Endophytic fungi associated with two *Suaeda* species growing in alkaline soil in China

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Suaeda species are annual halophytes growing in soils with high salinity and high concentration of irons where non-halophytes are unable to thrive. It might be expected that the endophytic fungi of *Suaeda* species might help their host to resist the environmental stress. Endophytic fungal communities associated with stems and leaves of healthy *S. microphylla* and *S. corniculata* plants were determined from June to October in 2009. The plants were growing in alkaline land in Songyuan City, Jilin Province in China. Endophytic fungal composition varied between the two plants, however *Alternaria* spp. and *Phoma* spp. colonized both plant species. The fungal diversity and colonization rate were highest in September. Different fungal species inhabited different tissues and a few species were overlapping between stems and leaves. Although species of *Phoma chrysanthemicola* were isolated from both stem and leaves in September, the isolation frequency in stems was significantly higher than in leaves. Pigmented dematiaceous endophytic fungi were particularly common in the halophytes and they may play an important ecological role for survival and stress resistance of the plant species.

Key words – *Suaeda microphylla* – *Suaeda conriculata* – fungal community – *Alternaria alternata* – plant phenology.

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Introduction

Suaeda species are annual halophytes, one of the most common plants in saline and alkaline soils, which can survive with salt concentration equal to or greater than 2% (Shao & Li 1998). *S. microphylla* and *S. corniculata* occupy large areas and are the dominant species in degraded grasslands in Songnen Plain. They are the most important vegetation species for the restoration process of alkaline meadows (Wang et al. 1996). Endophytic fungi have been reported to help their hosts overcome environmental stress (Redman et al. 2002, Shearer 2002, Gregory & Cheplick 2004, Gonthier et al. 2006, Parsaeian et al. 2007, Porras-Alfaro et al. 2008, Newsham et al. 2009). Therefore, endophytic fungi of the halophyte *Suaeda* are suspected to benefit their hosts in the high salinity environment.

Only a few halophytic plants have been studied for their endophytic fungal communities (Petrini & Fisher 1986, Fisher & Petrini 1987, Peláez et al. 1998, Suryanarayanan & Kumaresan 2000, Maria & Sridhar 2003,

Dorothy & Kandikere 2009). Okane (2001) examined the endophytic fungal community of *Salicornia europaea* in Japan and found that *Alternaria* spp., *Cladosporium* spp., *Stemphylium* spp. and *Pleospora* sp. (the sexual stage of *Stemphylium*) were dominant colonizers of the plants in all five sampling locations. El-Morsy (2000) isolated fungi from halophytes (*Avicennia marina, Arthrocnemum macrostachum, Halocnemum strobilecium, Limonastrum monopetalum, Zygophyllum album, Z. simplex, Tamarix nilotica, Zilla spinosa* and *Z. coccineum*) of the Red Sea Coast of Egypt after surface sterilization. The most common species were *Alternaria alternata, Cladosporium cladosporioides* and *Penicillium chrysogenum*. Dorothy & Kandikere (2009) investigated the assemblage and diversity of fungal of *Canovalia cathartica* in India. They found that *Aspergillus niger* was the dominant endophytic fungus. It appears that melanised dematiaceous fungi universally inhabit plants in soils with high salinity. In England and Canada, these pigmented dematiaceous fungi were also isolated from halophytes. Thus, these endophyic fungi may have an important ecological role for the survival of halophytes.

The tissue specificity and host specificity of endophytic fungi from halophytes have also been examined. Fisher & Petrini (1987) examined fungal endophytes in *Suaeda fruticosa* in England and found that *Colletotrichum phylachoroides* and *Camarosporium* spp. were dominant colonizers. The endophytic fungi showed tissue speicificity; *Colletotrichum phyllachoroides* was confined to leaves, while two species of *Camarosporium* were mainly isolated from stems. Host specificity of endophytic fungi has been also observed in some halophytes. Suryanarayanan & Kumaresan (2000) screened four halophytes, *Acanthus ilicifolius* (*Acanthaceae*), *Arthrocnemum indicum*, *Suaeda maritima* (*Chenopodiaceae*) and *Sesuvium portulacastrum* (*Aizoaceae*); *Camarosporium* species showed some degree of host preference as they were the dominant endophytes of the *Chenopodiaceae* but were absent from the other halophytes.

