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Ecological Studies on *Pythium* Species Associated with Some Plants Rhizosphere in El-Minia, Egypt

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The present study describes various population and ecological aspects of *Pythium* species in the rhizosphere of agriculture landscape in El-Minia, Egypt during the growing season of 2006-2008. The isolation of *Pythium* species was achieved by zoospores baiting technique (ZBT), direct isolation from soil (DIS) and soil serial dilution method (SSDM) using a selective agar medium (NARM). *Pythium* species were identified morphologically and sequencing of rDNA-ITS of the isolated species including the 5.8 S rDNA were analyzed to confirm the species identification. A total of 746 isolates were identified into 11 species: *P. aphanidermatum*, *P. aquatile*, *P. catenulatum, P. deliense, P. diclinum, P. graminicola, P. irregulare, P. myriotylum, P. oligandrum*, *P. spinosum*, *P. ultimum* var. *ultimum* and representatives of three heterothallic *Pythium* groups F, G and P. The most predominant species in all location was *P. aphanidermatum* fallowed by *P. ultimum* var. *ultimum* and *P. diclinum.* While *P. graminicola* was the lowest predominant species. Spread of *Pythium* species fluctuated seasonally and maximum number of isolates were recorded in spring fallowed by winter and least isolated could be found in summer.The study gives an overview of population status of *Pythium* species and possible crop infestation as well as its potential as biological control measure.

Key words: *Pythium*, Rhizosphere, Oomycetes, El-Minia.

The genus *Pythium* is an important fungus belongs to class oomycetes, family Pythiaceae, order Saprolegniales of the Chromista kingdom[1] or Kingdom Straminopila[2]. The genus spread worldwide with 305 described species (www.mycobank.org) and distributed throughout the world ranging from tropical to temperate and aquatic environmental habitats[3, 4]. *Pythium* exist as saprophytes or parasites in water, soil, on plants, insects, fish, animals and human beings[5,6]. Moreover, some other myco-parasitic *Pythium* species frequently was been used as biocontrol agent for some pathogenic fungi of different crop plants[7, 8, 9]. *Pythium* species commonly occur in rhizosphere, where humidity and root exudates encourage its growth[10,11]. The previous researches recorded highly occurrence and distribution of *Pythium* species in rhizosphere of most cultivated plants in the Nile valley of Egypt, which were responsible for many plant diseases[12, 13, 14]. Morphological features such as sporangia, antheridia, oogonia, oospores and their sizes and shapes were traditionally used as taxonomic tools for *Pythium* species identification[3, 4, 15]. Subsequently, molecular tools of identification facilitated more accurate taxonomy and classification of these species[16, 17, 18]. Amongst the

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molecular taxonomic parameters as interior transcribed spacer (ITS) using ribosomal DNA gene sequences have become significant tools for identification and characterization of *Pythium* species[19, 20, 21].

However, a similar study was scanty for *Pythium* in El-Minia, Egypt.The present work aimed to a comprehensive survey on the occurrence and distribution of *Pythium* species in the rhizosphere of some crop and vegetable plants cultivated in El Minia Governorate, Egypt, during the period of 2006-2008.

MATERIALS AND METHODS

Sampling sites

Four agriculture fields of nine El-Minia provinces as El-Edwa, Maghagha, Beni-Mazar, Matai, Samalout, El-Minia district, Abou-Qurqas, Mallawi and Der Mawas were chosen as geographical locations. The collection of rhizosphere soil samples were gathered from sixteen cultivated plant species during four seasons of two years (2006-2008). The total number of cultivated vegetables and crop plants was 1152 plant samples including, *Abelmoschus esculentus*, *Allium cepa*, *Brassica oleracea*, *Cucumis sativus*, *Eruca sativa*, *Glycine max*, *Lactuca sativa*, *Phaseolus vulgaris*, *Raphanus sativus*, *Sacharum spontanum*, *Sorghum bicolor*, *Spinacia oleracea*, *Trifolium alexandrinum*, *Triticum aestivum*, *Vicia faba* and *Zea mays*.

