation.

Fig. 2 Pathogenicity bioassay of *Alternaria* isolates using detached leaf method. A, Light brown lesion; B, Dark brown lesion; C, Dark brown with a yellow halo.

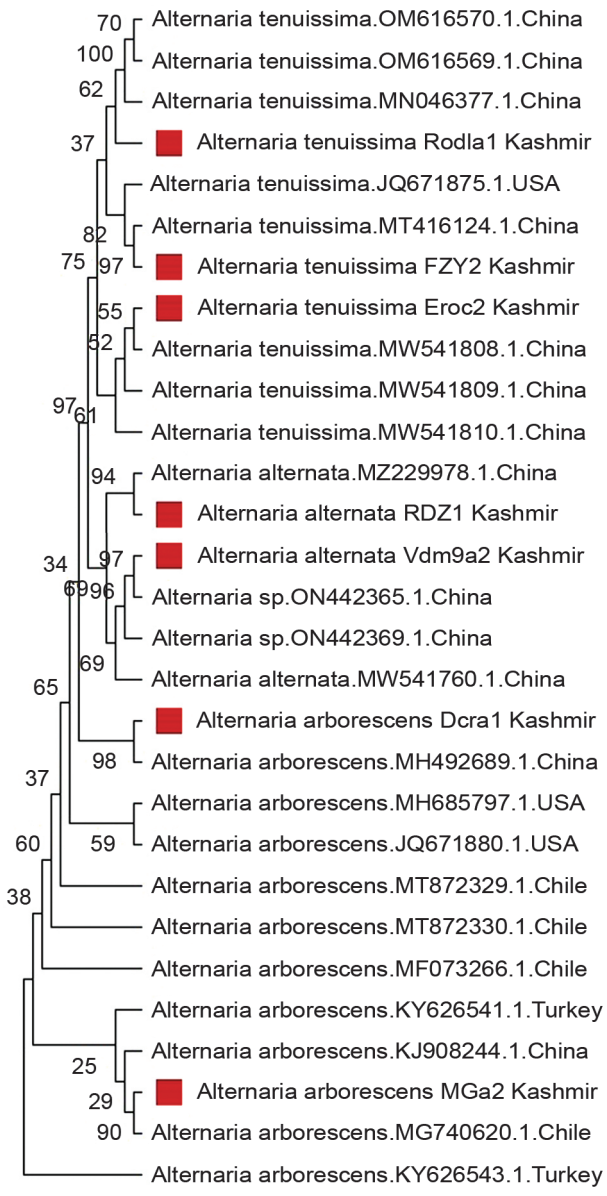

Fig. 3 The phylogenetic tree of *Alternaria* spp. derived from neighbour–joining analysis of the plasma membrane ATPase gene.

membrane ATPase gene in various isolates associated with apple leaf disease in India. Amplified products of the ATPase gene, ranging from 1195–1225 base pairs, were sequenced and analyzed at National Centre for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/nuccore) using programme BLASTn, revealing 94–99% resemblance with *Alternaria alternata*, *Alternaria tenuissima*, and *Alternaria arborescens*. The sequences were also submitted to Gene Bank and were given accession numbers after submissions for the ATPase were OQ355499, OQ222078, OQ333060- OQ333064. The ATPase gene categorized the isolates into three clades i.e. (Fig. 3) Clade-I (similar to *A. tenuissima* from China and the USA), Clade-II (similar to *A. alternata* from China), and Clade-III (similar to *A. arborescens* from Chile, the USA, and Turkey). The pathogens *A. alternata, A. tenuissima*, and *A. arborescens* were confirmed as the primary agents causing apple leaf disease in India, corroborating findings from similar studies *of* Zhu and Xiao (2015), who employed ATPase to discriminate *Alternaria* spp., specifically identifying *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. rosae*. In Italy, Rotondo *et al.* (2012) identified three *Alternaria* spp. associated with leaf and fruit symptoms; *A. tenuissima, A. arborescens,* and *A. alternata.* Likewise, in Australia, a variety of *Alternaria* spp. were linked to leaf blotch and fruit spot of apples as reported by Harteveld *et al.* (2013). Additionally, Elfar *et al.* (2023) complemented our findings by identifying *A. alternata* and *A. arborescens* as the causative agents of fruit spot and leaf blotch in apple in California.

The study reinforces the role of the ATPase gene as a reliable marker for identifying *Alternaria* spp. as already done by Elfar *et al.* (2018). Moreover, the study emphasized the limitations of relying solely on morphological characterization for species identification, highlighting the importance of multi-gene phylogeny for accurate molecular differentiation. The current study is the first to identify *A. arborescens* and *A. tenuissima* as being associated with apple leaf blotch in India.

The study concluded that Alternaria leaf blotch and spot in high density apple orchards of union territory of Jammu

and Kashmir, is caused by *A. alternata*, *A. tenuissima*, and *A. arborescens*. Symptom analysis revealed two distinct patterns i.e. spots and blotches which appear to be varying across cultivars. The pathogenicity of these three species was verified through the detached leaf method. Additionally, seven isolates were characterized culturally, morphologically, and molecularly, revealing the association of three species: *A. alternata*, *A. tenuissima*, and *A. arborescens*.

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