Little is known about the temporal changes in the endophytic fungal community. It has been suggested that the endophytic fungal community can be affected by seasonal changes of climate factors (Hata et al. 1998), and the seasonal occurrence of endophytes may correlate with plant phenology (Rodrigues 1994, Kaneko et al. 2003). Clarification of fungal species composition and changes of endophytes on a temporal scale is an important step in understanding the ecology and physiology of endophytes and host-endophyte interactions (Sahashi et al. 1999).

In the present study, fungi associated with *S. microphylla* and *S. corniculata* were examined during the growing season between June and October, 2009, in alkaline soil in China, and the endophytic fungal community in leaves and stems were compared.

Methods

Sampling site

Suaeda microphylla and *S. corniculata* were collected from an alkaline soil in Songyuan Guaibodian in Jilin (45°02'N; 124°36'E) (Fig. 1). This vast alkaline grassland covers 23925 km^2, about two-thirds of the meadow grasslands area of the Songnen Plain in north-eastern China and it is one of the biggest saline and alkaline areas in China. The evaporative demands are extremely high, about three times the annual precipitation, which tends to bring solutes dissolved in ground water and deep soil up to the surface. The topographical features and climatic conditions of the area result in a very slow primary soil alkalization and salinization. The monthly mean temperature in this area ranges from -15–23°C with the highest temperature in July. Rainfall is mainly distributed from July to August; the monthly average precipitation from June to October 2009 was 289–1611 mm, with the highest precipitation in July (Fig. 2).

Plant material

Samples of *S. microphylla* and *S. corniculata* were collected five times (Table 1). Four to five individual asymptomatic plants of each species were sampled randomly each time. Forty individual healthy leaves and 40 stems were randomly picked for fungal isolation. The shoot tip (terminal leaf) and withered cotyledon were not selected as it has been reported that fungi were not detected inside leaves just after leaf emergence (Kaneko et al. 2003). Samples

