ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (4): 651-659, 2008
ISSN 1682-296X
© 2008 Asian Network for Scientific Information

Morphological and Molecular Characterisation of Potato Cyst Nematode Populations from Tunisia and Survey of Their Probable Geographical Origin

[1]W. Hlaoua, [1]N. Horrigue-Raouani, [2]D. Fouville and [2]D. Mugniéry
[1]Institut Supérieur Agronomique de Chott Mariem, 4042 Sousse, Tunisie
[2]INRA, Domaine de la Motte-au-Vicomte, B.P. 35329, 35650 Le Rheu, France

Abstract: In order to investigate species and distribution of the Potato Cyst Nematode (PCN), *Globodera* spp., present in Tunisia, soil samples were collected from 118 different potato fields in 6 different regions. Identification of *Globodera* species was based on some morphological criteria and confirmed by PCR-RFLP. The RAPD was used to compare Tunisian and Europeans PCN populations to reveal a possible phylogeny between them and then determine the origin of infestations. This is the first report of molecular characterisation and polymorphism of PCN populations in Tunisia. Biological tests using resistant and susceptible cultivars of potato were done *in vitro* on *G. rostochiensis* populations to determine the pathotypes. Analysis showed that 37.28% of the investigated fields were infested by PCN. Morphological and molecular analysis, showed the presence of *G. pallida* and *G. rostochiensis* having different proportions according to regions. The RAPD revealed that Tunisian *G. pallida* populations correspond to Pa2/3 pathotypes group and biological results proved that the *G. rostochiensis* populations belong to the Ro1/4 pathotype. Results from the bootstrap analysis showed a great similarity with *G. pallida* and *G. rostochiensis* groups of European populations, evidence that Tunisian populations of PCN are derived from importation and exchange of potato seeds.

Key words: *Globodera rostochiensis*, *G. pallida*, PCR, RAPD, pathotypes, phylogeny

INTRODUCTION

Potatoes (*Solanum tuberosum*) are one of the main crops in the Tunisian cultivated agro ecological environments (B'Chir and Namouchi-Kachouri, 1993). Since its first introduction in the 18th century, the potato is cultivated in all regions of the country in different proportions. The most important production area is the north eastern Mediterranean coast, particularly the vicinities of Bizerte and Tunis (North), the Cap Bon (East north) and the stretch of coast from Sousse to Mehdia (East centre) (Horton *et al*., 1986). These regions annually produce more than 60% of the national potato crop (Rhoades *et al*., 2000; Glorenec, 1981). The total area planted has already increased to attempt 25000 ha in 2005 that represented 15% of the total market-garden products superficies. However, the yields per hectare are still lower than those reported in the European potato-producing countries. They are also restricted by fungi, bacteria and parasites, especially nematodes.

Potato Cyst Nematode (PCN), *Globodera rostochiensis* and *G. pallida* are important obligate parasites considered as a worldwide traditional potato pest (Ibrahim *et al*., 2000; Manduric *et al*., 2004; Mulder *et al*., 1997). They are generally associated with potato essentially in temperate areas (Mugniéry, 1984). The potential for damage by these nematodes is demonstrated in several countries and its economic damage is controversial for some authors. This is essentially due to disparity of climate and edaphic conditions of the region (B'Chir and Namouchi-Kachouri, 1993; Cunha *et al*., 2004).

In Tunisia, PCN was first identified in 1977 in the northern part of the country (Mai, 1977) specifically in the region of Cap Bon and Bizerte. B'Chir (1990) estimated an average of 45% of yield loss which is variable according to the regions, the seasons and the agricultural practices as well as the virulence of nematode species.

The fast identification and the diagnosis of *Globodera* spp. on potato is the key element for the management of this pest (Ibrahim *et al*., 2000) and for the application of reasonable control methods (Manduric *et al*., 2004). Several morphological characters are used to differentiate species of *Globodera*. The pattern of perineal region of cyst (number of ridges between vulva and anus and the Granek's ratio) and some second stage juveniles (J2) characters (stylet length and shape of stylet knobs) are the most common key features

Corresponding Author: Hlaoua Wassila, Institut Supérieur Agronomique de Chott Mariem, 4042 Sousse, Tunisie
Tel: +21697309293, +21673327544, +21673327546 Fax: +21673327591

for identification (Manduric *et al*., 2004). Morphological identification is convenient, but differentiation is quite difficult between some species like *G. pallida*, *G. tabacum* and *G. mexicana* (Thiéry and Mugniéry, 1996). Molecular technique is a useful diagnostic tool that is reliable and quick. DNA based analyses, such as Polymerase Chain Reaction (PCR) of Internal Transcribed Spacer (ITS) in ribosomal DNA (Ferris *et al*., 1995; Thiéry and Mugniéry, 1996), Restriction Fragment Length Polymorphic DNA (RFLP) (Burrows and Perry, 1988), random amplified polymorphic DNA (RAPD) (Folkertsma *et al*., 1994; Thiéry *et al*., 1997; Conceição *et al*., 2003; Sedlak *et al*., 2004; Bendezu and Evans, 2001), satellite DNA (Stratford *et al*., 1992) are the most commonly used diagnostic tools (Manduric *et al*., 2004; Zouhar *et al*., 2000; Burrows and Boffey, 1986). Due to the limited amount of DNA from a single nematode, the PCR seems to be the most appropriate for nematode characterisation and allows presentation of phylogenic relationships inside the *Globodera* genus (Thiéry and Mugniéry, 1996). Meanwhile, the ITS polymorphism was not sufficient to distinguish subspecies and populations in given species. This polymorphism can be revealed by the RAPD technique (Bendezu *et al*., 1998; Umarao *et al*., 2002). It is neutral, generates trees in accord with the ITS profile and presents a good correlation with the biological virulence results (Thiéry *et al*., 1997).

