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Studies on *Aspergillus* Section Flavi from Peanut in Iran

M. Houshiyarfard[1], H. Rouhani[1], M. Falahati-Rastegar[1], E. Mahdikhani Moghaddam[1], S.Malekzadeh Shafaroudi[2]*

1. Department of Plant protections, Collage of Agriculture, Ferdowsi University, Mashhad, Iran.
2. Department of Agricultural Biotechnology, Collage of Agriculture, Ferdowsi University, Mashhad, Iran.

Abstract

During 2010 and 2011, *Aspergillus* species from section Flavi were isolated from rhizosphere in main peanut-growing region of Iran, Astaneh-e Ashrafiyeh, Guilan Province (northern Iran). The soil samples were taken from fruiting zone of peanuts in 3 districts and 10 locations, during the pod-filling period. The populations of *Aspergillus* section Flavi showed significant differences in cfu g^{-1} among sampling locations. Individual assays averaged from 188 and 139 *Aspergillus* section Flavi population/g soil in two years, respectively. So that, we were unable to detect *A.* section Flavi on soil dilution plates at greater dilutions ($<10^{-2}$). The *A. parasiticus* was the dominant species isolated in fruiting zone of all peanut plants. There were significant differences ($p < 0.05$) in the ratio of L and S strains of *A. flavus* and *A. parasiticus* isolates in locations. In two locations, higher frequencies and prapagule densities of *Aspergillus* section Flavi in soil were found. Prapagule density levels of *Aspergillus* section Flavi for two years indicated that continuous planting of peanuts result in no variable *Aspergillus* species from section Flavi in Astaneh-e Ashrafyieh.

Key words: Aflatoxin, Aspergillus species, Iran, Peanut.

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is a unique among major crop plant that its flower is pollinated aerially, and the seed matures underground. The soil immediately surround the pods. Peanuts with their subterranean growth habit is exposed directly to soil populations of *Aspergillus* section Flavi (Horn 2005a). Soil is a source of primary inoculum for *Aspergillus flavus parasiticus* fungi that produces highly carcinogenic Aflatoxins in peanuts (Gilman, 1969; McDonald 1970; Porter et al. 1979; Diener et al. 1987; Payne 1998; Horn et al. 1995, Horn and Dorner 1998; Horn 2003). Aflatoxigenic fungi commonly invade peanut seeds during maturation. Aflatoxins (AFs) are secondary metabolites produced primarily by the fungi *Aspergillus parasiticus.* These *Aspergilli* are soil-borne and normally derive most of their nutrition from decaying plant and animal debris. The *Aspergillus* section Flavi fungi are ubiquitous in soil (Diener et al. 1982). *Aspergillus* species from section *Flavi* present in soil included *A. flavus* morphotypes L and S strains, *A. parasiticus, A. caelatus, A. tamarii* and *A. alliaceus* (Horn, 1997; Horn and Greene, 1995; Peterson, 2000). There were several reports from peanut fields that indicated the different soil *A. flavus* populations (Bell and Crawford 1967; Joffe 1969; McDonald 1969). Infection of peanut seeds predominantly by primary inoculum differs from infection of corn and cotton-seed in which secondary inoculum plays a dominant role (Horn 2003). Sclerotia are important survival structures in the life cycle of *Aspergillus* species (Hessletine et al. 1970; Saito et al. 1986). The phenotypic variation within *A. flavus* allows the species to be subdivided into groups based on morphology. One such characteristic is sclerotial size, the large strain (L) having sclerotia >400 µm in diameter and the small strain (S) with sclerotia <400 µm (Horn 2003). In Iran , many studies have been made on the identification and prevalence of *Aspergillus* species in soils of maize and pistachio (Moradi et al. 2004; Mirabolfathy et al. 2005, Mohammadi and Banihashemi, 2006, Razzaghi-Abyaneh et al. 2006; Cheraghali et al. 2007; Mohammadi et al. 2009). Determination of the populations of *Aspergillus* section Flavi in the fruiting zone of peanut is needed to obtain some data about the soil inoculum potential that exist for fungal fruit colonizations. The relative distribution of *Aspergillus* species section Flavi and the L and S strains in peanut soils have not yet been investigated. The present investigation was conducted to evaluate the soil population of *A.* section Flavi fungi for the first time in Iran and Aflatoxin production in the fruiting zone of peanut fields, near the mid pod-filling period.