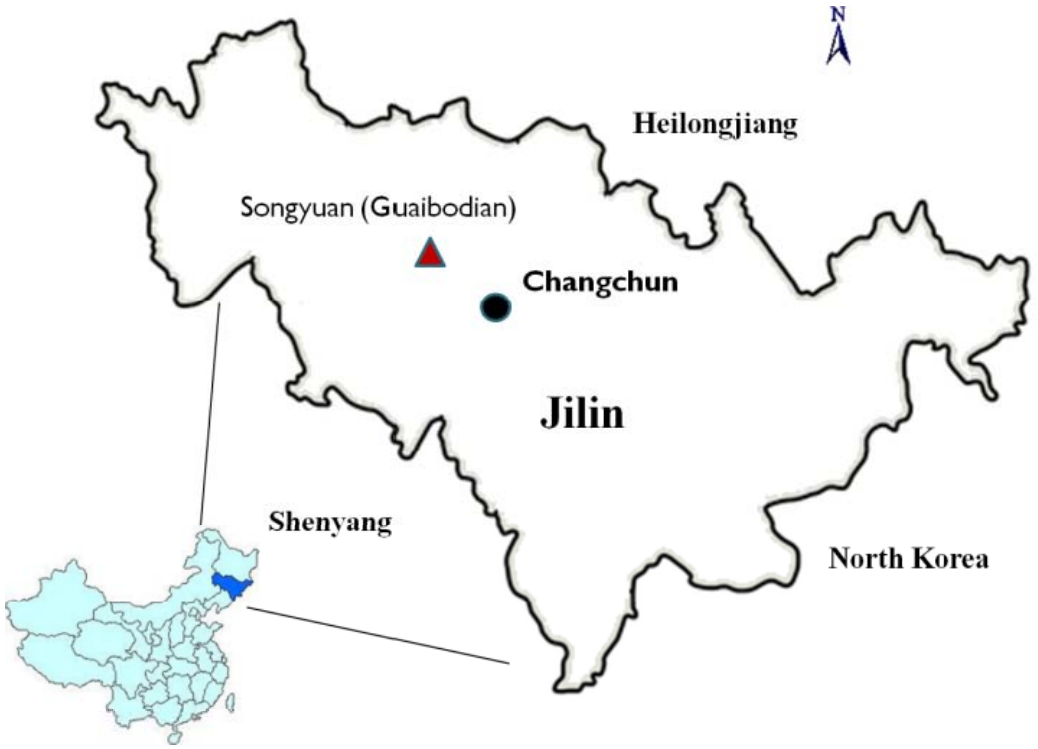

Fig. 1 – Map of sampling locations of the saline-alkaline soil area in Songyuan Guaibodian in Jilin. The saline-alkaline soil area is located north-west of Jilin (45°02'N; 124°36'E). It is 170 km away from the capital city Changchun.

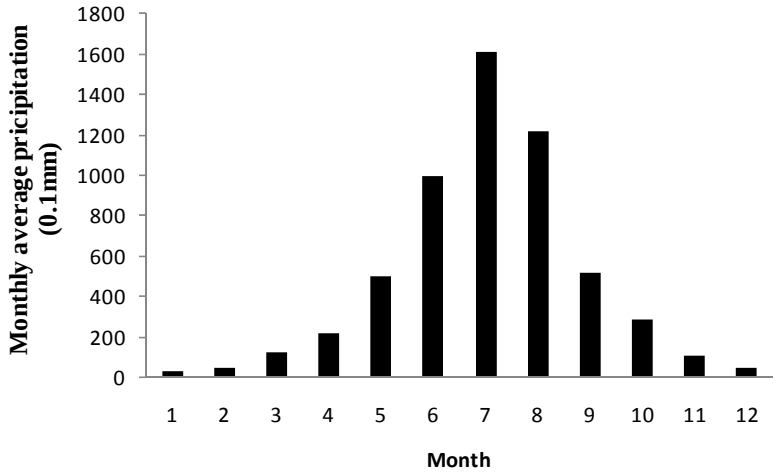

Fig. 2 – Monthly average precipitation at sampling site, Songyuan.

were taken to the laboratory, and stored at 4°C in a refrigerator; isolations were performed within 48 hours**.**

Isolation

The samples were first washed under tap water to remove sand. Surface sterilization followed the method described previously (Koga et al. 1993, Braun et al. 2003). Tissues were rinsed in 70% ethanol for 30 second, dipped in 2% sodium hypochlorite for 2–5 min, and washed 3–5 times with sterilized distilled water. Tissues were allowed to surface-dry over night under sterile conditions. They were then cut into segments of approximately 4–5 mm. Forty segment of each tissue were randomly selected for each host species. Ten segments were plated in each Petri dish (90 mm) containing CMA (2% corn-meal extraction, 15% agar, Nissui, Tokyo, Japan) and

Table 1 Plant materials collected for isolation of endophytic fungi.

Sample no.	Plant species	Tissues used for isolation	Sampling date
SM1	*Suaeda microphylla*	Leaf, stem	6.6.2009
SM2	*S. microphylla*	Leaf, stem	8.7.2009
SM3	*S. microphylla*	Leaf, stem	8.8.2009
SM4	*S. microphylla*	Leaf, stem	16.9.2009
SM5	*S. microphylla*	Leaf, stem	16.10.2009
SC1	*S. corniculata*	Leaf, stem	6.6.2009
SC2	*S. corniculata*	Leaf, stem	8.7.2009
SC3	*S. corniculata*	Leaf, stem	8.8.2009
SC4	*S. corniculata*	Leaf, stem	16.9.2009
SC5	*S. corniculata*	Leaf, stem	16.10.2009

incubated at 23°C for 4–8 weeks. Mycelia growing from the segments were transferred onto corn meal agar (CMA) media.