The objectives of this report are first to identify *Globodera* species and pathotypes with biological, morphological and molecular tools and secondly to investigate a possible phylogeny between Tunisian populations and European ones.

MATERIALS AND METHODS

Soil samples: Soil samples were collected from 118 different potato fields situated in the regions of North (Bizerte), East North (Cap Bon), East Centre (Sousse and Monastir) and centre (Kairouan and Mehdia). Regions are previously divided into zones attributed of numbers 1, 2, 3… prospected parcels in theses different zones are given the name of the region and the suffix of the zone (Table 1).

Each area is subdivided into seven different sites sampled separately. Sample usually consists of cores (10 to 30) collected from a number of locations within the site to represent the entire area. Cysts were extracted using Fenwick can after drying and sieving samples. Initially samples of 250 mL were processed, but this amount was later increased to 500 mL, when the number of the extracted cysts was fewer than 50. Twelve populations from four Tunisian locations (relatives to regions and zones) were studied (Table 1).

The *Globodera* species present in each population were identified following morphological and molecular procedures.

Morphological analysis: Three characters were selected to identify species: stylet length and stylet knobs shape for juveniles and Granek's ratio (quotient between anus and vulval basin and basin diameter) for cysts. Ten specimens from each infested sample were examined. Observations and measurements were made using Leitz Dialux light microscope.

Biological analysis: After morphological identification, biological tests were done *in vitro* on *G. rostochiensis* populations in order to distinguish pathotypes. Four different resistant cultivars carrying the *H1* gene from *Solanum andigena* (Naga, Santana, Mondial and Nicola) and one susceptible (Spunta) were used. Five replications were made in Petri dishes and inoculations were done after 3 days of root emission using seven J2. Development of females is the character of definition of pathotypes, since only Ro1/4 pathotypes are not able to develop on resistant cultivars (Mugniéry, 1976). Dishes were stored for 30 days between 16 and 18°C and observed weekly to determine the development of females.

Molecular analysis: Molecular analyses were carried out on 13 Tunisian populations from the 44 infested samples (in fact they are 12 but the population Sousse 3 is represented in two replications: one first in the *G. pallida* pool and a second in the *G. rostochiensis* group). Five European populations were studied in comparison with the Tunisian populations. RAPD analyses were used to

Table 1: Region, No. of samples taken from potato fields and cultivated varieties in the different zones during the growing season 2006

Regions	Sampling site (Populations)	Σ visited fields	Σ infested fields	Σ collected samples	Σ infested samples	Varieties used
Monastir	Monastir	16	2	7×16 = 112	7×2 = 14	Spunta
Sousse	Sousse1, 2 and 3	29	6	7×29 = 203	7×6 = 42	Spunta, Mondial, Safrane, Nicola
Cap Bon	Cap Bon 2, 3, 4 and 5	24	23	7×24 = 168	7×23 = 161	Spunta, Atlas
Bizerte	Bizerte 1, 2, 3 and 4	13	13	7×13 = 91	7×13 = 91	Spunta, Mondial
Kairouan		19	0	7×19 = 133	0	Spunta, Mondial
Mehdia		17	0	7×17 = 119	0	Spunta
Total		118	44	826	308	-

Table 2: Name, origin and reference numbers of different populations. (The populations were separated based on morphological analyses result)

Species	Location (code country)	Reference No.
G. rotochiensis	Monastir (TN)	1
	Sousse 1 (TN)	2
	Sousse 2 (TN)	3
	Sousse 3 (TN)	4
	Cap Bon 2 (TN)	5
	Cap Bon 4 (TN)	6
	Cap Bon 5 (TN)	7
	Cap Bon 3 (TN)	8
	Gerbier des Joncs (F)	9
	Ecosse (UK)	10
G. pallida	Sousse 3 (TN)	11
	Bizerte 4 (TN)	12
	Bizerte 2 (TN)	13
	Bizerte 1 (TN)	14
	Bizerte 3 (TN)	15
	Duddingston Pal (UK)	16
	Chavornay (CH)	17
	Noirmoutier (F)	18

separate pathotypes and to investigate a probable geographical origin of the Tunisian PCN. The names, origins and reference numbers of different populations were shown in Table 2.