Materials And Methods

Peanut Fields

This study was conducted from 2010 to 2011 to evaluate population of *Aspergillus* section *Flavi* in cultivated peanut soils at the fruit-forming period in Iran. Peanut fields 0.2-0.3 ha apart from 500-1000 m were selected arbitrarily in 3 districts and each 10 different locations in Astaneh-e Ashrafiyeh county, Guilan province, Iran (northern Iran, Fig. 1). The city is situated at latitude and longitude of 37°16' N and 49°56' E with an average altitude of 5m (above the sea level). This city is lies close the caspian sea and located in the alluvial palins of the Guilan province, on the Sefid Rood delta. The climate is temperate mediterranean with average annual rainfall of about 1500 mm and a mean relative humidity of 70%. The properties of these soils are shown in table 1.

Soil Sampling and Enumeration of *Aspergillus* section Flavi

***Corresponding Author:** E-mail: mhoushiarfard@yahoo.com

Soil samples were collected from each of fifty peanut fields in ten locations at the mid period of pod-filling (development of fruits and seeds). In each location, each field soil sample consisted of a composite of 10 subsamples approximately 300-500 g that were randomly taken within fruiting zone with an auger (cylindrical sample) to a depth of 30 to 35 cm, at random from two adjacent rows, and each of five 10-m intervals down the rows. Approximately, the same areas of the fields were sampled during 2010 and 2011. In some cases, whole plant was carefully removed from the soil and excess soil was collected after shaking the roots. Soil subsamples were placed into sterile plastic bags and were transported to the laboratory. For each location, soil subsamples from five fields were pooled, air dried for two days, passed through a 2 mm mesh screen and then stored at 4°C. Soil pH and EC were measured in laboratory. The five random combined subsamples of sieved soil (20 g) were removed and then placed into a sterile 500 ml flasks containing 180 ml 1% pepton-water , soil suspensions agitated for 1 h at 150 rpm on an orbital shaker.

Isolation and Quantitation of *Aspergillus* section Flavi

Population density of *A.* section Flavi (populations per g of soil) at each location was determined by dilution plate technique and averaging prapagule counts on plates (Cotty, 1994). The 0.5-ml aliquots of additional dilutions (0, 10^{-1},10^{-2},10^{-3} in each subsample per plate) were spread onto three 9-cm diam plates of Dichloran–Rose Bengal Chloramphenicol agar (DRBC agar) (Horn and Dorner 1998) and AFPA (Aspergillus flavus/parasiticus agar) supplemented with 50 mg chloramphenicol (Pitt et al. 1983; Horn and Dorner, 1998, Gourama and Bulerman, 1995). Plates were incubated for 3-4 days at 37 °C and for 2-3 days at 30 °C, respectively. *Aspergillus* section Flavi colonies were visually identified and enumerated. Mean population density was estimated on the basis of the number of colony forming units per gram soil dry weight (cfu/g soil) using the following equation:

cfu/g.soil = (no. of colonies x dilution factor) / volume inoculated

The colonies of *Aspergillus* species (section Flavi) developing on dilution plates from each soil subsample were identified directly according to Raper and Fennel (1965) and Klich and Pitt (1988). Also, colonies were transferred to slant Czapek agar (CZ) medium, incubated for 14 days at 25 C and were purified using single-spore method.

Identification of *Aspergillus* species (section Flavi)

All fungi belonging to *A.* section Flavi genus *Aspergillus* were identified by morphologically, based on various macro and micro morphological characters. The identification of *Aspergillus* species (Section Flavi) has been traditionally based on morphological characterization (Table 2) (Klich 2002; Samson et al. 2004; Kurztman et al. 1987). Before identification, discrete colonies were grown on MEA (Powdered Malt Extract 20 g, peptone 1g, Glucose 20g, Agar 20 g, DW 1L). In the darkness for 7 days at 25 ºC. From this culture, a loop full of spores was suspended in 500 µL of 0.2% agar with 0.05% Tween 80, and this suspension was used for three-point inoculations on 9 cm petri dishes containing approximately 20 ml of Czapeck yeast extract agar (CYA, Pitt, 1973, Kich and Pitt,1988) per plate (CYA: Sucrose 30 g/l, Powder yeast extract 5 g/l, K_2HPO_4 1 g/l, $NaNO_3$ 2 g/l, KCl 0.5 g/l, $MgSO_4.7H_2O$ 0.5 g/l, $FeSO_4.7H_2O$ 0.01 g/l, $ZnSO_4.7H_2O$ 0.01 g/l, $CuSO_4.5H_2O$ 0.005 g/l, Agar 20 g/l) and Czapek-Dox agar (CZ: Czapek Concentrate 10ml, K2HPO4 1g, Sucrose 30g, Agar 17.5g, DW 1L) at 42 °C. CYA cultures were incubated in the dark at 25 ºC (CYA25) for 7 days. Several 3-mm plugs of sporulating culture were transferred to vials containing 5 ml of sterile distilled H_2O. These conidial suspensions were stored for further study at 8°C.