Pythium isolation

Direct isolation from the soil (DIS)

Soil particles were removed from the plant roots and distributed over the surface of NARM plates medium (NARM) selective medium [Nystatin (10 mg L-1), Ampicillin (250 mg L-1), Rifampicin (10 mg L-1) and Miconazole (1 mg L-1) in corn meal agar (CMA)] for isolation of *Pythium* species [22, 23]. Three replicates for each sample were made and incubated at 25°C. The emerging hyphal tips were transferred to 2.5-3% water agar (WA) for purification and get a colony of about 1 cm diameter. For farther purification from bacterial contamination the whole WA medium in the plate was then turned upside down and incubated until the colony reached to the brim of the dish wall. The non-contaminated mycelia penetrated the agar medium and come to its peak. Purified isolates were then maintained on corn meal agar (CMA) slants till identified.

Soil serial dilution method (SSDM)

Pythium species were isolated from the collected rhizosphere soil samples by serial dilution method[24]. The soil samples were diluted from 10^{-1} to 10^{-5} dilutions, and the diluted soil samples were spread over NARM selective medium agar plates. The inoculated plates were incubated at 25°C for 3 days during which cultures were daily examined. Hyphal tips of the emerging colonies were transferred to a fresh WA plates and subculture was repeated several times to obtain pure culture which were preserved on CMA slant till identification.

Zoospores baiting technique (ZBT)

Rhizosphere soil (5 gm) was placed in sterilized Petri-dishes containing 10 ml of sterile distilled water. Autoclaved *Zea mays* leaf discs were used as baits. After 5 days of incubation at 25°C, baits were removed, washed thoroughly with sterile distilled water and dried with sterile filter paper. Four baits were then placed near the edge of a dish containing NARM selective agar medium. All plates were incubated at 20°C for 3 days and examined daily to detect the presence of fungal growth. The emerging hyphal tips of *Pythium* colony were moved to 2.5-3% WA for farther purification from bacterial contamination as described above. Slim pieces of the agar containing a single hyphal were taken and subcultured to CMA slants for preservation[25].

Morphological identification of *Pythium* species.

For identification of isolated *Pythium* species, the position, form and shape of sporangia, antheridia, oogonia and oospores were resolute in grass blade culture[23]. Morphological identification was carried out using two keys of[3, 15].

Molecular identification of *Pythium* species.

DNA extraction

Mycelia were developedin the V8 agar medium at 25ºC for 7 days or until adequate development was discerned. To extract the total genomic DNA, mycelia from the end of *Pythium* colony from a culture plate were emerged in 200µl of Prep Manultra specimen preparation reagent (Applied Biosystems, CN, USA) in a 2.0 ml microcentrifuge tube. Specimens were vortexed for 20 sec and then boiling for 10 min at 100ºC in water bath. Specimens were rotated for 30 min at 15000*g*.

Supernatants were moved into new microcentrifuge tubes and were preparedfor (PCR) amplification by the polymerase chain reaction[23].

DNA amplification and sequencing

The nuclear rDNA district of the internal transcribed spacer (ITS), encompassingthe 5.8S rDNA, was amplified with the universal primers ITS4 (5´TCCTCCGCTTATTGATATGC3´) and ITS5 (5´GGAAGTAAAAGTCG TAACAAGG3´). Depending on the experiment primers of ITS1 (5´ TCCGTAGGTGAACCTGCGG3´) and ITS2 (5´GCTGCGTTCTTCATCGATGC 3´) were utilized [26, 19]. The amplicons were 700-900 bp long. On the other hand, 563 bp of the *cox II* gene was magnifiedin certain Pythia with the primer pair FM66 (5´TAGGATTTCAAGATCCTGC3´) and FM58 (5´CCACAAATTTCACTAC ATTGA´)[27].

Amplification of the sequencing template was made by DNA Thermal Cycler 2700 with a cycling profile of pre-PCR at 94ºC for 5 min, pursuedby denaturation at 94ºC for I min, I min primer annealing at 55ºC for ITS, 52ºC for *cox* II and elongation at 72ºC, 2 min for 40 circuits, with a 7 min extension at 72ºC after the last cycle. To ascertain the presence of PCR products, 5 µl of the PCR reaction mixture was laden in 2% L03 (Takara Bio) agarose gel, electrophoresed at 100 V, 20-30 min, and stained with ethidium bromide. Sequencing templates were purified with Gen Elute PCR Clean-up kit (Sigma Chemical Co., St Louis, Missouri, USA), then the manufacturer's instructions. Sequencing was done using Big Dye Terminator v3.1 Cycle Sequencing Reaction kit utilizing the same primers in the primaryPCR step. After purifying the sequencing reaction blend through ethanol precipitation it was run on ABI 3100 DNA Sequencer (Applied Biosystems)[27].

