Density Fluctuations of Two Major *Aspergillus* Species Airborne Spores in Pistachio Growing Regions of Iran

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Abstract

Contamination of pistachio nuts by aflatoxigenic strains of *Aspergillus* species is the greatest constraint to production and export of pistachio nuts in Iran, the leading producer of pistachio nuts in the world. Kerman province is the main region in Iran where 85%, of pistachio nuts are produced. Determining the population density of *Aspergillus* spores in the orchards and terminals which are the main sources of contamination would provide information about critical control points throughout pistachio production process. Density fluctuations of airborne *Aspergillus flavus* and *Aspergillus niger* spores were investigated during 2000-2002. The monthly density fluctuations of *Aspergillus* spp. spores were determined by exposing open petri-dishes containing AFPA and Cz media for 15 minutes, between 11 and 13 hours at different localities in each orchard from spring to winter, and at pistachio nut processing terminals during harvesting time. The results showed that density fluctuations of *A. flavus* and *A. niger* rose from the beginning of spring reaching a peak in September. The population then gradually decreased and had little variation in relation to different periods and places. Population density of *Aspergillus* spores increased during processing stages (especially hulling and peeling) at different pistachio terminals. In most cases, there were positive correlations (r = 0.69 - 0.91) between environmental temperature and spore density. The peak of spore density coincided with pistachio nut maturation characterized by splitting and cracking**.**

Keywords: aflatoxin, *Aspergillus flavus*, *A. niger*, density fluctuations, pistachio nut

Introduction

Contamination of pistachio nut by *Aspergillus* species and their mycotoxins are the most serious challenge to pistachio production, consumption and exportation (Mojthahedi *et al.*, 1978 and 1979; Sommer *et al.*, 1986; Doster and Michailides, 1995, 1999; Moradi, 2005, Moradi and Javanshah, 2005; Moradi and Mirabofathy, 2007). The infection of pistachio nuts to *Aspergillus* species during maturation at pistachio orchards (Thomson and Mehdy, 1978; Mojthahedi *et al.*, 1979 and Doster and Michailides, 1995). Factors influencing infection of pistachio nuts include cracking of pistachio nuts (especially early splitting pistachios), environmental factors, cultural practices, frequency and time of irrigation, plant litter, animal manures, frequency of toxigenic strains and harvesting date (Thomson and Mehdy, 1978; Mojthahedi *et al.*, 1979, 1980; Sommer *et al.*,

1986; Doster and Michailides, 1995, 1999; Mirabolfathy *et al.,* 2005; Moradi, 2005, Moradi and Javanshah, 2005 and Moradi and Mirabofathy, 2007). These factors have been shown to be critical in infection especially in early splitting cultivars where the hull (Pericarp) gets split exposing the kernel to molds and insects increasing chances of aflatoxin production and contamination (Doster and Michailides, 1995; Moradi *et al.,* 2004; Moradi, 2005 and Moradi and Javanshah, 2005). Whereas the molds such as *Aspergillus* spp. may cause direct contamination resulting in aflatoxin production, insects may play the role of spreading fungal spores, which in turn infect exposed kernels. Finding the relationship between density fluctuations of *Aspergillus* species and maturation period of pistachio nuts could be helpful in reducing infection and addressing factors that affect this process. Density of airborne *A. flavus* conidial population has been found to fluctuate on other plants during the growing season and year and correlated with the amount of rainfall and hours of leaf wetness (Mojthahedi *et al.,* 1978; Jones *et al.,* 1980 and 1981; Diener *et al.,* 1987; Klick *et al.,* 1992 and Ahmad and Singh, 1996). In a study conducted in Missouri, the percentage of days on which spores were collected varied from year to year and spores were collected during more than 70% of the days at two locations and 40% of days at another location (Holtmeyer and wallin, 1981). Rodriguez–del–Bosque (1996) pointed out that *A. flavus* infection and aflatoxin concentration were undetectable in all treatments in fall growing season during 1991 and 1992, when average minimum temperatures were <16^{o}C during reproduction and maturation (November to December).

There are limited studies so far which have focused on aflatoxin contaminating fungi on pistachio nuts in relation to environmental factors and maturation period of pistachio nut. This study concentrated on density fluctuations of *Aspergillus* spp. in pistachio orchards and their relations with three environmental factors including; temperature, rainfall and relative humidity as well as *A. flavus* fluctuations density of pistachio nuts at the processing terminals.

