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Pathogenicity and RAPD analysis of *Phytophthora nicotianae* pathogenic to pepper in Tunisia

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Abstract

Nine isolates of *Phtophthora nicotianae* were isolated from infected pepper plants. Their pathogenicity was studied in *Capsicum annuum* in comparison with *P. nicotianae* isolates from tomato and tobacco. The pathogenicity test showed that pepper isolates of *P. nicotianae* are adapted to their host. Banding patterns obtained by RAPD analysis with six oligonucleotide primers revealed polymorphism that grouped the isolates independently of the plant host. The polygenic dendrogram showed that pepper isolates were more similar to tomato isolates than to tobacco isolates. The RAPD bands of 1300 and 1500 bp, detected with primers OPD-01 and OPD-10, respectively, appeared specific to the most pathogenic pepper isolates. The OPK-08-1950 seems specific to the isolates of *P. nicotianae* from tomato. These results suggest that host specified might occur in *P. nicotianae* and that may be due to interspecific hybridization events resulting in novel pathogenic behavior.
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Keywords: *Phytophthora nicotianae*; RAPD pathogenicity

1. Introduction

Phytophthora species are responsible for economically important disease of a wide range of agronomic and ornamental crops. *Phytophthora nicotianae* has been described as a pathogen of numerous hosts including citrus [1] and carnation. In Tunisia [2] and in Northwest Spain [3] this species has been isolated also from pepper. In Tunisia, *P. nicotianae* is a major disease causing up to 100% crop losses [2]. The pathogenic behavior of the isolates was studied in *Capsicum annuum* in comparison with that of *Phytophthora capsici* [3]. The characteristic symptoms of *P. nicotianae* are as dry necrosis of roots and collar, rarely progressing along the stem and never affecting fruits or leaves. The death of the plants occurs late at the stage of full production. *P. capsici*, on the other hand, produces soft, water-soaked, dull green spots that rapidly elongate under favorable conditions to cover more than one-quarter of the plant [3]. Species identification is based primarily on the shape of the sporangia and the morphological features of the sexual structures. Other criteria widely used to distinguish species are cardinal growth temperature, growth rate, morphological (growth) characteristics in culture, and mating behavior. Due to intraspecific variation and overlapping characters, accurate identification of isolates is often difficult, even for specialists. Moreover, some species (e.g., *Phytophthora megasperma*, *Phytophthora cryptogea*) probably consist of species complexes rather than single taxa [5].

Neither *P. nicotianae* nor *P. capsici* produced sex organ on potato dextrose agar (PDA) with daylight. When crosses were made between isolates of different mating types to obtain oogonia and antheridia, *P. nicotianae* produced anphigynous antheridia. They confirmed the presence of abundant chlamydospores on these strains. *P. capsici* produced very few or no chlamydospores [3]. *Phytophthora* species are either homothallic or heterothallic, with two mating types, A1 (*P. capsici*) and A2 (*P. nicotianae*), in the latter case. Mating types are universal in that an A1 of one

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species is compatible with A2 of another species. Homothallic species contain at least one, but mostly both, mating hormones and their compatible receptors, and hence, outcrossing between homothallic species cannot be excluded theoretically [5]. In fact, it is proven that in homothallic *Phytophthora sojae* outcrossing actually does occur [6,7]. Hybridization has shown possible between the heterothallic *P. nicotianae* and the homothallic *Phytophthora cactorum* [5].

Possible exchange of genetic materials in hybrid oospores may be an important factor in the evolution of natural *Phytophthora* populations [5]. The hybrid's marked developmental instabilities, unusual morphological variability, and evidence for recombination in their internal transcribed spacer profiles indicates that, they are of recent origin and that their evolution is continuing [8].

A preliminary cytological study of an alder *Phytophthora* isolate revealed abnormal nuclear behavior. This observation led us to examine the sequences of the internal transcribed spacer (ITS) regions in its rRNA genes (rDNA). These genes occur in multiple arrays, and mutations in their noncoding regions occur at a rate that approximates the rate of species emergence. Over time, such mutations become fixed through unequal crossing over and gene conversion, a process that is commonly termed concerted evolution [8].

Interspecific hybrids are more likely to survive if they have a fitness advantage over the parent species, such as increased aggressiveness or the ability to exploit a new host [9].

Molecular methods have proved to be a useful tool to study pathogenic relationship within the genus *Phytophthora*. This genus has been characterized using isozyme analysis, analysis of random amplified polymorphic DNA, analysis of the ITS region of the ribosomal DNA gene repeat (rDNA), and AFLP polymorphisms [10] to confirm the hybrid status of the isolates.

RAPD markers generated with single primers of arbitrary nucleotide sequence have been showed useful in detecting intraspecific polymorphisms among fungi [11,12]. They have a great potential to characterize the genetic variability of a population of *P. nicotianae* isolates from China [13]. Moreover, RAPD analysis suggested that races occur in *Phytophthora cinnanomi* [14].