DNA extraction: DNA extraction was performed by adding one juvenile (1 egg) tissue into 5 μL Proteinase K buffer (Tris 100 mM pH = 8.8; EDTA; TERGITOL MP40; Proteinase K 100 μg μL^{-1}; dH_2O).

Samples were rapidly frozen at -20°C or treated 60 min at 55°C followed by 10 min at 95°C to activate the Proteinase K.

PCR-RFLP: PCR primers were designed to amplify ITS1, ITS2 and 5.8 sec gene positioned between 18 and 26 sec genes (Thiéry and Mugniéry, 1996). Then the amplification reaction mixture contained 8.38 μL dH_2O; 2.5 μL 10XPCR buffer Qbiogène; 1.1 μL $MgCl_2$ 10 mM; 2.5 μL of each ribosomal primer (18S and 26S genes); 0.066 μL Taq polymerase 15 U μL^{-1} and 0.1 DNTP (4×25 mM). Amplification was performed in a Peltier Thermal cycler 100 (PTC100) programmed for 30 cycles of denaturisation at 94°C for 1 min, annealing at 60°C for 50 sec and polymerisation at 72°C for 1 min. PCR was done by adding 17.14 μL of PCR Mix to the 5 μL treated DNA.

The ITS amplification does not present differences between species, digestion of DNA by a restriction specific enzyme have to be achieved. The digestion Mix was composed of 2 μL $MgCl_2$ 1 mM, 2 μL enzyme buffer 10X, 5.5 μL dH_2O and 0.5 μL of the restriction enzyme 10 U μL^{-1} (The ACCII: isoschizomers, Bsh1236I (CG/CG)). Digestion was performed at 37°C for 2 h 30 min and visualisation of results was achieved on 2% gel (standard Agarose-Agarose small fragment, proportions: 1-1).

RAPD procedure: Nine primers randomly chosen from the kit L, G and K of Operon Technologies Inc. (Alameda, CA, USA) were used (L3, L4, L5, G2, G3, G5, G16, K4, K12). Amplifications were performed on 12.6 μL reactions, in a PTC 100 thermocycler programmed for an initial denaturisation step at 94°C for 5 min, followed by 40 cycles of 1 mn at 94°C, 1 mn at 35°C and 1.30 min at 72°C. Samples were loaded on a 1.5% agarose electrophoretic gel (standard Agarose-Agarose small fragment, 2-1) in 1X Tris Boric Acid-ethylenediamino-tetra-acetate (TBE) pH 8. Electrophoresis was carried in 1X TBE at 120 V. Results were visualized by sybersafe (0.5 μg mL^{-1}) staining and UV transillumination.

Four replications of each amplification were performed independently to check the reproducibility of the reactions. Population markers were chosen only when present without ambiguity in the repeated amplification. Differences in band brightness were not taken into account. A negative control was included as a test for contaminations.

Computing results: Results, the matrix data containing population names followed by the number 0 or 1 to indicating presence or absence of bands with each primer were put on and used as data for the phylogeny inference package (Phylip, version 3.5°C) program. Genetic distances of Nei and Li (1979) and cluster analyses with Unweighted Pair as well as Group Method with Arithmetic mean (UPGMA) were obtained with the Gendist and Neighbor programs.

RESULTS

Infestation proportions: Forty four of the total 118 investigated fields (37.28%) in the different regions were infested by PCN (Table 1). Infestation proportions of visited fields varied according to regions, 20.7% in Sousse, 12.5% in Monastir, 95.83% in Cap Bon and 100% in Bizerte (Table 1).

Morphological identification: Altogether, 440 specimens of *Globodera* sp. were analysed by morphological characters combining cysts perinea (Granek's ratio) and their relative J2 stylet length and knobs shape. Analysis showed the presence of two species: *G. pallida* and *G. rostochiensis*. In the juveniles, there were clear differences between the mean of the *G. rostochiensis* and those of *G. pallida* populations for the stylet length (20.51 and 22.73 μm) and knobs shape. In the cysts, the Granek's ratio mean is as expected higher for the *G. rostochiensis* populations (4.12) than for the *G. pallida* populations (2.14). The region of Bizerte (Bizerte 1, 2, 3 and 4) presented 100% of *G. pallida*, whereas the regions

