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DIAGNOSTIC OF PLANT PARASITIC NEMATODES USING MOLECULAR BIOLOGY TECHNIQUES

by

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Abstract

The potato cyst nematodes (PCN) *Globodera pallida* (Stone) and *G. rostochiensis* (Wollenweber) are economically important pests and distributed worldwide. In Lebanon, the potato crop is economically important and cultivated on a large scale. The current study confirms the recent discovery of the potato cyst nematode *G. rostochiensis* in Lebanon and demonstrated its distribution throughout the country. All 204 soil samples collected from 38 different locations were infested with PCN. Of the samples tested 72.5% contained live eggs. The population density ranged between 0.18-4.1 cyst/g soil and 0.13 -15.5 eggs/g soil. Of the infestation found, 76.1% had a population density of less than 10 eggs/g soil. The PCR results confirmed the presence of the potato cyst nematode *G. rostochiensis* in Lebanon. The three set PCR primers readily identified field samples in a single PCR reaction. PCR amplification of *G. rostochiensis* gave a 265 base pair (bp) fragment, while *G. pallida* had a 434 bp fragment. The origin of this infestation is unkown. No *G. pallida* was found in the samples tested. The results of the occurrence and distribution of *G. rostochiensis* in each area are discussed.

Key words: nematodes, PCN, *Globodera pallida*, *G. rostochiensis*, PCR, rITS.

Introduction

Nematodes have occupied a wide range of ecological niches and in addition to parasitizing higher plants, fungi, invertebrates and vertebrates, there are free-living forms in soil, marine and fresh water habitats (Croll, 1970). The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* originate from the Andean region of South-America (Evans and Stone, 1977), and were introduced into Europe around 1850 (Jones, 1970). The estimated annual yield loss that they cause to potato production in the European Community is valued at 300M ECU ($342M) (Mulholland *et al.*, 1996). In the United Kingdom alone annual yield loss caused by PCN is estimated £15-56 million (Williamson, 1995).

The PCN are the subject of stringent quarantine regulations in most countries where they occur (Spears, 1968). For seed potato production, PCN are quarantine pests within the European Union and legislation imposes a minimum 10 years ban on the production of seed potatoes if a single cyst of PCN is found in a field (Anon, 1991).

In Lebanon, the potato crop is economically important to the local economy, as it is cultivated on large areas throughout the country. Approximately 21% of Lebanese land is in arable rotation and the potato crop is grown on 14,000 ha yielding 265,000 t of tubers. Lebanon imports between 18,000 and 22,000 t of seed potatoes every year.

A recent survey in Rayak area in the central Bekaa Valley revealed that 17% of surveyed field were infested with *G. rostochiensis* (Ibrahim, *et al.*, 2000). This study was the first to report the presence of PCN in Lebanon, establishing its origin and distribution throughout the potato growing areas as an important pest (Fig.1). In Lebanon, no extensive and systematic surveys have been done on plant parasitic nematodes for the last 20 years. In a survey on plant parasitic nematodes conducted during the early 1970s (Taylor, *et al.*, 1972), PCN was not reported in Lebanon. Their survey concentrated on the coastal areas, whereas the present survey was conducted in the main areas of agricultural production throughout the country. The distribution of *Globodera rostochiensis* and *G. pallida* is worldwide and they rarely occur alone but more often in combination in one field (Ibrahim, *et al.*, 2001, Minnis, *et al.*, 2002).

The PCR technique offers the prospect of a simple, rapid and reliable diagnostic tool for plant parasitic nematodes, which will enable advisers to identify and quantify populations of PCN from field samples (Burrows & Perry, 1988; Stratford *et al.* 1992; Ibrahim, *et al.*, 1997, Ibrahim, *et al.* 2001, Bates, *et al.*, 2002),

The main objectives of this study were: 1) to determine the relative distribution of PCN throughout the potato growing areas in the country, 2) to quantify the population level in field samples and 3) to determine species identification using PCR techniques.