Materials and Methods

Five commercial pistachio (cv.Ohadi) orchards in Kerman, Rafsanjan (two different orchards during 2 year study period), Sirjan and Zarand were selected as representatives of different geographical areas to trap spores during 2000-2002. Trapping was carried out regularly in Kerman, Sirjan and Zarand at 14-day interval and in Rafsanjan at 14 and 7 days interval in 2000-2001 and 2001-2002, respectively. The semi-selective agar media were *A. flavus* and *A. parasiticus* agar (AFPA) (Gourama and Bullerman, 1995) and the modified Czapek-dox agar medium (Cz). The modified Cz medium comprised of 50g Czapek, 100mg choloramphenicol, 50mg streptomycin and 5 mg dicoloran in 1 liter distilled water (Raper *et al.,* 1965). Twenty petri-dishes (10 AFPA and 10 Cz at each orchard) were exposed to a platform, 1.5 m above the ground for 15 minutes, between 11 and 13 hours at different geographical localities in the same distance in each orchard.

Fluctuation density of *A. flavus* spores at the five different terminals was studied during processing

stages in two successive years. Spores were trapped by exposing petri-dishes (five replicates) 1m above ground platform at hulling spaces for 5 minutes and other spaces of terminals for 10 minutes. The plates were incubated at 28 °C for 8 – 10 days. Relative density and frequency of *A. flavus* and *A. niger* were recorded after the incubation period. Identification of *Aspergillus* species was performed based on the micro and macro- morphological features, reverse and surface characters of colonies using the genus *Aspergillus* (Raper *et al.,* 1965 and Klich, 2002). Only data of *Aspergillus niger* (because of high isolation frequency) and *Aspergillus flavus* (because of its importance due to aflatoxin production) are shown. Other species were not present in all of the times when sampling was done.

All statistical analyses were done using SASS software and the means were compared using Duncan's Multiple Range Test. To determine the individual relationship between the dependent variable (*Aspergillus* spore concentration) and the independent variables, the Pearson correlation coefficients and regression analysis were calculated and P values <0.05 regarded as significant.

Weather data on temperature, eva-transpiration and relative humidity in different regions of Kerman province where the study was carried out, were taken from Kerman metrological office. It should be mentioned that there was no data for the Zarand region.

Results

During 2000-2002 *A. flavus* and *A. niger* spores were presented in all the studied regions. However, their population densities varied during different months. Their airborne spores were viable and could infect pistachio nuts or other substrates in artificial inoculations (In experiments to determine toxigenic and atoxigenic strains, data not shown), which was manifested by fungal contamination and aflatoxin production in the pistachio nuts at different orchards. Overall in all regions, population density of *A. flavus* and *A. niger* increased from early spring till mid of September, then the density decreased gradually (Fig. 1). However, more detailed inspections showed that it is actually a quite short period during August and September while the general increase and often drastic decrease takes place for almost all regions, year, and species. *A. flavus* spores were trapped in 63% and 79% of days out of all sampling days in Kerman; 69% and 89% in Zarand; 79% and 85% in Sirjan; and 84% for Rafsanjan during 2000-2001 and 2001-2002 respectively. Spores of *A. niger* group were not trapped during a few days in2000-2002 (Table 1). Spore density increased during harvesting time in the terminals with the greatest increase being recorded at the hulling terminals (Table 2). The mean of *A. flavus* and *A. niger* colony counts in hulling spaces were 96.5 and 348 on 9 cm diameter plates or 0.006358 m^3 of space during 5 minutes respectively. Based on the above quantity of exposure for 1 hour (the time needed for processing 1 ton of pistachio nuts or peeling 2.5 m^3 pistachio), spore quantities were approximately 4.5×10^5 for *A. flavus* group and 1.6×10^6 for *A. niger* group (Table 2).