Sclerotia Production from Soil Isolates

Plates 6 cm in diameter (three replicate per isolate) containing Czapek-Dox agar ($NaNO_3$ 3%) were inoculated with mycelia and incubated at 30 °C in darkness for three weeks. Sclerotia were obtained according to Novas and Cabral (2002) by scraping the surface of the plate (three replicate plates per isolate) over a N° 2 What man filter paper during irrigation with water containing Tween 20 (100 µl/l), followed by rinsing with running tap water. Sclerotia were further cleaned in a beaker with repeated rinses and decanting, and later were air-dried. S- (<400 µm) and L- (>400 µm) Sclerotia diameters and the number of sclerotia produced per square centimetre was determined by measuring the diameter of the sclerotia using a reticle in a 400× Microscope for each isolate (Cotty 1989; Chang et al. 2001).

Detection of Aflatoxigenic Fungi and Aflatoxin B_1

Microbiological Method

All isolates of *Aspergillus* identified as belonging to section Flavi were cultured on *Aspergillus flavus/parasiticus* Agar (AFPA; Sigma) for 3 to 5 days at 25 ºC, in the dark, to confirm identification at the section level by colony reverse colour. In order to determine relative frequency of aflatoxin producing and non-producing strains of *Aspergillus flavus/parasiticus* isolates, the Yeast extract sucrose agar (YES) supplemented with 0.3% methyl- β -cyclodextrin and 0.6% sodium desoxycholate (Sigma, St. Louis, MO, USA) were used. (Fente et al. 2001; Ordaz et al. 2003; Rojas et al. 2004). The cultures were incubated for 5 days in dark at 28°C .The reverse side of cultures were periodically examined under longwave UV (365nm) for blue fluorescence. Isolates were scored as positive or negative by presence/absence of fluorescence ring in the agar surrounding the colonies.

TLC Method
Production of Aflatoxin B_1 from each isolate was verified by thin-layer chromatography using the agar plug and chloroform method with some modifications (Filtenborg et al. 1983; Lamanaka et al. 2007). Fungal mycilium was extracted with chloroform in stomacher for 3 min, filtered and concentrated at 60° C to near dryness and dried using vacuum evaporator (Heidolph WB 2000). The 20 µl of chloroform extracts (samples) and 20 µl of B_1 aflatoxin (reference standard solution) in methanol (Sigma-Aldrich, Germany) were spotted on imaginary line 1cm from the bottom edge of TLC chromatoplates (silica gel 60, 20×20 cm, 250 mm, E. Merck, Germany) by development with ethyl ether/methanol/water (96:3:1) and run at room temperature. Aflatoxin spots were visualized under UV light at 365 nm.

Statistical Method
All statistical analyses of data were performed using MSTAT-C software with Duncan's Multiple Range Test for a randomized complete block design with five reolications. A P-value less than 0.05 (95 percent accuracy) was considered significant. Data from counts of colony-forming units (cfu) and percent frequencies of *Aspergillus* spp. were transformed as $\log_{10}(x + 1)$ and arcsine square root (%) to obtain homogeneity of variance, respectively.