RESULTS

Identification of *Pythium* species and their occurrence percentage.

Seven hundred and fourty-six isolates belonging to eleven species and three represented groups of *Pythium* were identified as *P. aphanidermatum* (Edson) Fitzp, *P. aquatile* Hohnk**,** *P. catenulatum* Matthews, *P. deliense* Meurs, *P. diclinum* Tokunaga, *P. graminicola* Subramaniam**,** *P. irregulare* Buisman, *P. myriotylum* Drechsler, *P. oligandrum* Drechsler, *P. spinosum* Sawada var. *spinosum*, *P. ultimum* Trow var. *ultimum*, *Pythium* "group F", *Pythium* "group G" and *Pythium* "group P".These were isolated successfully from the rhizosphere soil of different cultivated plants, previously mentioned. These *Pythium* species were identified based primarily on the morphological characteristics and confirmed by molecular identification as a result of sequencing of rDNA-ITS including the 5.8 S rDNA of *Pythium* species isolates from GenBank (Table, 1). All isolates identified had 100% similarity with known *Pythium* species in Gen-Bank data. Only isolate of MS51 showed 99% similarity with Gen-bank accession number, AY598690.1 as *P. diclinum*. Data in Table (1) also demonstrated that *P. aphanidermatum* was generally the most dominant species with frequency of 19.03%. On the other hand, *P. graminicola* and *P. myriotylum* were the lowest frequent species with 1.47% and 2.01% respectively. Moreover, *P. catenulatum* and *Pythium* "group F" were found to have a similar frequency.

***Pythium* species frequency in different localities of El-Minia Governorate:**

The frequency of *Pythium* species in the nine provinces of El-Minia Governorate was recorded in table (2). It was proved that *P. aphanidermatum*, *P. ultimum* and *P. diclinum* were isolated from all locations. *P. aphanidermatum*, *P. ultimum* and *P. deliense* were the most frequent of *Pythium* species in El-Minia district with frequency percentage of 23.23, 20.52 and 19.18% respectively, while *P. graminicola*, *P. catenulatum* and *Pythium* "group P" were the predominant species in Matai. In contrast, *P. catenulatum*, *P. graminicola* and *Pythium* "group P" were not recorded in Samalout as well as *P. deliense*, *P. myriotylum* and *Pythium* "group F"were not foundin Abou-Qurqas too.

Distribution of *Pythium* species in rhizosphere soil of cultivated plants in El-Minia governorate

Table (3) showed the occurrence of *Pythium* species in rhizosphere soil of 16 plants cultivated in the studied locations. It was observed that *P. aquatile***,** *P. catenulatum*, *P. myriotylum* and *Pythium* "group G" were the most frequent species in rhizosphere soil of *S. spontanum* plants with occurrence percentage of 16.33, 14.29, 10.20 and 12.24% respectively while the highest frequency of *P. oligandrum* and *Pythium* "group P" were recorded in rhizosphere soil of *R. sativus*. On the

other hand *P. irregulare* and *P. spinosum* were the most frequent species in rhizosphere soil of *T. alexandrinum*. In contrast, *P. aphanidermatum* was only predominant species isolated from in rhizosphere soil of *G. max*. Moreover, *P. deliense*, *P. diclinum* and *P. ultimum* were the most frequent species isolated from in rhizosphere soil of *Z. mays*, *C. sativus* and *P. vulgaris* respectively.

Seasonal variation of isolated *Pythium* species in El-Minia governorate

The seasonal fluctuation of *Pythium* species and total number of *Pythium* isolates during agricultural seasons of 2006-2008 was tabulated in table (4). It was proved that *P. ultimum, P. spinosum, P. irregulare, P. catenulatum,* and *P. aquatile* were the most frequent Pythia in winter while *P. oligandrum, Pythium* "group P" and *Pythium* "group F" were the most frequent species in spring**.** On the other hand, *P. aphanidermatum* and *P. deliense* were the dominant species in summer. Meanwhile *P. diclinum* was the most frequent species in autumn. Moreover *P. catenulatum, P. graminicola* and *P. myriotylum* were absent during summer. It was also concluded that the frequency of *Pythium* spp. were dominant in winter while the lowest occurrence of *Pythium* spp. were recorded in summer.