This study was shown that the identification of *P. nicotianae* as a severe fungus, frequently observed on pepper crops on Tunisia, required confirmation based on pathogenicity characteristics. The molecular study was based to examine usefulness of RAPD analysis in determination of pathogenic groups specific in *P. nicotianae* isolates. This may be due to interspecific hybridization events resulting in novel pathogenic behavior.

2. Materials and methods

2.1. Fungal isolates

Isolates were obtained from infected plants using a selective pythiaceous medium [15]. Infected plants were collected from commercial cultivation sites in Tunisia. Twelve fungal isolates were studied, nine of which were obtained from wilted plants of pepper showing collar and root rot. Three other isolates, two from tomato and one from tobacco were used as a reference (Table 1). The plant species and varieties used in pathogenicity tests are listed in Table 2. Seedlings at the six-leaf stage were used; the plants were grown in a growth chamber at 25–28 °C. The rooting medium was a 1:1 mixture of peat and sand previously sterilized at 120 °C for 45 min. Fungal cultures were grown on pea broth in Petri dishes under illumination (30.000 lx) using a 16 h photoperiod at 24 °C for 8 days, to stimulate sporangium formation [3]. When abundant sporangia were formed, the growth medium was replaced with sterile distilled water. The Petri dishes were cooled at 4 °C for 30 min, and then left at 24 °C for 2 h to stimulate the discharge of zoospores. The zoospores suspension was filtered and vortexed for a period of 1 min and adjusted to the inoculation dose of 20,000 zoospores/ml. Each plant was inoculated at the six-leaf stage by dripping 5 ml of zoospore suspension onto the collar of each plant. Disease symptoms were rated at 12 days after inoculation according to the following scale of [15]: 0 (healthy plant), 1 (necrosis only on the lower half of primary roots), 2 (necrosis on all the primary roots), 3 (necrosis reaching the crown and the lateral roots), 4 (rotten hypocotyls) and 5 (whole plant dead). The experiments were performed using a randomized design with three replicates per isolate and 10 plants per replicate.

2.1.1. Statistical analysis

The mean comparison test, analysis of variance (ANOVA) two factors (variety and isolates) were performed using the STATISTICA software system.

Table 1
Isolates of *Phytophthora nicotianae* used in pathogenicity and molecular tests

Stains codes	Host and geographic origin	Year of isolation
Pnt 317	*Capsicum annuum*, from Oued Ellil	06/6/2000
Pnt 319	*Capsicum annuum*, from Tazarka	03/7/2002
Pnt 320	*Capsicum annuum*, from Tazarka	03/7/2002
Pnt 322	*Capsicum annuum*, from Tazarka	03/7/2002
Pnt 327	*Capsicum annuum*, from Korba-beach Km 9 Nabeul	03/7/2002
Pnt 328	*Capsicum annuum*, from Korba-beach Km 9 Nabeul	03/7/2002
Pnt 329	*Capsicum annuum*, from Korba-beach Km 9 Nabeul	03/7/2002
Pnt 336	*Capsicum annuum*, from Tazarka	23/7/2002
Pnt 338	*Capsicum annuum*, from Tazarka	23/7/2002
T3	*Solanum lycopersicum* from Korba Km 9 Nabeul	23/7/2002
T4	*Solanum lycopersicum* from Korba Km 9 Nabeul	23/7/2002
Pnn374	*Nicotiana tabacum* from South of Africa	1992

Table 2
Solanaceous varieties used in pathogenicity tests

Species plant	Variety	Origin and date of harvest	Features
Solanum lycopersicum	Marmande verte Frantic (ES 97-100)	INRA, Montfavet, 1989 Esasem, Italy, 2001	Susceptible to *P. nicotianae* var. *parasitica* isolated from tomato
Solanum melongena	Black Beauty	Quarantaine, Tunisia, 2002	Susceptible to *P. nicotanae* var. *parasitica* and to *P. capsici*
Nicotiana tabacum	Samsun	Phytopathologie, FUSA de Gembloux, 1991	Susceptible to *P. nicotianae* var. *nicotianae* isolated from tobacco
Capsicum annuum	Beldi	INRAT (Tunisia), 2000	Susceptible to isolates of *P. nicotianae* var. *parasitica* isolated of pepper [4]

2.2. DNA extracts

Fungal strains were grown on pea broth in Erlen Meyer flasks (200 ml per flasks) at 25 °C for 14 days. Before the extraction of the DNA, the mycelium was frozen at −80 °C and ground to a fine powder in liquid nitrogen with a mortar and pestle. Total cellular DNA was prepared from the powdered mycelia. DNA has been extracted according to the CTAB method described by Ref. [16] (CTAB 2%, EDTA 20 mM, NaCl 1.4 M, PVP 1%, Tris 0.1 M, 1% B-Mercaptoéthanol, pH 8) with few modifications.