Identification

Fungal identification was based on morphology of the fungal culture, the mechanism of spore production and characteristics of the spore, following standard mycological manuals (Ellis 1971, Ainsworth et al. 1973, Carmichael et al. 1980, Sutton 1980, Udagawa et al. 1980, Klich 2002, Barnett & Hunter 2003, Boerema et al. 2004, Simmons 2007). Isolates which did not sporalate were placed under near UV light (black light for 12h dark: 12h light) in an attempt to stimulate sporulation. Isolates which did not produce spores were treated as sterile mycelium (*sensu* Lacap et al. 2003). All isolations were deposited in Mycological Herbarium of Jilin Agricultural University (HMJAU).

Data analysis

Isolation frequency (IF) and colonization rate (CR) of the fungi species were calculated using the following formula:

Isolation frequency (IF) = Ni/Nt ×100

Colonization rate (CR) = Nc/Nt × 100

Where Ni is the number of segments from which a given species was isolated; Nc is the total number of segments from which fungi were isolated in a sample and Nt is the total number of segments used for isolation.

Results and Discussion

Endophytic fungal composition of two plants

One hundred and four isolates belonging to 25 species (including one unidentified species) and 26 sterile mycelia were obtained from leaves and stems of *S. microphylla*. Eight species of *Alternaria* and seven species of *Phoma* were isolated, along with *Cladosporium* spp., *Fusarium* spp., *Ulocladium* sp., *Camarosporium* sp. and an unidentified ascomycete. The most commonly isolated species were *Alternaria alternata* (in 25% of leaf tissues in June and 17.5% of leaf tissues in August) and *Phoma chrysanthemicola* (in 22.5% of stems in September).

One hundred and thirty two isolates belonging to 21 species and 24 sterile mycelia were obtained from *S. conriculata*. *Alternaria* (10 spp., *Fusarium* (3 spp.), and *Phoma* (7 spp.) were frequently isolated. *Ulocladium* spp., *Cladosporium* sp., *Bipolaris* sp. 2 and an unidentified ascomycete were also isolated. *Alternaria alternata* was isolated from 12.5% of leaf tissues in August and *P. chrysanthemicola* from 17.5% of stems in September.

The results showed that pigmented fungi colonized in these two halophytic plants. *Alternaria alternata*, *A. angustiovoidea*, *A. brassicicola*, *Cladosporium* sp. 1, *Fusarium* sp. 1, *Fusarium* sp. 2, *P. chrysanthemicola*, *Phoma* sp. 1 and *Phoma* sp. 2 were obtained from both plant species, and *A. alternata* and *P. chrysanthemicola* were dominant in both. *Fusarium* spp. were also isolated from both plants, but the isolation frequency was lower.

The endophytic fungi of halophytes have been investigated on some plant species. *Salicornia europaea* were screened for endophytic assemblages in five saline lands in Japan (Okane 2001). The results showed that the pigmented fungi *Alternaria* spp., *Cladosporium* spp. and *Stemphylium* spp. dominantly and universally inhabited the plants in five sampling

localities. *Fusarium* spp. were also obtained from parts of the samples, but their occurrences were lower. Similarly, pigmented fungi were detected in other halophytes. Fisher and Petrini (1987) found *Camarosporium* spp. (85.3% in stems) and *Colletotrichum gloeosporioides* (30% in leaves) endophytically colonized in *Suaeda fruticosa* in England. Crabtree & Gessner (1982) found that the salt-marsh fungus *Camarosporium roumeguerii* occurring on plants of the Chenopodiaceae produced a dark green pigment. Other fungi such as *Pleospora* spp., *Stemphylium* spp., *Cladosporium* spp. and *Camarosporium* sp. were also always isolated from plants in desert and saline land (Petrini & Fisher 1986, Muhsin & Booth 1987, Okane et al. 1998). A high production of pigment was characteristic of these fungi (Leach 1971). These results suggest that these pigmented dematiaceous fungi universally inhabit halophytes and they are considered to play an important ecological role for stress resistance of halophytes.