DISCUSSION

The current comprehensive study of the *Pythium* provides some interesting data in diverse climatic and land use regimens around the Nile valley. Three techniques for *Pythium* isolation (DIS, SSDM and ZBT) were used to attain the maximum isolation probability of *Pythium* species present in concerned soil rhizospher. DIS and SSDM isolation methods enhanced the isolation of mycelial fragments and the dormant stages of *Pythium* like oospores while ZBT method was used to encourage the growth of zoosporic *Pythium* species. For purification and morphological identification of isolated pythia, the NARM selective medium was found to prevent the growth of bacteria in *Pythium* cultures while not influencing *Pythium* itself. Eleven species and three groups of *Pythium* were recorded from 746 *Pythium* isolates which collected from the rhizosphere soil of agriculture fields.

P. aphanidermatum is typical plant pathogenic species in warm regions that isolated from soil rhizosphere of numerous diseased plants such as Gramineae, vegetables and ornamental plants[3, 28]. Our data displayed that *P. aphanidermatum* was the most common species

Table 1. Match of the ITS sequence of El-Minia *Pythium* isolates with GenBank Database and their occurrence percentage

Isolate	*Pythium* species	Accession No.	Max. Identity	Isolates number of *Pythium* sp.	Occurrence of *Pythium* sp. (%)
MS14	*P. aphanidermatum*	AB274404.1	100%	142	19.03
MS09	*P. aquatile*	AF597536.1	100%	23	3.08
MS27	*P. catenulatum*	AY597756.1	100%	29	3.89
MS26	*P. deliense*	AY598689.1	100%	73	9.79
MS51	*P. diclinum*	AY598690.1	099%	88	11.80
MS42	*P. graminicola*	AB597526.1	100%	11	1.48
MS43	*P. irregulare*	AF452142.1	100%	58	7.78
MS36	*P. myriotylum*	AB989556.1	100%	15	2.01
MS31	*P. oligandrum*	AY986954.1	100%	62	8.31
MS87	*P. spinosum*	AY598701.1	100%	48	6.43
MS133	*P. ultimum* var. *ultimum*	AY598657.1	100%	117	15.68
MS112	**Pythium* "group F"			28	3.75
MS102	**Pythium* "group G"			20	2.68
MS152	**Pythium* "group p"			32	4.29
	Total number of isolates				746

* Species only morphologically identified

Table 2. Frequency of isolated *Pythium* species in different locations of El-Minia Governorate

S. No.	Location	Frequency percentage (%) of *Pythium* species isolates													
		P.aphanidermatum	*P. aquatile*	*P. catenulatum*	*P. deliense*	*P. diclinum*	*P. graminicola*	*P. irregulare*	*P. myriotylum*	*P. oligandrum*	*P. spinosum*	*P. ultimum* var.*ultimum*	*P.* "group F"	*P.* "group G"	*P.* "group p"
1	El-Edwa	7.04	8.69	6.89	9.59	10.23	-	-	13.33	3.23	10.42	7.69	-	20.00	18.75
2	Maghagha	9.89	-	6.89	8.22	9.09	18.18	6.89	13.33	9.68	-	16.24	10.71	30.00	-
3	Beni-Mazar	4.92	8.69	17.25	13.69	19.32	-	8.62	20.00	14.52	-	8.54	21.43	-	15.62
4	Matai	9.16	21.74	24.14	10.96	11.36	27.27	-	13.33	9.68	22.91	8.54	-	15.00	21.88
5	Samalout	10.56	13.05	-	15.08	10.23	-	18.97	13.33	16.12	10.42	11.97	21.43	20.00	-
6	El-Minia city	23.23	8.69	10.34	19.18	18.18	18.18	12.07	-	14.52	10.42	20.52	10.71	-	6.25
7	Abou-Qurqas	14.08	13.05	6.89	-	10.23	-	22.41	-	11.29	25.00	6.84	-	15.00	12.50
8	Mallawi	5.63	-	10.35	9.59	3.41	27.27	15.52	-	8.06	8.33	10.26	14.29	-	18.75
9	Der Mawas	15.49	26.09	17.25	13.69	7.95	9.10	15.52	26.68	12.90	12.50	9.40	21.43	-	6.25