Materials and Methods

Field soil sampling

Two hundred and four soil samples were collected from 82 fields from 38 different locations (Table 1 & Fig.2). The samples were collected between March and May 2001. Soil samples were taken from a randomly selected field that potatoes were cultivated in the previous two years. Samples were collected using a "cheese-corer" style auger with a half-cylindrical blade. Soil samples consisting of approximate 50 cores (2.5 x 20 cm) were collected from each field in "Z" pattern and bulked together to give approximately 1 kg of soil. Soil samples were labeled and stored at 20-22 °C for cyst extraction.

Cyst extraction, detection and estimation of cyst contents

The soil samples were air-dried and cysts were extracted using a modified container based on the Fenwick can technique (Fenwick, 1940). Each soil sample was thoroughly mixed and a 250 g sub-sample was weighed and the cysts were extracted. The tank was filled with water before the sample was introduced. The sample was stirred with a plastic rod for five minutes. Cysts were then collected in sieves and transferred into muslin, labeled and left to dry. The number of cysts and their content were estimated by standard methods (Southey, 1970). The PCN cysts present in each extract were counted under a stereomicroscope and 50 cysts (all cysts if there were less than 50) were removed for egg estimation before crushing. The PCN cysts were soaked in water overnight and the eggs were counted in 1 ml aliquots from the resulting suspension. The population density (egg /g) of dried field soil was calculated for each sample in which PCN was found. Samples that contained cysts other than *Globodera* species were noted (results not shown).

Species identification

DNA Extraction

DNA was extracted as described by Ibrahim *et al.*, (2001). The suspensions of crushed cysts remaining from the egg counts were used as a source of DNA template for the PCR reactions. The remaining suspension was centrifuged at 500 rpm for 5 minutes, the pellet was re-suspended in water in a 1.5 ml microcentrifuge tube and centrifuged at 12 000 rpm for 5 minutes. The excess water was removed and 20 µl of lysis buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 1% Nonidet P-40, 100 µg/ml proteinase K) were added. The pellet was homogenised with a micro-homogeniser (Biomedix, UK) and the extract incubated at 95 °C for 5 minutes. The crude DNA extracts prepared in this way were suitable for PCR amplification without further treatment.

PCR Amplification and agarose gel electrophoresis

The PCR amplification was used as described by Mulholland *et al.*, (1996), except Taq polymerase thermostable DNA, (Advanced Biotechnologies) was used. The set of primers is designed to bind to the ITS1 region and the 5.8S rRNA gene. The *G. rostochiensis*-specific primer was 5'-TGTTGTACGTGCCGTACCTT-3', the *G. pallida*-specific primer 5'-GGTGACTCGACGATTGCTGT-3' which bind to the ITS1 region and the universal primer was 5'-GCAGTTGGCTAGCGATCTTC-3' located in the 5.8S gene. Each sample was run at least three times. PCR products were separated on 1.2 % agarose gel buffered in 1X TEB, which contained 0.02-µg/ml ethidium bromide. DNA was visualised under UV light and records made with a digital camera attached to a Gel Doc-1000 box (Bio-Rad Ltd). The data were subjected to analysis of variance(Genstat 5^{TM}
, Version 3.2, Lawes Agricultural Trust, Oxford Press, NY).

Results

Distribution and percent occurrence of PCN in the potato field is presented in Table 1. The counts revealed that all soil samples tested (204) contained PCN cysts, but only 148 (72.5%) of the samples contained live eggs. The results also showed that PCN were present in all the sampled areas, but the level of infestation was varying from one area to another. The percentage distribution of PCN with live eggs ranged from 54.5% to 78.8%. The highest distribution was detected in the northern Lebanon. In some samples other nematodes cysts (*Heterodera* spp) and fungal fruiting bodies (both of which may be confused in appearance with PCN cysts) were detected, but were either removed or not counted where possible.

Distribution of PCN in north Lebanon

The data presented in Table 2 show that all the sampled regions are infected with PCN. However, the level of infestation differed significantly (P <0.001) from one area to another. The highest number of

cysts(2.7 and 1.9 cysts /g soil) were found at the Aarka and Halba area respectively. The lowest infestation (0.5 cysts/g soil) was found in Al Hissa region. There was no correlation between the number of cysts and the egg contents. The highest level of egg content (7.7 eggs/g soil) was detected in the Aarka area while the lowest egg content (0.3 eggs/g soil) was observed in the Tal Aabbas soils. There was no significant difference in the PCN populations with live egg contents collected from different areas.