The density of species decreased in the order of Rafsanjan, Sirjan, Kerman and Zarand for 2000-2001 and Sirjan, Zarand, Kerman, and Rafsanjan for 2001-2002 (Table 3), respectively, which indicates significant differences between years, regions and species. For the spores of *A. flavus* the density was significantly lower in Kerman and Zarand compared to Rafsanjan and Sirjan regions whereas for *A. niger*, infection was significantly higher in Sirjan and Zarand compared to Rafsanjan, which in turn was significantly higher than in Kerman. *A. niger* spores density was significantly higher than that of *A. flavus* in pistachio orchards and terminals (Table 2 and 3).

Generally temperature, relative humidity, eva-transpiration were significantly correlated to population density of spores either in positive or negative way with different linear regressions (Table 4). The spore density increased with raising the temperature and eva-tanspiration ($r > 0.69$). Temperature decreased in August, whereas spore density increased till September and then decreased where the monthly mean temperature was less than 25 ºC (Figs. 1 and 2).

Discussion

Aspergillus flavus and *A. niger* were common airborne spores in orchards of Kerman province during 2000-2002 with varying densities. *A. niger* population density was more than that of *A. flavus*, However, *A. flavus* has greater importance due to its aflatoxin production. It has been documented that the abundance of *A. niger* in pistachio nuts, plant debris, soil, processing terminals and different manures is significantly higher than that of *A. flavus* (Mojthahedi *et al.*, 1978; Doster and Michailides, 1995, 1999; Moradi *et al.*, 2004, 2005; Moradi and Mirabolfathy, 2007). A positive correlation was observed among increasing spore density and reproduction and maturation period of pistachio nuts in spring and summer seasons. The results indicated that the peak of spore density coincides with cracking and harvesting of pistachio nut in the orchards. Therefore, late harvesting (after the second half of September) not only causes an increase in cracking of pistachio nuts but also provides further exposure to high spore density of *A. flavus*, resulting in more aflatoxin production and contamination of kernels in the orchards (Doster and Michailides, 1995, 1999). Damage of birds, pests, shell discoloration, and falling pistachio nuts from trees on contaminated soil surface are other risk factors associated with late harvesting (Sommer *et al.*, 1986; Doster and Michailides, 1995, 1999; Moradi, 2005; Moradi and Javanshah, 2005; Moradi and Mirabofathy, 2007).

Positive and negative correlations of spore density with agro-climatically parameters especially temperature can explain the fluctuation of spore density as well as biological and ecological requirement of the *Aspergillus* spores during the year. The monthly mean temperatures were equal or higher than 25 ºC in all regions excepts for Kerman in 2000-2001 during June to September, while in the October the temperature means deceased 5 to 7 degrees in all areas, which may explain drastic reduction in spore density in all regions. There was no evidence to indicate temperature would be a limiting factor for growth and sporulation of *A, flavus* and *A. niger* during summer, which could be supported by the wide

temperature range under which *Aspergillus* species grow. This indicates the temperature may act as a positive factor for contamination of nuts by *Aspergillus* species in orchards (Mojthahedi *et al.,* 1978 and 1980; Gourama and Bullerman, 1995). The main reason could be mentioned for increasing the spore density in the orchards during August and September is harvest operation accompanied with processing of nuts in processing plants distributing a large number of spores in the orchards. In the summer, other factors such as falling pistachio nuts on the ground, cracking of pistachio green hulls, insects and bird damages, plant debris and animal manures from the previous year as well as horticultural practices play an important role in increasing *Aspergillus* species population densities in pistachio orchards (Thomson and Mehdy 1978, Sommer *et al.,* 1986, Doster and Michailides 1995 and 1999, Mirabolfathy *et al.,* 2005, Moradi 2005, Moradi and Javanshah 2005 and Moradi and Mirabofathy 2007). A study by Jones *et al.,* (l980 and 1981) showed that date of planting, humidity stress, insect damages, delay at harvesting time, late season rainfall and pericarp damages are effective factors for aflatoxin corn contamination and totals of viable trapped *A. flavus* spores (15^{th} May-15^{th} August) were negatively correlated with the amount of rainfall and hours of leaf wetness in the field.

Low temperature during fall and winter seasons could be one of the factors causing decrease in spore density. Michailides and Morgan, (1990) showed that the specific temperature requirements for some fungi explain their seasonal prevalence. For instance, *Botrytis cinerea* is a low temperature fungus and is more common in spring. In contrast *Aspergillus niger* and other *Aspergillus* spp. prefer high temperatures, and so are common during summer and early fall. Rodriguez-del-Bosque (1996) investigated on the factors affecting *A. flavus* infection and aflatoxins contamination of corn, and reported that aflatoxins were undetectable in all treatments in fall growing season during 1991 and 1992, when average minimum temperatures were <16°C during reproduction and maturation (November to December).