Table 3. Occurrence *Pythium* species associated with cultivated plants in El-Minia governorate

S. No.	Cultivated plants species of(1152) samples	Occurrence percentage (%) of *Pythium* species associated with cultivated plants													
		P.aphanidermatum	*P. aquatile*	*P. catenulatum*	*P. deliense*	*P. diclinum*	*P. graminicola*	*P. irregulare*	*P. myriotylum*	*P. oligandrum*	*P. spinosum*	*P. ultimum* vir.*ultimum*	*P.* "group F"	*P.* "group G"	*P.* "group p"
1	*A. esculentus*	15.56	6.67	6.67	8.89	8.89	-	4.44	4.44	11.11	6.67	15.55	6.67	.	4.44
2	*A. cepa*	20.83	8.33	-	20.83	12.50	-	-	-	16.67	-	12.51	8.33	-	-
3	*B. oleracea*	20.93	-	-	13.95	20.93	-	13.95	-	11.63	4.65	-	6.98	-	6.98
4	*C. sativus*	31.04	-	-	10.34	20.69	-	10.34	-	6.90	-	20.69	-	-	-
5	*E. sativa*	-	7.32	9.76	4.88	14.63	4.87	4.88	-	34.15	-	12.19	-	-	7.32
6	*G. max*	64.71	-	-	23.53	-	-	-	-	-	-	11.76	-	-	-
7	*L. sativa*	15.28	-	4.17	6.94	8.33	2.78	6.94	4.17	11.11	9.72	11.11	6.95	5.56	6.94
8	*P. vulgaris*	18.37	-	-	10.20	16.33	-	10.20	-	-	6.12	38.78	-	-	-
9	*R. sativus*	14.82	-	-	7.41	14.81	-	7.41	-	33.33	-	-	-	3.70	18.52
10	*S. bicolor*	44.44	-	11.11	18.52	11.11	-	-	-	-	-	7.41	-	-	7.41
11	*S. spontanum*	4.08	16.33	14.29	-	-	4.08	12.25	10.20	-	6.12	12.25	-	12.24	8.16
12	*S. oleracea*	17.24	-	-	5.17	17.24	-	8.62	-	3.45	12.07	20.69	5.17	3.45	6.90
13	*T. alexandrinum*	12.70	-	3.18	11.11	11.11	3.17	14.29	-	6.35	15.87	19.05	3.17	-	-
14	*T. aestivu*m	14.62	4.49	3.37	8.99	13.48	3.37	5.62	3.37	2.25	8.99	19.10	4.49	3.37	4.49
15	*V. faba*	7.27	5.46	7.27	-	18.18	-	9.09	3.64	9.09	9.09	20.00	10.91	-	-
16	*Z. mays*	41.46	-	-	24.39	-	-	7.32	-	4.88	-	12.19	-	9.76	-
Percentage of *Pythium* sp. associated with total number of plant samples		12.37	2.00	2.52	6.34	7.64	0.96	5.04	1.30	4.35	4.35	10.16	2.43	1.74	2.78

in rhizosphere soil of all studied plants with occurrence percentage of 19.03% of the total Pythia isolates. Few isolates of *P. aquatile* were isolated with occurrence percentage of 3.08%. Similar results have been reported that, *P. aquatile* was recorded few times all over the world[29, 30]. *P. deliense* proved to be pathogenic to maize [3, 31]. These results were coincident with our reported data as *P. deliense* was isolated from most locations especially from Z. mays fields with occurrence percentage of 9.78%. Previous studies reported that, *P. diclinum* was isolated from flooded young rice plants[32]. The same species was isolated from rhizospher soil of diseased wheat seedling in Egypt[33].