Distribution of PCN in Bekaa Valley

The distribution of the PCN in Bekaa Valley is presented in Table 3. These results also showed that all sampled areas were infected with PCN. There was significant difference (P <0.05) between infested areas. The highest level of cysts was found in the Soultan Yaacoub (2.3± 0.7 cysts/g soil) and Al Kaa (2.3 ± 0.7 cysts/g soil) area, while the highest egg content were detected in Tal Amara (15.8± 26.5 eggs/g soil) and Terbol (7.7eggs/g soil), respectively. Although there was a high level of cyst counts collected from Chlifa and El Kaa area, no eggs were detected. Lower level of egg counts (<2 eggs /g soil) was also observed in Btedai, Deir el ahmar and Yate. There was a high degree of variability of cyst ranging between 32 and 497 cysts/g soil among the areas and within the field samples.

Distribution of PCN in mount-Lebanon

Table 4. presents the distribution of the potato cyst nematodes in mount-Lebanon. The results showed that there was no significant difference between the means of cysts in the samples collected from various potato growing regions, however, there was significant difference (P<0.05) in the egg content. For example, Halat (15.5±9.2 eggs/g soil) and Aakoura (9.7±15.0 eggs/g soil) regions showed the highest mean of egg content in comparison to the level of cysts present in the samples from Hrajel. No eggs were detected in the cysts collected from Halatte area. The average distribution from one area to another ranged from 131 to 390 cysts/g soil.

Distribution of PCN in south Lebanon

Although data in Table 5 did not show any significant difference between the mean of samples collected from south Lebanon, there was significant difference (P<0.05) in the egg contents between the two areas. Samples collected from Marjouyoun showed degree of variability than those collected from Sahl Al Khiam .

Distribution of PCN in different regions of Lebanon

The percentage distribution of PCN with live egg content in Lebanon is presented in Fig 3. Although, there was no significant difference in the number of cysts among different regions, the north Lebanon (39%) showed the highest level of cysts with live eggs in comparison to the Bekaa Valley (17%) and the south Lebanon (19%).

Population densities

The population densities of PCN are presented in Fig 4 and expressed in eggs/g soil. The most common population density was less than 10 eggs/g soil (76.1% of infested samples) (0.3 ±2.4 standard deviation). Only 3.1% of infested sites showed the highest population density (62.3 eggs/g^{1} soil). The highest mean population density was 15.5 eggs/g soil and the lowest mean was 0.13 eggs/g. The average population density was 3.3 eggs/g soil.

Molecular identification

The species of PCN were determined in each sample that was found to contain eggs. One hundred and forty eight population identifications were made by PCR techniques. The results showed that all the

samples tested (72.5%) were infested with *G. rostochiensis* (Fig 5.) No *G. pallida* population was detected by PCR techniques among the tested samples. The PCR amplification was successful using crude DNA of single or bulk nematodes as templates. In combination, the species-specific primers of *G. pallida* (PITSp4) and *G. rostochiensis* (PITSr3) and the ITS5 primer readily differentiated between all the populations tested from Lebanon and from known British populations of *G. pallida* and *G. rostochiensis* (Fig 5). *G. rostochiensis* had a PCR product of 265 bp using PITSr3 primer, whereas *G. pallida* showed 434 bp with PITSp4 primer. Some populations (7% of the total sample tested (11 samples)) failed to amplify with PCR.

Discussion

Globodera sp. cysts (100%) were found in all samples tested by standard detection methods. This figure is significantly higher than 17% of the last survey (Ibrahim *et al.*, 2000), indicating a significant increase in distribution. The previous survey was concentrated in a small area (Riakk), while the current one covers almost all cultivated areas throughout the country. Recent survey (484 field samples) carried out in England and Wales revealed that 64% of samples were infested with PCN (Minnis, *et al.*, 2000). Our survey demonstrated that all soil samples tested (204) contained PCN cysts, however, only 72.5% of these samples contained live eggs. This may indicate the wide distribution of PCN throughout the country. Although, there was no significant difference found between different regions, the north Lebanon showed the highest level of cysts containing live eggs (39%) in comparison to the Bekaa Valley (17%) and south of Lebanon (19%). The difference of infestation level among the areas could be due to several factors, such as cultural practice, absence of crop rotation, use of susceptible cultivars, different soil types and chemical application.