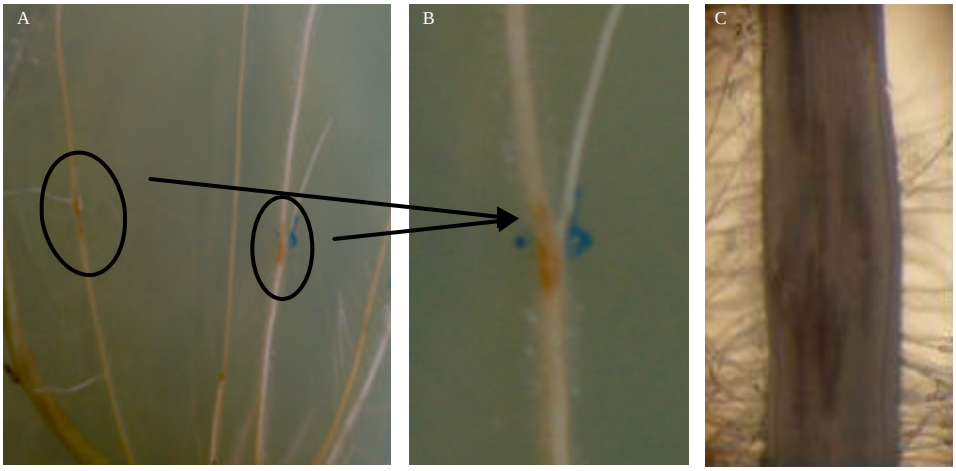

Fig. 1: Characteristic necrosis of resistant cultivar (Naga) to *G. rostochiensis* (A and B): Observed on Petri dish experiment, (C): Stereoscopic observation of the necrosis (x20)

of Monastir and Cap Bon (Cap Bon 2, 3, 4 and 5) showed 100% of *G. rostochiensis*. The region of Sousse presented a mixture of the two species with 83.3% of investigated fields infested by *G. rotochiensis* (Sousse 1, 2 and 3) and 16.6% infested by *G. pallida* (Sousse 3). The fields of Kairouan and Mehdia did not present infestation by potato cyst nematode.

Biological analysis: Analysis of virulence towards the H1 gene showed the development of *G. rostochiensis* J2 into females on the susceptible variety (Spunta) and not on the resistant cultivars (Naga, Mondial, Santana and Nicola). Characteristic necrosis on the roots of the resistant cultivars resulted from the penetration of the J2 (Fig. 1). This result confirms that the Tunisian *G. rostochiensis* populations belong to the Ro1/4 pathotype.

PCR: The amplification of the ITS region of each population gave one type of fragment of approximately 1.25 kb characteristic of *Globodera* spp. (Fig. 2). Use of the ACCII enzyme to digest the amplification product generated a polymorphism and distinguished *G. rostochiensis* and *G. pallida* characteristic profiles. Conform to the morphological analyses, the PCR showed that *G. pallida* was present in both regions of Bizerte and Sousse, whereas *G. rostochiensis* characterized the regions of Cap Bon, Monastir and a part of Sousse fields (Fig. 3).

RAPD: The amplified DNA fragments were in the range between 100 and 2000 bp, with 9 to 19 amplification

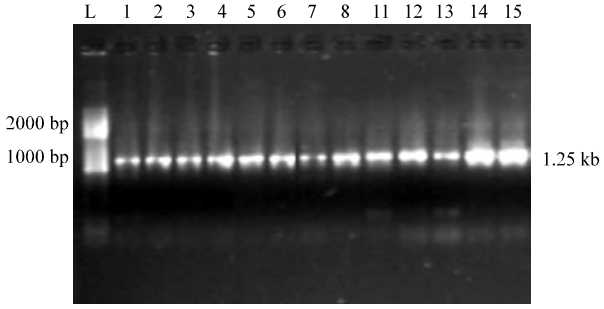

Fig. 2: Amplified ITS region of the 13 Tunisian *Globodera* spp. populations (Ethidium bromide stained 1% agarose gel, molecular weight markers (L) = 100 bp) (Names of populations were reported in Table 2)

products per population for each of the 9 primers. A total of 125 markers for the 18 PCN populations were generated.

The three L kit primers (L3, L4 and L5) gave 37 markers, the four G kit primers (G2, G3, G5, G16) gave 69 markers and the two last K kit primers gave 19 markers.

The RAPD profiles that resulted from one of performed amplifications illustrate the banding pattern observed (Fig. 4). Few markers were monomorphic between the 18 populations of the two species, but the majority showed inter and intraspecific polymorphism.

Trees were derived by computing the 125 RAPD markers with the Nei distances of Gendist and UPGMA (Fig. 5A) and after 1000 bootstraps using the consensus procedure (Fig. 5B).

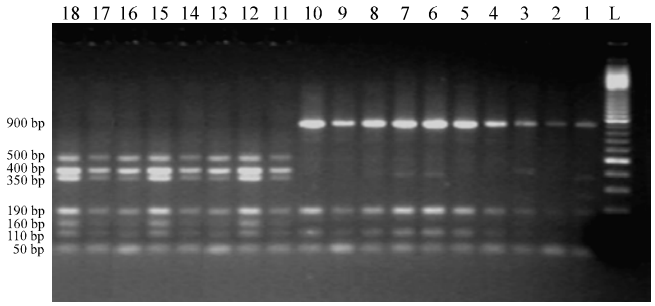

Fig. 3: Molecular differentiation of 13 Tunisian PCN populations compared to the 5 European ones (9, 10, 16, 17 and 18). Molecular weight markers (L) = 100 bp DNA ladder are indicated in base pairs. Populations 1-10 = *G. rostochiensis* and populations 11-18: *G. pallida* (Names of populations are reported in Table 2)