Results

Aspergillus (section Flavi) Populations

Aspergillus section Flavi was detected in soil from each of the 10 sampling locations. There are no significant differences in the *A.* section Flavi populations between two sampling years (Table 3). Significant differences among locations were seen in the quantity of propagules (CFU) per gram of soil (Tables 3). Mean CFU and incidence of *A. flavus/parasiticus* L-strain isolates differed significantly among locations (Tables 3 and 4). The *A. parasiticus/flavus were* present in soil in nearly 2:1 proportions during the mid periode of the growing season in fields sampled from different locations (Table 4). So that, among a total of 298 *Aspergillus* colonies obtained from 50 peanut fields, 182 showed yellow-orange reverse coloration on AFPA of which *A. parasiticus* was predominant (54% of section Flavi), followed by *A. flavus* (30%) and *A.nomius* (10%) (Table 2). In this study, six percent of the isolates were belong to *A. terreus*, *A. niger*, and *A. fumigatus* and *A. tamarii* species, other most prevalent aspergilli in soil from the three fields. The mean populations of *Aspergillus* species (section Flavi) were generally low. Mean analysis of CFU revealed significant differences between locations (Table 4). The mean populations of *Aspergillus* species section Flavi were mostly averaged from 188 to 139 cfu/g soil in 2010 and 2011, respectively. So that, we were unable to detect *Aspergillus* populations on soil dilution plates at greater dilutions ($<10^{-2}$).

Soil populations of the *Aspergillus* species (section Flavi) shown in Table 4 were generally higher in the heavy soils than in the sandy soils (Tables 1 and 4). Otherwise, populations of these *Aspergillus* species often varied considerably between locations with various soil types.

The lowest population of *Aspergillus* species (section Flavi) occurred in Keshel-e Azad Mahalleh and Estakhr-e Bijar locations with a mean less than 105 cfu/g soil (Table 4). Griffin and Carren (1974) found that *A. flavus* population in Virginia peanut field soils was around 0.5-57.3 propagules/g soil. Bell and Crawford (1967) reported significantly greater amount of propagules in naturally infested soils in Georgia (1.5×10^4 propagules/g soil). Soil population levels of *Aspergillus* (section Flavi) from locations remained fairly constant over two years.The distribution of sclerotial types and no aflatoxin production potential of isolates differed according to the sampling locations (Tables 3 and 4). The *A. flavus/parasiticus* (Flavi section) fungi were two species with the highest frequency in ten sampling locations. This showed variation in the population in various locations and soil types (Tables 1 and 4).

Aspergillus section Flavi isolates, predominately *A. parasiticus/flavus* L strain isolates from different sampling locations differed significantly in production sclerotia. For all soils, two types of *A. flavus/parasiticus* fangal colonies developed on CZ plates. Some produced larg (>400 µm) and small (<400 µm) sclerotia, but the others produced none. Sclerotia are germinated to produce additional hyphae or conidia (asexual spores) which can be dispersed more in soil. A total of 153 *A. parasiticus/flavus* colonies transfered from AFPA to CZ containing NaNO3 3%. About 78.6% of the *A. parasiticus* and 64% of the *A. flavus* isolates produced varying sizes and numbers of sclerotia (Table 4). Of the *A. flavus/parasiticus*, 80% and 76.6 % were L strain isolates, respectively. Although *A. parasiticus/flavus* fungi were detected in all 10 locations, S strain isolates were found in only 3 locations(data not shown). An important aspect of the presence of *A. parasiticus/flavus* fungi in the field soil is the potential of fungus for producing carcinogenic aflatoxins. About 94 (96%) of *A. parasiticus* and 51(92%) *A. flavus* isolates recovered from peanut soils produced aflatoxin. Of 104 L strain isolates tested, 46 were positive for aflatoxin B_1 production (data not shown).

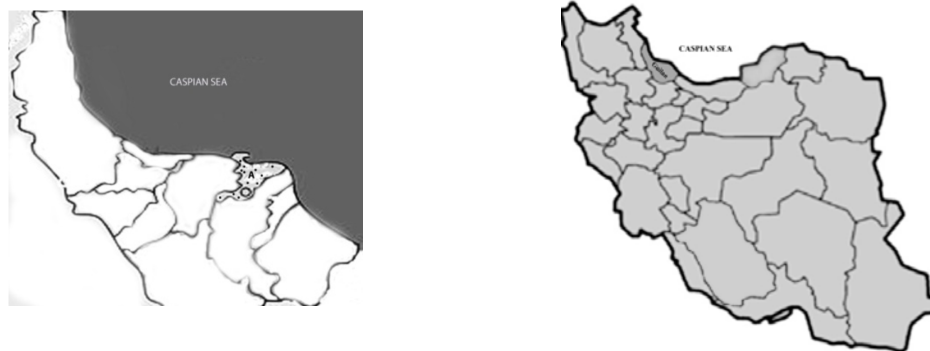

Fig 1. Map of Guilan province (northern Iran) and Astaneh-e Ashrafiyeh (A) with locations where soil samples were collected