As potato production has become more specialized and there is reduction in the number of growers, it has been concentrated on a smaller area of land leading to shorter rotations, which have encouraged build-up of PCN infestations. Discussions with growers included in the survey indicated that they were unaware of the presence of nematodes and repeated potato production on the same field may contribute to accumulation of high levels of PCN and thus result in yield loss. In addition, the sugar beet production has become very restricted or even absent; forcing the growers to shift to potato production. Potato production has been carried out all over the Bekaa plain, Akkar, Marjouyoun as well as in certain localities in Mount Lebanon without crop rotation. This may explain the build–up of PCN infestation. Also, there are other factors that may contribute to this large distribution of PCN in Lebanon, such as the use of susceptible varieties, contamination of equipment, uncertified cultivars or seeds, soil type, irrigation methods and absence of quarantine.

The species identification by PCR found only *G. rostochiensis* present in the soil samples tested. The PCR data are also in agreement with an earlier report (Ibrahim *et al.*, 2000) confirming the presence of *G. rostochiensis* in Lebanon. The origin of *G. rostochiensis* detected in Lebanon is not known. However, it may have been introduced on potato seed tubers imported from Europe. The distribution of *G. pallida* and *G. rostochiensis* is worldwide and they rarely occur as separate species but more often as a mixture species in one field. The current study found only *G. rostochiensis* present in the soil samples tested. However, the absence of *G. pallida*, cannot be ruled out as only a small proportion of potato growing land in the Lebanon has been sampled and, in fields that have been infested relatively recently containing unevenly distributed populations, the probability of detecting cysts in a soil sample is low (Haydock & Evans, 1998).In the contrary, a recent survey carried out in England and Wales revealed that *G. pallida* was the dominant species in the field samples tested. The PCR results indicated that 66% of field samples contained pure *G. pallida*, 8% contained pure *G. rostochiensis* and 26% contained mixtures of the two species (Ibrahim *et al.*, 2001).

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Table 1. Areas of Lebanon, number and percentages of samples found to contain cysts and eggs of *Globodera rostochiensis*

Region	Number of locations	Number of samples tested	Number of samples containing cysts	Number of samples containing eggs	% samples containing eggs
North-Lebanon	10	66	66	52	78.8
Bekaa-Valley	19	101	101	75	74.3
Mount-Lebanon	7	22	22	12	54.5
South-Lebanon	2	15	15	9	60.0
Total	38	204	204	148	72.5

Table 2. Distribution and infestation level of the potato cyst nematodes *Globodera* spp. in north Lebanon

Region	X ±S Cysts/g soil	Maximum Cysts/g soil	Minimum Cysts/g soil	X± S Eggs/g soil
Bellanet Al Hissa	0.4 ± 0.2 [a]	0.59	0.3	4.5 ± 6.1
TalAabbas Al Gharbi	0.6 ± 0.2 [a]	1.0	0.4	0.25 ± 0.5 [a]
Tal Hayat	0.9 ± 0.3 [a]	1.4	0.6	6.9 ± 17.5 [ab]
Minieh	1.1 ± 0. 6 [a]	2.0	0.3	4.9 ± 9.8 [a]
Al Abdeh	0.9 ± 0.7 [a]	2.7	0.2	4.9 ± 11.6 [a]
Aarka	2.7 ± 1.0 [b]	4.0	1.2	7.7 ± 14.9 [ab]
Halba	1.9 ± 1.5 [ab]	4.1	0.4	0.3 ± 0.7 [a]
Al Klayaat	0.8 ± 0.3 [a]	1.1	0.4	1.1 ± 2.2 [a]
Al Hissa	0.5 ± 0.4 [a]	1.3	0.2	0.7 ± 1.1 [a]
Al Aaboudieh	0.6 ± 0.2 [a]	1.2	0.3	1.8± 3.5 [a]

Means ± Standard deviation followed by a common letter are not significantly different at $P<0.05$.