How the pigmented fungi benefit their hosts, if at all, remains unknown. Crabtree & Gessner (1982) suggested that the darker mycelia produced by *C. roumeguerii* would absorb more UV radiation than white mycelia. An unidentified *Alternaria* species on cherries was noted for its resistance to UV radiation which was partly through its dark pigmentation (English & Gerhardt 1946). Bell &Wheeler (1986) and Carlos et al. (2008) suggested that the pigments are melanins, which increase fungal resistance to microbes and hydrolytic enzymes.

Diversity and colonization rate of endophyic fungi

The diversity and colonization rates of endophytic fungal isolated from *S. corniculata* in September were higher than the other months (Tables 2 and 3). Sixteen fungal taxa were isolated in September, with a colonization rate 55% in leaves and 65% in stems, which is significantly higher than the other months. Some species such as *A. alternata*, *A. brassicicola*, and *Fusarium* sp. 1 also appeared in June or July, while, 12 species were only isolated in September. The isolation frequencies of *P. chrysanthemicola* (17.5% in stems) and *Torula* sp. (12.5% in stems) were relatively high in September. In *S. microphylla*, the number of endophytic taxa was higher in June and September. Fourteen and 9 taxas were isolated in June and September respectively. *Alternaria alternata* (25%) (Fig. 3) showed the highest isolation frequency in leaves in June, while *P. chrysanthemicola* appeared in September with high isolation frequency (22.5%) (Fig. 4).

The results showed fungal composition and isolation frequency of the fungal species varied among months, and most of these species did not colonize the tissues consistently, but appeared in different periods or only in a given month during the growing season. The growth of mycelia within plants and new infection of spore were considered to be the major reasons for increased isolation frequency of endophytic fungi (Kaneko 2002). Studies concerning seasonal change of endophytes in various tree species leaves suggested that endophyte infection increased during the growing season (Helander et al. 1993, Helander et al. 1994, Wilson & Carroll 1994, Faeth & Hammon 1997). Therefore, colonization by endophytic fungi inhabiting plants is considered to increase over time. However, the abundance of endophytic fungi in *S. corniculata* and *S. microphylla* varied among sampling times, and did not increase over time. The sampling location Songyuan is one of the largest saline and alkaline land areas in China. *S. corniculata* and *S. microphylla* can survive and thrive in soils with high salt concentration, and thus process growth, physiological and biochemical adaptations that allow them to grow in saline habitats (Desai & Chavan 2011). The evaporative demands in Songyuan are extremely high with about three times the annual precipitation, which tends to bring solutes dissolved in ground water and deep soil up to the surface. The topographical features and climatic conditions of the area result in a very slow primary soil alkalization and salinization.

It has been reported that precipitation may influence the infection of endophyic fungi (Sahashi et al. 2000, Göre & Bucak 2007). The study of seasonal influence on distribution of endophytic fungi in *Lautus nobilis* showed that more fungal endophytes established in the plant tissues in spring as compared to autumn and it was considered that the greater amount

Table 2 Isolation frequency and colonization rate of the endophytic fungi associated with *S. microphylla* over the growing season.