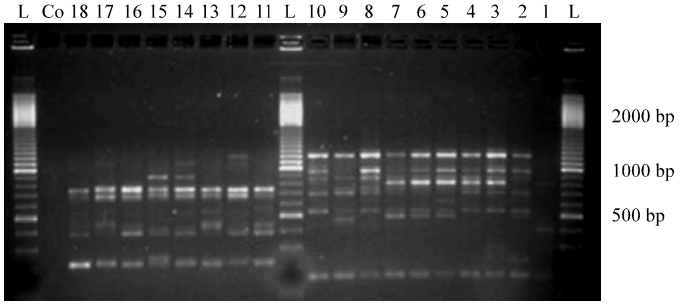

Fig. 4: RAPD patterns of 18 *Globodera* spp. populations with OPG2. Co: Control sample without template DNA. Molecular weight (L) = 100 bp DNA ladder. Populations 1-10 = *G. rostochiensis* and populations 11-18: *G. pallida* (Names of populations are reported in Table 2)

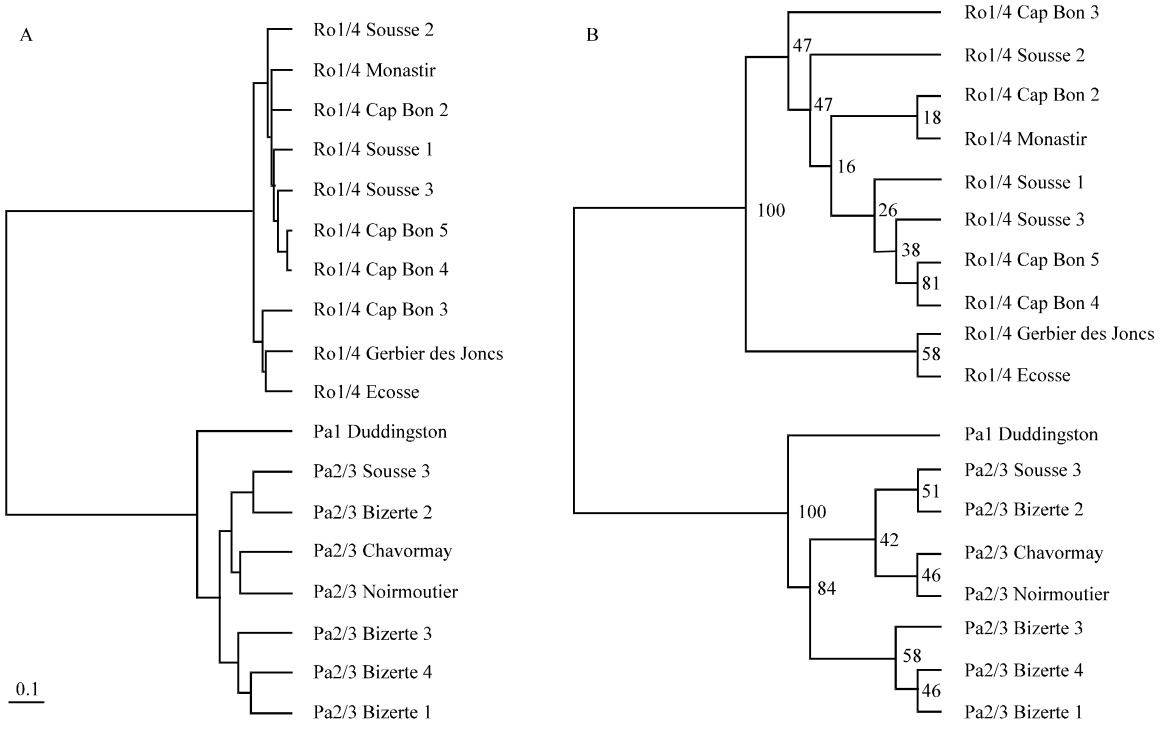

Fig. 5: UPGMA tree derived from RAPD data (A) and the consensus tree obtained after 1000 bootstraps (B)

G. rostochiensis clusters were well separated from *G. pallida*. There were several groups in the two species clusters corresponding to different subgroups. In the *G. pallida* cluster, the Duddingston population from UK, a Pa1 pathotype, was far from the other populations belonging to Pa2/3 pathotypes. This latter group is divided in two distinct subgroups. One group included the European population Pa2/3 Chavornay, Pa2/3 Noirmoutier, Pa2/3 Sousse 3 and Bizerte 2. The other group included the three Pa2/3 populations of Bizerte 1, Bizerte 3 and Bizerte 4.

In the *G. rostochiensis* gathers the two European Ro1/4 pathotypes Gerbier des Joncs and Ecosse, separately from the Ro1/4 Cap Bon 3 and more far from the rest of Ro1/4 Tunisian populations. This Tunisian group presents tow different sub cluster. The first is containing Ro1/4 Sousse 1, 2 and 3, Ro1/4 Cap Bon 2 and the Ro1/4 Monastir pathotypes. The second sub cluster is presenting Ro1/4 Cap Bon 4 and 5 and seems to be less polymorphic than the first.

Results from the bootstrap analysis were also conclusive, separating *G. pallida* from *G. rostochiensis*. Although, similarity between the two species tended to be small, the general variation in similarity among *G. pallida* and *G. rostochiensis* groups is too great to allow a dissuasive separation of Tunisian populations from the European ones, except from Duddingston in *G. pallida* group.