Table 3. Distribution and infestation level of the potato cyst nematodes *Globodera* spp. in the Bekaa Valley

Place	X ± S Cysts/g soil	Maximum Cysts/g soil	Minimum Cysts /g soil	X ± S Eggs/g soil
Tel Al-Aamara	0.7 ± 0.2 [a]	1.0	0.5	15.8±26.5
Terbol	0.7 ± 0.3 [a]	1.2	0.3	7.7±7.3
Barr Elias	1.4 ± 0.9 [ab]	2.6	0.2	0.7±1.3
Talia	0.9 ± 0.05 [a]	1.0	0.9	3.0±4.7
Nabi Chit	0.7 ± 0.3 [a]	1.2	0.5	0.2±0.2
Riak	0.7 ± 0.4 [a]	1.2	0.2	0.2±0.2
Btedai	1.0 ± 0.583 [a]	2.5	0.5	0.8 ±1.5
Deir Al Ahmar	1.3 ± 0.672 [ab]	2.7	0.7	0.4±0.7
Chlifa	0.8 ± 0.452 [a]	1.1	0.3	0
Yaat	0.8 ± 0.381 [a]	1.2	0.6	0.8±1.4
Al Kaa	2.3 ± 0.7 [ab]	3.3	1.7	0
Koub Elias	1.7 ± 0.9 [ab]	2.3	1.0	7.1 ± 4.0
Aamic	0. 9 ± 0.3 [a]	1.2	0.6	1.4 ± 2.0
Mansoura	1.3 ± 1.0 [ab]	3.0	0.3	3.3 ± 4.3
Jib Jannin	1.1± 0.7 [ab]	1.7	0.06	0.4± 0.4
Kamed Al Laouz	0.7± 0.3 [a]	1.0	0.3	0.3 ± 0.2
Lucy	1.0± 0.6 [ab]	2.0	0.4	0.3 ± 0.2
Al Khiara	0.7 ± 0.3 [a]	1.0	0.3	4.9 ± 7.1
Soultan Yaacoub	2.3 ± 0.7 [b]	2.8	1.6	2.5± 6.1

Means ± Standard deviation followed by a common letter are not significantly different at $P<0.05$.

Table 4. Distribution and infestation level of the potato cyst nematodes *Globodera* spp. in mount Lebanon

Region	X ± S Cysts/g soil	Maximum Cysts/g soil	Minimum Cysts/g soil	X± S Eggs/g soil
Jbeil	0.5 ± 0.3 [a]	0.7	0.2	3.7 ± 6.4
Halatte	0.5 ± 0.3 [a]	0.8	0.2	0
Aamchit	1.6 ± 0.5 [a]	2.2	1.1	0.2 ±0.3
Mahrin	1.0 ± 0.4 [a]	1.4	0.8	1.5±2.1
Blat	0.6 ± 0.4 [a]	0.7	0.2	15.5±9.2
Hrajel	1.5 ± 0.8 [a]	2.1	0.6	0.5±0.1
Aakoura	1.5 ± 0.8 [a]	2.4	0.8	9.7±15.0

Means ± Standard deviation followed by a common letter are not significantly different at $P<0.05$.

Table 5. Distribution and infestation level of the potato cyst nematodes *Globodera* spp. in south Lebanon

Region	X ± S Cysts/g soil	Maximum Cysts/g soil	Minimum Cysts/g soil	X ± S Eggs/g soil
Sahl Al Khiam	1.5 ± 0.4 [a]	2.1	0.8	51±4.7 [a]
Marjouyoun	1.4 ± 0.6 [a]	2.3	0.7	0.17 ± 0.4 [b]

Means ± Standard deviation followed by a common letter are not significantly different at $P<0.05$.