A study focusing on population density of *Aspergillus flavus* and *A. niger* during 1998-2001 in pistachio refuse and leaf litter, soil and three kinds of animal manures in Kerman province indicated that *A. niger* was recovered more frequently than *A. flavus*, and pistachio refuse was the major source of soil contamination. Sheep manure had the highest population density among other kinds of manures such as cow and poultry manure commonly used in Kerman pistachio orchards (Moradi *et al.,* 2004).

Gardening and soil fertilization during fall and winter seasons, spreading of plant litter (especially rubbish and other plant materials remaining from pistachio nuts processing) and animal manures were shown to cause increasing of the spore density in pistachio orchards through this study (Fig. 1). *Aspergillus* species were soil borne in pistachio growing areas and their spore density is greatly affected by the fungal population in the soil, any gardening and soil practices would result in increasing the population density of spores in pistachio orchards during nut growing season (Mojthahedi *et al.,* 1978 and Moradi *et al.,* 2004). Further more their

population was influenced by frequency and time of the irrigation.

The results indicated that population density of *A. flavus* and *A. niger* varied in different years and regions. Variations in spore density among different agro-ecological zones may be related to the variation in prevailing climatic conditions and orchard management (soil cultivation, pest management, different irrigation regimes and so on) in the different zones. For example, the monthly temperature means of Kerman, Rafsanjan, Zarand and Sirjan regions showed that Kerman had the lowest monthly temperature during the years of study, which could account for the lower *Aspergillus* species population densities of this region. Setamou *et al.,* (1997) pointed out that there was a trend of higher rate of aflatoxin contamination and *A. flavus* infection from south to north, which may affect infection and contamination incidences of *Mussidia nigrivenella* cultivars grown in each zone.

Fluctuations of spore density in the terminals during peeling stage were influenced by the spores released during processing. Early splitting, cracking, shrunk and dried hull, damage by insects and birds and remaining the fallen pistachio nuts on the ground have been found to increase spore density throughout peeling and processing stages (Moradi 2005) which cause high surface infection of healthy pistachio nuts during processing.

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Table 1. Frequency of days (%) that *A. flavus* and *A. niger* spores were collected using settle plate method in pistachio orchards during 2000-2002

	Rafsanjan		Zarand		Kerman		Sirjan	
Year	2000-2001	۲۰۰1-2002	2000-2001	۲۰۰1-2002	2000-2001	۲۰۰1-2002		
2000-2001 2001-2002								
A. flavus	84	84	69	89	63	79	79	85
A. niger	100	95	95	95	100	95	95	100

Table 2. Fluctuation density of *A. flavus* spores in pistachio processing terminals (spore/plate)

Region	Rafsanjan		Rafsanjan		Zarand		Kerman		Sirjan	
	Other parts	Hulling	Other parts	Hulling	Other parts	Hulling	Other parts	Hulling	Other parts	Hulling
Year										
2000-2001	5.2 f	127 bc	13.2 f	122 bc	0.4 f	62 e	2.25 f	146 b	7 f	92 d
2001-2002	12.6 f1	22 bc	8 f	21.6 f	5 f	188 a	7.4 f	20 f	10.25 f	100 dc
Mean	8.9C	124 A	10.6 C	71.8 B	2.7 C	125 A	4.8 C	83 B	8.6 C	96 A

Data with the same letter(s) are not significantly different at p=0.05 (Duncan). Capital letters for mean values; small letters for differences among years, sampling area and region values.

Table 3. Population Density of *A. flavus* and *A. niger* in different regions of Kerman province (spore/plate).