Taxa	June		July		August		September		October
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Stem
Alternaria alternata	25	7.5	10	10	17.5	7.5	12.5	12.5	15
A. angustiovoidea	2.5								
A. brassicicola								2.5	
A. calycipyricola						2.5			
A. hemuli		7.5							
A. pellucida	2.5								
A. tangelonis	2.5								
A. vaccariicola		2.5							
Ascomycete sp.1								2.5	
Bipolaris sp.1	2.5								
Camarosporium sp.			10	10					
Cladosporium sp.1		2.5						2.5	
Cladosporium sp.2							2.5		
Fusarium sp.1									
Fusarium sp.2									
Penicillium sp.	10	7.5							
Phoma chrysanthemicola	2.5								
P. jolyana								2.5	
Phoma sp.1	2.5		2.5				2.5	22.5	
Phoma sp.2		5						5	
Phoma sp.3	5	2.5							
Phoma sp.4		2.5							
Phoma sp.7								2.5	
Sporomiella sp.				2.5					
Ulocladium sp.					5	5			
Sterile mycelium	15	25	5	2.5		2.5		2.5	12.5
Colonization rate	70	62.5	27.5	25	22.5	17.5	17.5	55	27.5

of rainfall in spring may promote more effecttive dispersal of fungal spores. These authors suggested that infection by endophytic fungi increased along with increase of precipitation (Göre & Bucak 2007).

Endophytic fungal community among tissues

As shown in Table 3, leaves and stems were colonized by different fungal species. In *S. corniculata*, 11 fungal species were recorded in stems and 15 species were obtained in leaves. Only *A. alternata*, *Fusarium* sp.1, *P. chrysanthemicola*, and *Phoma* sp.5 overlapped in stems and leaves. Although *P. chrysanthemicola* was isolated from both leaves and stems, the isolation frequencies of *P. chrysanthemicola* isolated in September were signifycantly different in leaves and stems. The isolation frequency of the fungus in stems was 17.5%, while in leaves was 2.5%. The occurrence of this fungus in stems was higher than that in leaves. *Alternaria* species were the most common species in both of the tissues, but only one species of *A. alternata* was obtained in both leaves and stems. The other, *A. angustiovoidea*, *A. brassicicola*, *A. daucicaulis*, *A. molesta* and *A. yaliinficiens* were isolated from leaves; *A. citriarbusti*, *A. franseriae*, *A. hibisci* and *A. smyrnii* were isolated from stems. *Ulocladium* sp. was obtained from leaf and *Cladosporium* sp.1 and *Torula* sp. were isolated from stems.

In *S. microphylla*, totally, 13 fungal species were isolated in leaves and 18 species were obtained in stems. *Alternaria alternata*, *Camarosporium* sp., *Fusarium* sp.1, *P. chrysanthemicola*, *Phoma* sp.3 and *Ulocladium* sp. were overlapped in both stem and leaves. Similar with *S. corniculata*, the isolation frequency of *P. chrysanthemicola* isolated from stems (22.5%) of *S. microphylla* was significantly higher than that in leaves (2.5%). *A. angustiovoidea*, *A. pellucid*, *A. tangelonis*, *Bipolaris* sp.1 and *Cladosporium* sp.2 were isolated from leaves; *A. brassicicola*, *A. calycipyricola*, *A. hemuli*, *A. vaccariicola*, *Ascomycete* sp.1, *Cladosporium* sp.1, *Penicillium* sp. and *Sporormiella* sp. were isolated from stems.

Table 3 Isolation frequency and colonization rate of the endophytic fungi associated with *S. corniculata* over the growing season.

Taxa	Jun		Jul		Aug		September		October
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Stem
Alternaria alternata	10	2.5	7.5	7.5	12.5	5	2.5	7.5	10
A. *angustiovoidea*	2.5								
A. *brassicicola*		2.5					2.5		
A. citriarbusti								2.5	
A. daucicaulis							2.5		
A. franseriae									2.5
A. hibisci								2.5	
A. molesta							2.5		
A. smyrnii								2.5	
A. yaliinficiens			2.5						
Cladosporium sp.1		2.5							
Fusarium sp.1	5	2.5	5	2.5			2.5		
Fusarium sp.2							2.5		
Phoma chrysanthemicola							2.5	17.5	
Phoma sp.1							5		
Phoma sp.2							2.5	7.5	
Phoma sp.5	5	2.5	2.5	2.5					
Phoma sp.6	5								
Phoma sp.8							7.5		
Torula sp.								12.5	
Ulocladium sp.							2.5		
Unidentified species			2.5				7.5		10
Sterile mycelium		7.5	5	10	7.5	2.5	12.5	12.5	2.5
Colonization rate	27.5	17.5	27.5	22.5	20	7.5	55	65	25