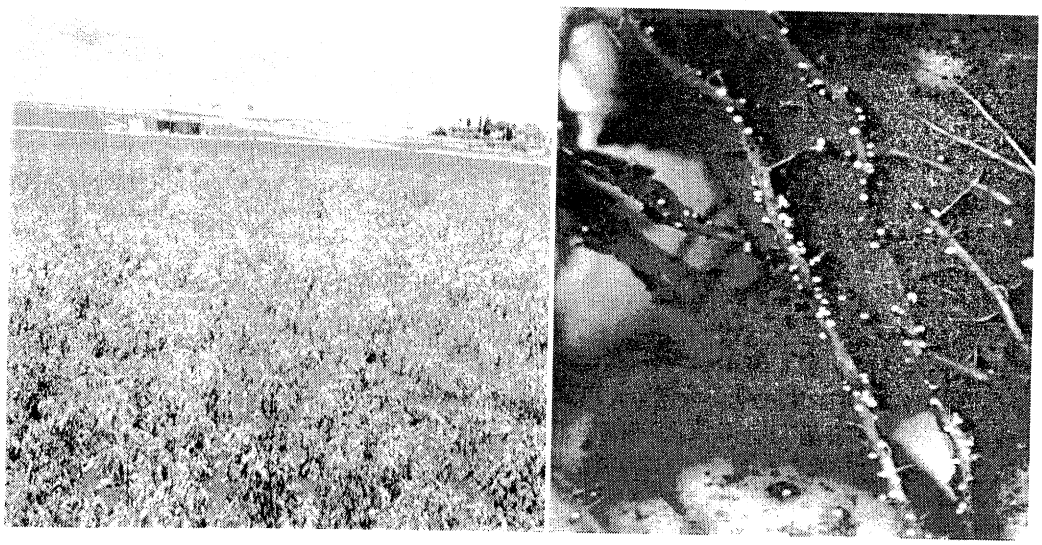

Fig. 1. Potato cyst nematodes *Globodera* spp; a) field symptoms (Abdeh area), b) infected root system and potato tuber.