Table 1. Mean properties of peanut field soils in 3 districts and 10 locations in Astane -e Ashrafiyeh, Guilan province, Iran

Districts	Location	PH	EC (ds/m)	Texture
Kiashahr	Sahid Rejaii	7.97	0.345	Silt-loam Sandy- loam
	Dehsar	7.08	0.226	Silt loam
Kisoom-e Chahardeh	Salestan	7.585	0.226	Loam Loam-sand Sandy- loam
	Kisoom-e Joikol	7.785	0.217	Silt-loam Loam Sandy- loam
	Amshal	7.815	0.334	Silt-loam Clay loam
Central	Tazeh-Abad-e Khoshkarvandan	8.35	0.381	Silt-loam Sandy-loam Loam
	Parkaposht-e Yavarzadeh	7.905	0.259	Silt-loam Loam
	Keshel-e Azad Mahalleh	7.795	0.236	Sandy- lom Loamy-sand
	Estakhr-e Bijar	7.84	0.224	Silt-loam Sandy-loam
	Koorka	7.725	0.341	Silt-loam loam

Table 2. Characterization of *Aspergillus* Section Flavi isolates

Species	Sclerotia on CZ30	Seriation on CYA25	Conidia on CYA25	Reverse on AFPA	Diameter on CZ42	Colony colour on CYA25	Fluorescence on YES
A. flavus	> 400 and <400	n.d./b/u	smooth	orange	1.4–2.9	yellow-green	+/-
A. parasiticus	> 400 and <400	n.d./u or u/b	n.d/Rough	orange	0.5–3.1	dark-green	+/-
A. nomius	+	n.d	Rough	cream		dark-green	+
A. tamarii	+	b/n.d.	Thick/Rough	brown		brown	

Table 3. Combined analysis of variance for CFU per g of soil, percent toxigenic *Aspergillus* spp., and L strain isolates of *A. parasiticus/flavus* (2010-2011)

S.O.V	df	MS				
		CFU/g soil	%Toxigenic *A.parasiticus*	%Toxigenic *A. flavus*	% L-strain of *A. parasiticus*	% L-strain of *A. flavus*
Year	1	69.408ns	51.234ns	73.028 ns	95.307ns	81.655ns
Replication×Year	8	26.156	17.926	18.230	31.984	20.605
Location	9	25.375*	16.33ns	16.26ns	28.423**	19.36**
Location×Year	9	4.352*	2.374ns	3.482ns	6.282*	4.603*
Error	72	460.579	320.991	331.14	55.24	41.151
C.V		23.6	21.7	19.4	20.6	17.2

Ns, * and **: Non significant, significant at 5% and 1% probability levels, respectively

Table 4. Mean comparison of CFU for *Aspergillus* section Flavi, percent toxigenic *Aspergillus* spp., and L strain isolates of *A. parasiticus* and *A. flavus* in the fruiting zone for 50 peanut fields in 10 locations in Astaneh-e Ashrafiyyeh (Guilan, Iran) in combined analysis (2010-2011)

Location	CFU per g of soil	%Toxigenic *A.parasiticus*	%Toxigenic *A. flavus*	% L-strain of *A. parasiticus*	% L-strain of *A. flavus*
Amshal	2.73 a	12.3 a	14.5 a	23.4 a	17.2 a
Dehsar	2.48 abc	10.6 a	12.6 a	7.9 bc	8.5 bc
Estakhr-e Bijar	1.98 bcd	9.9 a	9.0 a	4.0 c	5.7 bc
Keshel-e Azad Mahalleh	1.69 d	9.1 a	5.3 a	3. 7 c	2.8 c
Parkaposht-e Yavarzadeh	2.22 abcd	10.7 a	9.1 a	6.5 bc	8.7 bc
Kisoom-e Joikol	2.00 bcd	9.2 a	9.1 a	2.6 c	8.4 bc
Salestan	2.12 bcd	8.2 a	3.5 a	3. 8 c	2.9 c
Koorka	2.10 bcd	10.6 a	12.7 a	16.8 a	11.4 ab
Sahid Rejaii	2.52 ab	8.2 a	9.0 a	5.3 bc	8.6 bc
Tazeh-Abad-e Khoshkarvandan	2.17 abcd	9.2 a	7.2 a	2.6 c	5.8 bc

[a] Log10(CFU) used in analysis, untransformed data presented. [b] Based on analyses of samples from fruiting zone for five fields in each location. Means within a column followed by a different letter are significantly different ($\alpha = 0.05$).