DISCUSSION

Detection of the species pathotype and occurrence of *Globodera* sp. seems to be one of the most important steps in understanding the nematode distribution within potato growing area in Tunisia. Such information is very helpful for choosing an optimal method of integrated pest management in relation to PCN's incidence regulation. The present study showed different infestation levels which varied according to regions from 100% of the investigated fields in Bizerte to 12.5% in Monastir.

Identification was first done with the morphological characters since a number of comparative studies have been made to estimate the reliability of the morphology identification method (Ambrogioni and Irdani, 2001). Present result are conform to those of an earlier report of B'Chir (1990), where he indicated the dominance of *G. pallida* in the region of Bizerte and that *G. rostochiensis* is more frequent in Cap Bon. The high proportions of infested fields in these two regions are explained by their temperate cold climate. Nevertheless, our study showed the presence of the two species in regions characterised by a hotter climate (Schlûter, 1976). Besides, 20.7 and 12.5% of fields are infested in Sousse and Monastir, respectively, where annual mediate temperature is upper than 20°C. The dominance of *G. rostochiensis* in these regions, substantiate the results of Mugniéry (1978), who showed that *G. rotochiensis* was more competitive than *G. pallida* in high temperature at 24°C and reciprocally in low temperature.

Morphological identification isn't as reliable unless combined results referring to both cyst and juveniles characters are adopted. Moreover, both cyst and juveniles identification could be considerably improved when the two characters are combined and the probability of making correct identification can be considerably increased by increasing the number of investigated specimens (Manduric *et al*., 2004).

Morphological characters are still used to differentiate some species as with experience because it is fast and no advanced procedures or chemicals are needed. However theses methods couldn't be used to separate closely related species *G. mexicana* and *G. pallida* or the three subspecies of *G. tabacum* (*G.t. tabacum*, *G.t. virginiae* and *G.t. solancearum*) where morphological polymorphism is low (Conceição *et al*., 2003; Mota and Eisenback, 1993a-c; Marché *et al*., 2001; Thiéry and Mugniéry, 1996).

Molecular analyses were used to avoid doubt about effect of mixed population and to obtain more precise information about Tunisian species polymorphism of the *G. rostochiensis* and *G. pallida* populations. PCR-RAPD provided powerful methods for detecting the polymorphism between and within species (Bendezu *et al*., 1998; Bendezu and Evans, 2001; Conceição *et al*., 2003; Folkertsma *et al*., 1994; Roosien *et al*., 1993; Thiéry *et al*., 1997). This is the first report of molecular characterisation and polymorphism of the Tunisian PCN populations.

PCR amplification of specific regions of the PCN genome (ITS1 and ITS2) is a sensitive method which can be used for taxonomic purposes of cyst samples which vary in age (Pylypenko *et al*., 2005). This technique could be used for nematode populations which were directly sampled from potato fields and resulted in both young and old cysts in the soil. Although the PCR-RFLP is highly effective as a species diagnostic tool, it couldn't reveal the existence of a trend of subspecies in both *G. rostochiensis* and *G. pallida*.

The RAPD technique presents a great number of advantages: it is neutral, the dendrogram generated is in accord with ITS results (Thiéry *et al*., 1997). Such specific molecular assays would lead to greater precision in the identification of *Globodera* in commercial fields (Syracuse *et al*., 2004) and for separation of pathotype,

since the origin of PCN detected in Tunisia is unknown. Present results show that RAPD is suitable for studying variability within species. The great polymorphism observed between the two species could be explained by the use of European population as reference in this study. On the other hand, the small variability observed within these two species can explain the closeness or the probable origin of the populations among one subgroup. RAPD analysis showed that there are two subgroups within *G. rostochiensis* cluster and three among the *G. pallida* group. The bootstrap values are extremely variable between the two species clusters and the homogeneity of five subgroups is similar. In addition, the type of population clustering can reveal a possible geographic origin of these populations. The two European *G. rostochiensis* and *G. pallida* populations cluster separately in two different groups. One Tunisian *G rostochiensis* population (Cap Bon 3) huddles with the first and two *G. pallida* comes together with the second (Bizerte 2, Sousse 3). Combining this result with the history of potato crop beginning in Tunisia it seems that the two Tunisian PCN species are introduced from the European area. It may be reported to the period of colonialism or to the latter procedures of seed importation from Europe.

The number of isolates or populations is important in the molecular studies. Syracuse *et al.* (2004) using RAPD, showed an intraspecific variability within *G. tabacum solanacearum* with a large number of isolates, whereas other reports indicated little or no intraspecific variability among much smaller sets of isolates based upon RAPD data (Thiéry and Mugniéry, 1996; Thiéry *et al*., 1997). We would have better localised the Tunisian populations in relation to those European if we have used more European isolates especially from the Netherlands, knowing that the majority part of importations of potato were from this part of Europe.