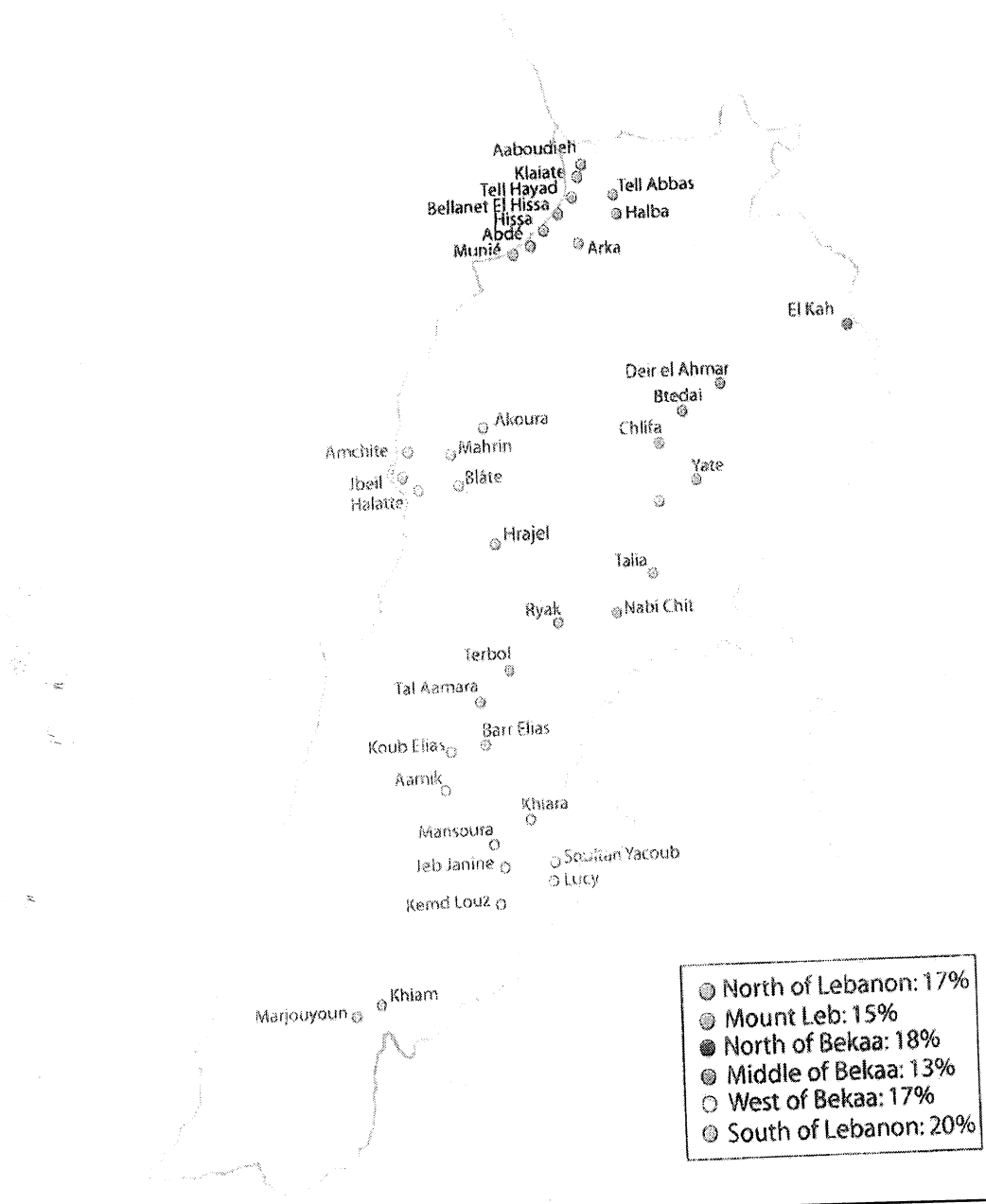

Fig.2. Map of Lebanon showing sites where PCN cysts were found.

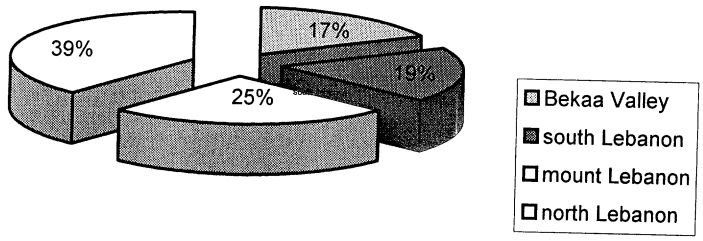

Fig.3: Percentage distribution of PCN in Lebanon

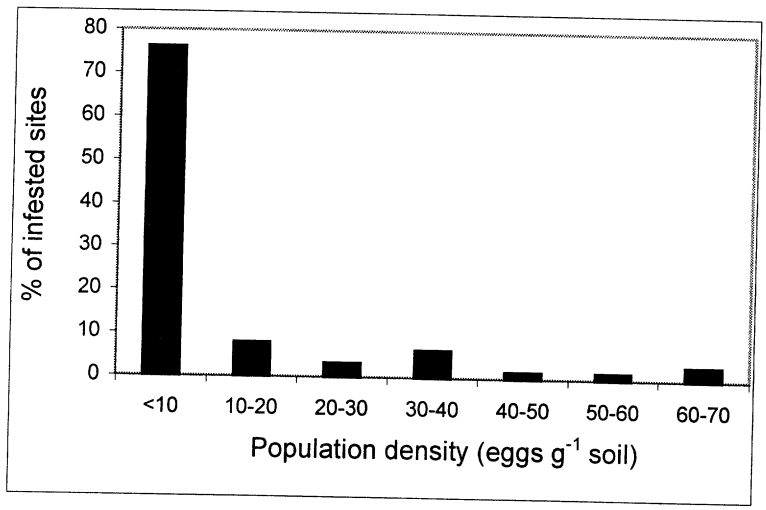

Fig.4. The percentage of infested sites with different population densities (eggs/g soil).

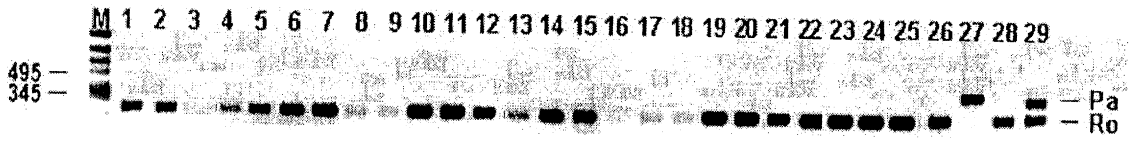

Fig. 5. PCR Differentiation of potato cyst nematodes (PCN) *Globodera rostochiensis* (Ro) and *G. pallida* (Pa). Lanes 1-26 from soil samples that contained PCN, Lanes 27-29 known species of PCN (control), M: molecular marker (ΦX174 DNA Hinc II).