Region	Sirjan			Kerman			Zarand			Rafsanjan		
Species	*A. flavus*	*A. niger*	Mean	*A. flavus*	*A. niger*	Mean	*A. flavus*	*A. niger*	Mean	*A. flavus*	*A. niger*	Mean
2000-2001	1.28 f	3.40 c	2.34 D	0.69 hg	3.46 c	2.07 DE	0.59 h	3.11 c	1.84 ef	1.80 e	6.01 a	3.93 A
2001-2002	1.34 f	4.60 b	2.97 B	0.53 h	2.64 d	1.59 F	0.93 g	4.40 b	2.67 C	0.77 h	2.40 d	1.58 F
Mean	1.31 D	4.0 A		0.61 E	3.05 C		0.74 E	3.76A		1.07 D	3.48 B	

Data with the same letter(s) are not significantly different at p=0.05 (Duncan). Different capital letters indicate differences between mean values in each row, small letters among years, fungi and region values

Table 4. Correlation between spore density of *A. flavus* and *A. niger* with temperature, relative humidity and eva-transpiration in Sirjan, Kerman and Rafsanjan provinces

Temperature (°C), RH, relative humidity (%); Eo, eva-transpiration (mm)

R, Pearson correlation coefficients; e, equation for dependent and independent variables; s, significant levels

			A. niger		*A. flavus*	
			2000-2001	2001-2002	2000-2001	2001-2002
Sirjan	T °C	r	0.69	0.85	0.72	
		e	y = 0.1723x + 0.27	y = 0.3573x - 1.6564	y = 0.0931x - 0.379	
		s	0.03	0.01	0.02	ns
	RH %	r		0.83		
		e		y = -0.2114x + 11.926		
		s	ns	0.002	ns	ns
	Eo	r			0.74	0.88
		e			y = 0.0059x - 0.1605	y= 0.0186x + 0.1
		s	ns	ns	0.035	0.0039
Rafsanjan	T °C	r		0.76		
		e		y = 0.3624x - 0.7638		
		s	ns	0.01	ns	ns
	RH %	r		-0.81157		
		e		y = -0.2528x + 13.375		
		s	ns	0.004	ns	ns
	Eo	r		0.90		
		e		y = 0.026x - 3.3562		
		s		0.0021		
Kerman	T °C	r	0.91	0.76	0.85	
		e	y = 0.295x - 1.3713	y = 0.1917x - 0.5256	y = 0.0576x - 0.2614	
		s	0.0001	0.01	0.001	ns
	RH %	r	-0.92755	-0.77356	-0.66103	
		e	y = -0.2533x + 11.492	y = -0.1077x + 6.2282	y = -0.0379x + 1.8789	
		s	0.0001	0.0087	0.0374	ns
	Eo	r	0.97	0.72	0.82	
		e	y = 0.021x - 1.4596	y = 0.0111x + 0.0354	y = 0.0038x - 0.2004	
		s	0.0001	0.05	0.0124	ns