The application of quarantine standards is a key objective to improve production of potato. There is also a need to improve regulatory control to prevent the introduction of pests or to control those that are already established which adversely affect potato production. The presence of *G. pallida* in Monastir cannot be excluded as it's close to the region of Sousse and as only a small proportion of the potato growing land was sampled. Moreover, in recently infected fields, populations are unevenly distributed and the probability to detect cysts in the soil sample is low (Ibrahim *et al*., 2000).

The use of resistant varieties like Mondial and Nicola (Table 1) was the simpler solution for farmers to protect yield from PCN in infested sampling sites that was estimated at 45% (B'Chir and Namouchi-Kachouri, 1993). The growing of resistant potato cultivars with H1 gene, which is effective against many populations of *G. rostochiensis*, is likely to be an efficient management tool to reduce population densities and yield losses given that these populations belongs, according to the biological test, to the Ro1/4 pathotype. However, careful monitoring would then be necessary to ensure that the deployment of such cultivars does not simply cause *G. pallida* to become predominant (Cunha *et al*., 2004) as it is the case of Bizerte.

CONCLUSION

The present study showed different infestation levels varying according to regions. We used for the first time molecular (PCR and RAPD) and biological tools to identify PCN species, pathotypes and polymorphism within Tunisian populations and with European ones. We showed the presence of the two species *G. pallida* and *G. rostochiensis* belonging respectively to Pa2/3 and Ro1/4 pathotypes. These species are present in regions characterised by an annual mediate temperature is upper than 20°C and especially a very hot summer. Combining the results of RAPD that demonstrated a great phylogeny between Tunisian and European populations, with the history of potato crop in Tunisia reveals that the two Tunisian PCN species are introduced from the European area. It may be reported to the period of colonisation and get worsen by the latter procedures of seed importation from Europe.

ACKNOWLEDGMENTS

This study is a part of the thesis of the first author Hlaoua W., who gratefully acknowledges Pr. D. Mugniéry for supplying the European PCN populations and for supervising this work in the UMR BiO_3P INRA of Rheu, France. Especially thanks are also due to D. Fouville for his collaboration and assistance.

REFERENCES

Ambrogioni, L. and I. Irdani, 2001. Identification of *Heterodera schachtii* group species in Italy by morphometric and RAPD-PCR. Nematol. Med., 29: 159-168.

Bendezu, I.F., K. Evans, P.R. Burrow, D. de Pomerai and M. Canto-Saenz, 1998. Inter and intra-specific variability of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* from Europe and South America using RAPD-PCR. Nematologica, 44: 49-61.

Bendezu, I.F. and K. Evans, 2001. Pathotype characterization of population of potato cyst nematodes *Globodera pallida* and *G. rostochiensis* by RAPD-PCR. Int. J. Nematol, 11: 200-208.

Burrows, P.R. and S.A. Boffey, 1986. A technique for the extraction and restriction endonuclease digestion of total DNA from *Globodera rostochiensis* and *G. pallida* second stage juveniles. Revue Nématol, 9: 199-200.

Burrows, P.R. and R.N. Perry, 1988. Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis*. Revue Nématol, 11: 441-445.

B'Chir, M.M., 1990. Evaluation de la nuisibilité de quelques groupes de nématodes en fonction des particularités agro-météorologiques régionales. Symposium OMM/OEPP/NAPPO, December 4-7, Florence, Italy, Joint, WMO EPPO NAPPO Symposium on Practical Applications of Agrometeorology in Plant Protection, pp: 88-89.

B'Chir, M.M. and N. Nammouchi-Kachouri, 1993. Evaluation de la nuisibilité réelle de *Globodera* spp. dans les conditions d'une culture de pomme de terre de saison en tunisie. Med. Fac. Landbouww. Univ. Gent, 59/1: 25--36.

Conceição, I.L.P.M. Da, M.C.V. Dos Santos, I.M. De, O. Abrantes and S.N.D.A. Santos, 2003. Using RAPD maker to analyse genetic diversity in Portuguese potato cyst nematode populations. Nematol., 5: 137-143.

Cunha, M.J.M. Da, I.L.P.M. Da Conceição, I.M. De O. Abrantes, K. Evans and S.N.D.A. Santos, 2004. Characterisation of potato cyst nematode populations from Portugal. Nematology, 6: 55-58.

Ferris, V.R., L.I. Miller, J. Faghihi and J.M. Ferris, 1995. Ribosomal DNA comparisons of *Globoedera* from two continents. J. Nematol., 27: 273-283.

Folkertsma, R.T., J.N.A.M. Rouppe Van Der Voort, M.P.E. Van Gent-Pelzer and K.E. De Groot *et al*., 1994. Inter and intraspecific variation between populations of *Globodera rostochiensis* and *G. pallida* revealed by Random Amplified Polymorphic DNA. Phytopathology, 84: 807-811.

Glorenec, N., 1981. La culture de pomme de terre en Tunisie. B.T.S «TRADICOPA 1981/1982» Lycée Agricole, Morlaix, France.

Horton, D., R. Cortbaoui, H. Hattab and A. Monares, 1986. Impact of Agricultural Research and Development: A Seed Potato Project in Tunisia. International Potato Centre, Lima.