Discussion

It would seem that in the small geographic area studied, different A. section Flavi populations are isolated from particular soil. Densities of section Flavi fungi in soil vary lowly among fields and may not influence the severity of peanut infection. Although Astaneh-e Ashrafiyyeh was cultivated in peanut for several long years (continous crop system), the mean section Flavi populations are low. Also, because of no crop sequence find in the studied peanut fields, there is no influence of crop sequence on *Aspergillus* species (section Flavi) populations. The results of this study indicated that these *Aspergillus* populations in the fruit and seed development stage were low. We had a little increase or decrease in *Aspergillus* species (section Flavi) populations during two years (data not shown). Peanut fields were not drought stressed during two years in the growth stages. Data from this study suggest that with adequate rainfall during the generative growth, soil populations of *Aspergillus* species (section Flavi) remain nearly constant in cultivated fields of Guilan province (Astaneh-e Ashrafiyeh). It is not known how these population levels are maintained in soil, nor is it understood whether these fungi reside mostly as conidia or hyphae.

In addition, possibly, low soil temperature in Guilan peanut growing area, northern-most province in the Iran, may be responsible for the low section Flavi populations. S strain isolates were least abundant in a limited area of Guilan province and suggested that a few population was related to soil environmental factors. In a study in which soil was inoculated with conidia from a color mutant of *A. parasiticus*, soil populations decreased little during a 10-month period (Horn et al. 1994). A certain proportion of conidia in soil may be quiescent. Griffin (1969) demonstrated that under laboratory conditions soil fungistasis prevents *A. flavus* conidia from germinating. The slow loss of conidium viability in soil (Wicklow et al. 1993) may be offset by colonization of crop debris (Angle et al. 1982; Griffin

and Garren 1976). It is not clear why *A.* section Flavi populations are generally low. It may be *Aspergillus* species from section Flavi stimulated less than other microbial groups (fungi and bacteria) in the zone around developing pods. Otherwise fungi and bacteria are favored over section Flavi in rhizosphere. Although sclerotia *A. flavus/parasiticus* are an important source of primary inoculums, but the survival of *Aspergillus* spp. particularly aflatoxigenic species in Guilan soils still is not well understood. The majority of Aflatoxin-producing isolates in our study except some isolates produced L-type sclerotia. This is very promising because in accordance to data from Cotty and Cardwell (1999) the L-type isolates usually produce less aflatoxins compared to S-type isolates. There was a non-random relationship between the sclerotial type and the production of aflatoxin; strains that did not produce sclerotia were significantly less likely to be toxigenic than strains that produced large sclerotia.
Because *A. flavus*/*parasiticus* populations commonly reach the soil surface through spillage at harvest, sclerotia may represent an important source of these fungal inoculum in field soils where peanut is grown. As we know, seeds from pods may be invaded by aflatoxigenic fungi when plants are drought stressed (Sanders et al*.* 1981), but data from this study suggest that with adequate rainfall during the growing season soil population levels of *Aspergillus* species from section Flavi from 10 districs remained nearly costant over the sampling period in cultivated fields of Astaneh-e Ashrafiyeh. Peanut seeds vary considerably in water activity (Aw) and exposure to soil temperatures. Water activities of individual seeds range from 0.75 to 1.00 (Dorner et al*.* 1989), and soil temperatures in the pod zone range from 20 °C in warm temperate regions to 38 °C in semi-arid tropical areas (Craufurd et al*.* 2003, Hill et al.1983). Peanuts are invaded by aflatoxigenic fungi primarily under conditions of late-season drought and elevated soil temperatures (Blankenship et al*.*1984, Hill et al*,* 1983; Sanders et al*.* 1981; Sanders et al*,* 1985b).

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