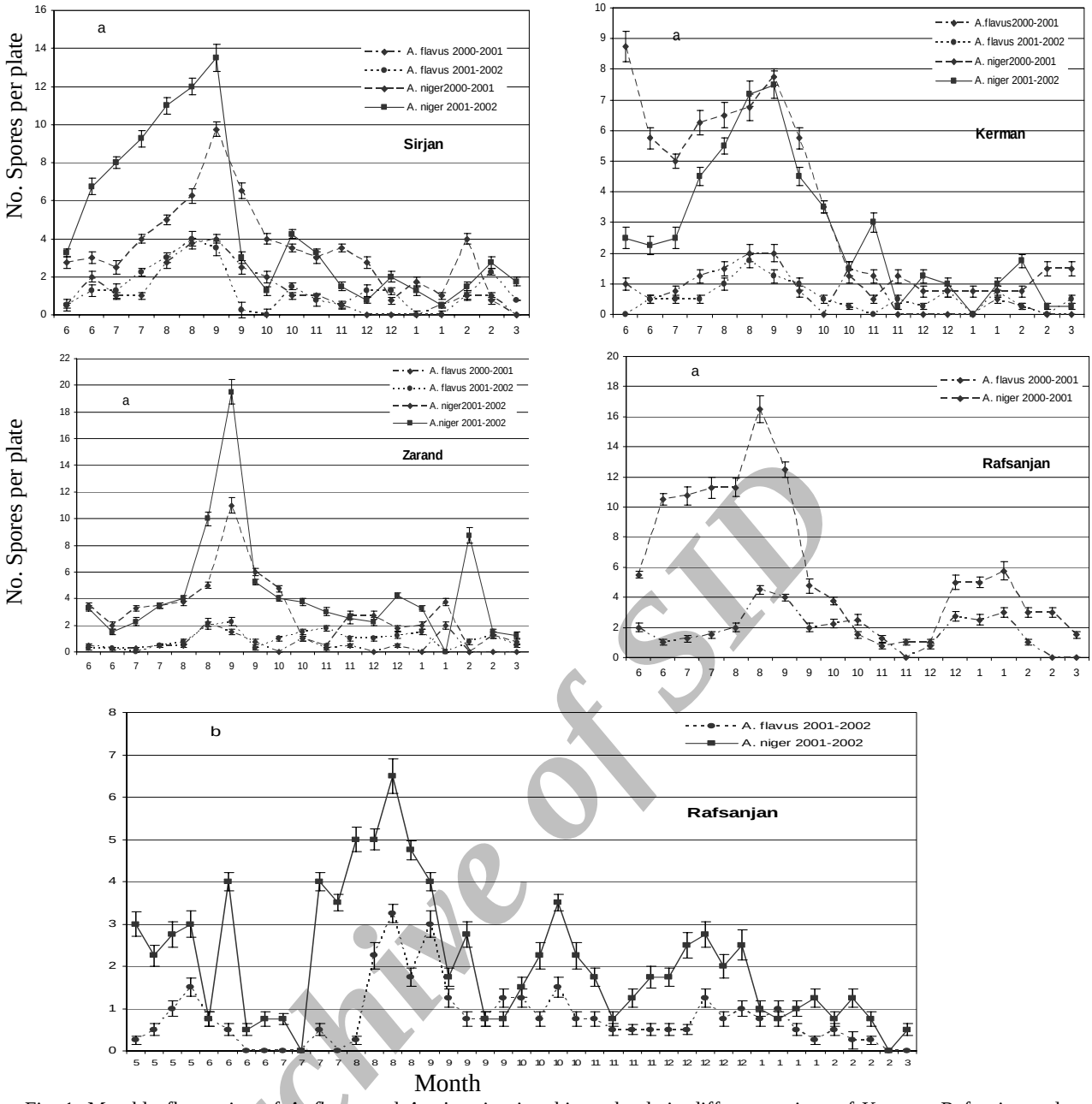

Fig. 1: Monthly fluctuation of *A. flavus* and *A. niger* in pistachio orchards in different regions of Kerman, Rafsanjan and Sirjan provinces
a: 14 days sampling interval
b: 7 days sampling interval

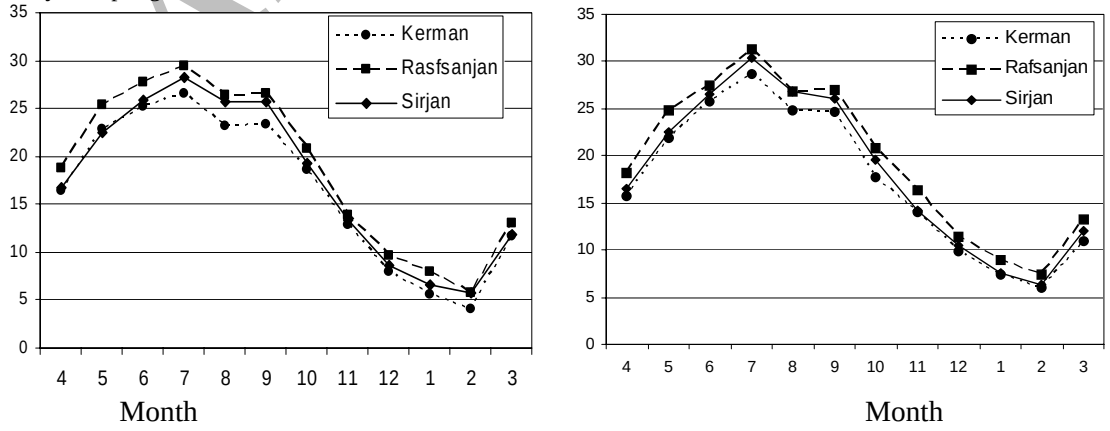

Fig. 2: Monthly fluctuations of temperature in Kerman, Rafsanjan and Sirjan provinces during 2000-2002

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