Ibrahim, S.K., A.T. Saad, P.P.J. Haydock and Y. Al-Nasri, 2000. Occurrence of the potato cyst nematode *Globodera rostochiensis* in Lebanon. Nematology, 2: 125-128.

Mai, W.F., 1977. Worldwide distribution of potato cyst nematodes and their importance in crop production. J. Nematol., 9: 30-34.

Manduric, S., E. Olsson, J.E. Englund and S. Andersson, 2004. Separation of *Globodera rostochiensis* and *G. pallida* (Tylenhida: Heteroderidae) using morphology and morphometrics. Nematology, 6: 171-181.

Marché, L., V. Valette, E. Grenier and D. Mugniéry, 2001. Intra-specific DNA polymorphism in the tobacco cyst nematode complex (*Globodera tabacum*) using AFLP. Gen., 44: 941-946.

Mota, M.M. and J.D. Eisenback, 1993a. Morphology of females and cysts of *Globodera tabacum tabacum*, *G. virginiae* and *G. t. solanacearum* (Nemata: Heteroderinae). J. Nematol., 25: 136-147.

Mota, M.M. and J.D. Eisenback, 1993b. Morphology of second stage juveniles and males of *Globodera tabacum tabacum*, *G. t. virginiae* and *G. t. solanacearum* (Nemata: Heteroderinae). J. Nematol., 25: 27-33.

Mota, M.M. and J.D. Eisenback, 1993c. Morphometrics of *Globodera tabacum tabacum*, *G. virginiae* and *G. t. solanacearum* (Nemata: Heteroderinae). J. Nematol., 25: 148-160.

Mugniéry, D., 1976. Etablissement d'un model de dynamique de population d'*Heterodera pallida* Stone : Application d'un cas pratique de lutte intégrée. Ann. Zool. Ecol. Anim., 8: 315-329.

Mugniéry, D., 1978. Vitesse de développement, en fonction de la température de *G. rostochiensis* et *G. pallida* (Nematodae). Revue. Nématol, 1: 3-12.

Mugniéry, D., 1984. Les nématodes de la pomme de terre. Agronomie, 3: 45-50.

Mulder, A., A.F. Van Der Wal and R.A.J. Velema, 1997. Relationship between potato cyst nematode and their host. III. consistency of nematode density-yield descriptors as influenced by seasonal variation. Potato Res., 40: 335-344.

Nei, M. and W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci., 76: 5269-5273.

Pylypenko, L.A., T. Uehara, M.S. Phillips, D.D. Sigareva and V.C. Blok, 2005. Identification of *Globodera rostochiensis* and *G. pallida* in the Ukraine by PCR. Eur. J. Plant Pathol., 111: 39-46.

Rhoades, R.E., R.J. Hijmans and L. Huccho, 2000. Potato in Tunisia. In World Potato Atlas.

Roosien, J., P.M. van Zandvoort, R.T. Folkertsma, J.N.A.M. Rouppe van der Voort and A. Goverse *et al*., 1993. Single juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* differentiated by Random Amplified Polymorphic DNA. Parasitology, 107: 567-572.

Schlûter, K., 1976. The potato cyst eelworm *Heterodera rostochiensis* Woll. in Morocco: Its distribution and economic importance. Z. Pflanzenkr. Pflanzenschutz, 83: 401-401.

Sedlak, P., M. Melounova, S. Skupinova, P. Vejl and J. Domkarova, 2004. Study of European and Czech populations of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) by RAPD. Method Plant, Soil Environ., 50: 70-74.

Stratford, R., R. Sields, A.P. Goldsbrough and C. Fleming, 1992. Analysis of repetitive DNA sequences from potato nematodes and their use as diagnostic probes. Phytopathology, 82: 881-886.

Syracuse, A.J., C.S. Johnson, J.D. Eisenback, C.L. Nessler and E.P. Smith, 2004. Intraspecific variability within *Globodera tabacum solacearum* using Random Amplified Polymorphic DNA. J. Nematol., 36: 433-439.

Thiéry, M. and D. Mugniéry, 1996. Interspecific rDNA restriction fragment length polymorphism in Globodera species, parasites of Solanaceous plants. Fundam. Applied Nematol., 19: 471-479.

Thiéry, M., D. Fouville and D. Mugniéry, 1997. Intra and interspecific variability in *Globodera*, parasites of solanaceous plants, revealed by Random Amplified Polymorphic DNA (RAPD) and correlation with biological features. Fundam. Applied Nematol., 20: 495-504.

Umarao, K.S.K., Prasad, A.K. Ganguly and K.K. Kaushal, 2002. Utility of a molecular diagnostic method for potato cyst nematode (*Globodera pallida* and *G. rostochiensis*) of Nilgiri Hills. Indian J. Nematol., 32: 165-168.

Zouhar, M., O. Rysanek and M. Kocova, 2000. Detection and differentiation of the potato cyst nematode *Globodera rostochiensis* and *G. pallida* by PCR. Plant Prot. Sci., 36: 81-84.