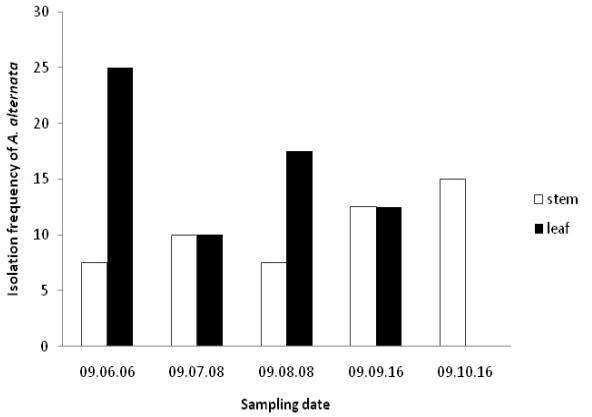

Fig. 3 – Seasonal occurrence of *Alternaria alternata* in leaves and stems of *Suaeda microphylla*.

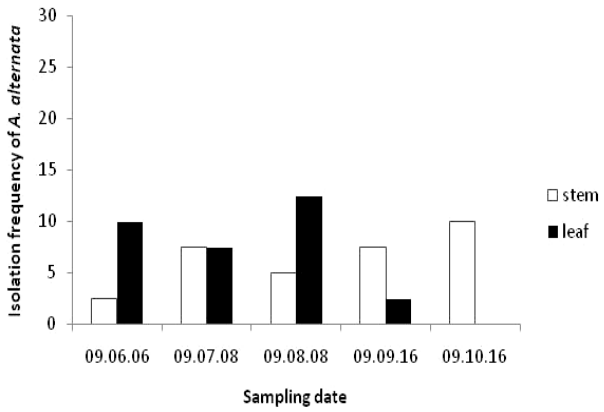

Fig. 4 – Seasonal occurrence of *Alternaria alternata* in leaves and stems of *Suaeda corniculata*.

Tissue specificity of endophytes has been indicated by other authors (Petrini 1986, 1991, Petrini & Fisher 1988, Fisher & Petrini 1990, 1992, Fisher et al. 1991, 1995, Sieber et al. 1991, Bettucci & Saravay 1993, Fröhlich et al. 2000, Photita et al. 2001). Photita et al. (2001) found that fungi isolated from *Musa acuminata* have an affinity for different tissue types: Xylariaceous taxa and *Guignardia cocoicola* were the most frequently isolated from leaves; *Pyriculariopsis parasitica* and *Dactylaria* sp. were most common in the pseudostem; *Colletotrichum musae* and *C. gloeosporioides* were most common in the midribs and petioles. Organ specificity was also demonstrated in another halophytes *Suaeda fruitcosa* (Fisher & Petrini 1987). *Colletotrichum phyllachoroides* was only isolated from leaves and *Camarosporium* sp.1 was significantly more common in stems than in leaves. The difference in

endophytic assemblages in different tissue types might be a reflection of tissue preferences of individual dominating taxa (Lugin buhl & Müller 1980, Widler & Müller 1984, Rodrigues & Samuels 1990, Halmschlager et al 1993), and might reflect their capacity for utilizing or surviving within a specific substrate (Rodrigues 1994).

In present study, some species such as *A. angustiovoidea*, *Fusarium* sp.2 were only isolated from leaf, and *Cladosporium* sp.2 was only isolated from stem. Although *P. chrysanthemicola* was isolated from both stem and leaf, the frequency was obviously high in stem. These results showed tissue specificity of endophytic fungi. However, because of the small sampling size, more sampling in different geographic localities is needed to make further assumption about the organ specificity of endophytic fungi with *S. corniculata* and *S. microphylla***.**

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