Several *Pythium* species considered as important fungal pathogen of higher plants causing series damage to agricultured crops[28]. The present study concerned with isolates of Pythia from 16 different cultivated plants. Our data indicated that, *P. aphanidermatum, P. ultimum* and *P. diclinum* were isolated from 12.37, 10.16 and 7.64% of total number of rhizospher soil samples respectively. The above *Pythium* species were isolated as pathogenic plant diseases[34, 35]. In addition, *P. irregulare* was isolated from 5.04% of rhizospher soil samples and this results were in accordance with [3, 36] who reported that *P. irregulare* is a common soil and plant inhabiting species. The same species was isolated in Egypt from rhizosphere of maize[33]. On the other hand, *P. spinosum* was initially isolated from seedling of *Antirrhinum magus* in Taiwan and recorded as a causal agent of plants damping-off diseases[3, 35]. Results in table (3) showed that, the isolation of *P. spinosum* from 4.35% of soil rhizosphere of studied plants especially during winter and spring. *P. catenulatum, P. graminicola* and *P. myriotylum* were isolated from 2.52, 0.96 and 1.3% respectively of rhizospher soil of the concerned plants particularly during cool season[37, 38].

Like most *Pythium* species, the mycoparasitic pythia are common inhabitants of cultivated soils survied. *P. oligandrum* was the most common mycoparasitic Pythia species isolated from 4.35% of rhizospher soil samples. Most of them were isolated during spring and autumn, while a few isolates of *P. oligandrum* were isolated during summer. Previous studies supported the occurrence of *P. oligandrum* in various climates including tropical, subtropical and cool countries. *P. oligandrum* was used as a

Table 4. Seasonal variation of Pythium species isolated from rhizosphere soil of El-Minia during agricultural seasons of (2006-2008).

S No.	*Pythium* sp.	Occurrence percentage (%) of *Pythium* species				Total number of *Pythium* sp. isolates
		Winter	Spring	Summer	Autumn	
1	*P. aphanidermatum*	10.55	12.96	45.80	18.88	142
2	*P. aquatile*	5.86	2.78	-	1.40	23
3	*P. catenulatum*	6.25	4.63	2.29	-	29
4	*P. deliense*	5.86	6.48	19.85	12.59	73
5	*P. diclinum*	11.33	12.04	6.87	16.78	88
6	*P. graminicola*	2.73	1.85	-	-	11
7	*P. irregulare*	9.77	6.48	4.58	9.09	58
8	*P. myriotylum*	3.91	2.32	0.00	-	15
9	*P. oligandrum*	4.30	13.43	3.05	12.59	62
10	*P. spinosum*	10.15	7.87	-	3.49	48
11	*P. ultimum* var. *ultimum*	17.97	14.81	12.98	15.39	117
12	*Pythium* "group F"	4.69	5.09	0.00	3.49	28
13	*Pythium* "group G"	3.51	2.78	3.05	0.71	20
14	*Pythium* "group p"	3.13	6.48	1.53	5.59	32
	Seasonal number of *Pythium* isolates	256	216	131	143	746
	Percentage of *Pythium* spp. Isolates	34.32	28.95	17.56	19.17	---

biocontrol agent against many fungal plants diseases[39,40,41,42]. On the other hand, Groups of *Pythium* represent the fungi which have unknown sexual stages, so far; they can only be identified according to their kinds of zoosporangia. These groups were scanty in the rhizosphere soil of few crop plants with occurrence percentage ranged from 1.7 – 2.5 % of the studied rhizospher soils. Seasonal fluctuation of total *Pythium* species in the present study was proved that, frequency of *Pythium* species were dominant in winter and spring, these results were coincident with previous studies that recorded their highly abundance cooler seasons[3,12,43].

In conclusion, the rhizosphere soil could be considered as a favorable environment for the occurrence of *Pythium* spp. indicating that the plant root exudates enhance the growth and proliferation of *Pythium*. In general, *Pythium* may be considered as one of the main components of the rhizosphere soil mycoflora. This may be due to the presence of organic matters, which support the saprophytic ability of these Pythia. Hence, *Pythium* species are facultative saprophytic organisms, where they live saprophytic on organic materials but when infected conditions become available, *Pythium* fungi become very dangerous causing plant diseases especially in seedling stages. There are many isolates of *Pythium* species in this study have historical facts as causal agents for plant diseases such as *P. aphanidermatum, P. deliense, P. diclinum, P. irregulare, P. spinosum, P. ultimum* var. *ultimum* [31, 33,39,44,45] so the presence of this fungi in the soil of Nil valley in El-Minia Governorate caused many disaffects in increasing the chances of disease to crop plants in this area of Egypt.

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