2.2.1. RAPD analysis

Polymerase chain reaction (PCR) amplification of DNA was performed with each of six decamer primers (Table 3) obtained from Operon Technologies [17]. Each 25 µl of PCR reaction mixture in 50 mM Tris–HCl buffer (pH 9) containing 50 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mg/ml BSA, 200 µM of each of the dNTPs: dATP, dCTP, dGTP, and dTTP (Promega), 4 µl of primers to 20 mM, 50 ng of template DNA, 0.25 µl of Taq polymerase (5 unités/µl) and 7.75 µl of sterile H_2O. Amplifications were performed in Biometra, DNA Thermal cycle programmed for the following parameters: 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 34 °C for 30 s, and extension at 72 °C for 30 s and a final cycle of extension at 72 °C for 10 min. Amplification products were separated by electrophoresis at 110 V for 2 h on ethidium bromide-stained (0.5 µg/mm), 1.2% agarose gels run in 0.5 × Tris–borate EDTA buffer and visualized under UV light. The experiment was conducted twice.

2.2.2. Data analysis

Each isolates was scored for the presence or absence of each amplicon. When a primer revealed polymorphism, all amplified DNA fragments were used for analysis and treated as binary characters. The character state of each RAPD band was scored for each individual isolate of *P. nicotianae*. Genetic similarities were computed between all pairs of isolates using the method of Ref. [18]. Dissimilarities were computed as genetic distances and the data were used to construct a phenogram, recording to the unweighted pair group method with arithmetic average (UPGMA).

Table 3
Nucleotide sequence of Operon primers used in the experiment

Code	Nucleotide sequence (5′–3′)
OPD-01	ACCGCGAAGG
OPD-03	TCTGGTGAGG
OPD-06	GTGTGCCCCA
OPD-07	TTGGCACGGG
OPD-10	GGTCTACACC
OPK-08	GAACACTGGG

3. Results and discussion

3.1. Pathogenicity test

The aim of pathogenicity tests was to compare the ability of *P. nicotianae* isolates to infect homologous and heterologous hosts. The percentage of mortality observed after the inoculation of the pepper plants with isolates *Pnt336* and *Pnt329* from pepper ranged from 70% to 80% and was associated with generalized root necrosis (values of disease index ranging between 2 and 5). Conversely, no tomato plant mortality was recorded after the inoculation with pepper isolates. The mild root necrosis observed on pepper plants inoculated with T3 and T4 was similar to that observed in the un-inoculated controls while both T3 and T4 isolates incite a strong necrosis on tomato plants (mean disease index = 4.2). The mortality of tomato plants inoculated with these isolates was 100%. In contrast tomato isolates did not cause mortality on plants of pepper, eggplant and tobacco. This strains (classified as *P. nicotianae* specific to tomato) behaved uniformly, producing collar and root rots 4 days after inoculation.

The isolates *Pnn374* produced on tobacco a mortality of 10% and the mean disease index was 2.3. Tobacco plants (var. Samsun) were not affected with the pepper isolates of *Phytophthora*.

This isolates of *P. nicotianae* were not able to infect tomato, host that is susceptible to *P. nicotianae* but still could infect pepper, a plant susceptible to *P. capsici.* These

Table 4
Pathogenicity of isolates of *Phytophthora nicotianae* inoculated on different solaneous plants

Isolates	Pepper Var. Beldi	Tomato Var. Marmande verte	Eggplant Var. Black Beauty	Tobacco Var. Samsun
Pnt 336	4 (2–5)[a] e	0.5 (0.5–1) ab	0.32 (0–1) ab	0 a
Pnt 329	3.5 (0.5–5) d	0.2 (0–0.5) ab	1.25 (0–5) ab	0.2 (0–1) a
Pnt 317	0.2 (0–0.5) a	0 a	0 a	0 a
T3	0.1 (0–0.5) a	4.2 (2–5) e	0.2 (0–0.5) a	0 a
T4	0.05 (0–0.5) a	4.2 (2–5) e	0.3 (0–5) a	0 a
Pnn 374	0.05 (0–0.5) a	0 a	0 a	2.3 (0–5) d
Control	0	0 a	0 a	0 a

Figures followed by the same letter do not significantly differ recording to the ANOVA's multiple range test ($P = 0.05$).
[a]Disease index 21 days after inoculation. Disease index rated from 0 (0% disease) to 5 (100% disease) (average of 10 replications and extreme values in brackets).

result are in agreement with previous observations made by Ersek et al. [19] who created species hybrids of the fungal plant pathogens *P. capsici* and *P. nicotianae* that exhibited expanded host ranges compared with parent organisms and one hybrids lost its ability to infect host plants specific to either species. Changes in pathogenicity in species hybrids may be a consequence of the exchange of intact chromosomes, recombination, or differences in ploidy levels [19]. The pathogenicity trait of the isolates from pepper could suggest that are somatic species hybrids of *P. capsici* and *P. nicotianae*. Host specificity commonly has been used to recognize parasitic fungal species. This specificity is bound to mechanisms of resistance that the plant develops to protect themselves against the attacks from pathogens. Among the elicitors of plant defence mechanisms, elicitins are unique [20]. These small proteins [21] are secreted by the phytopathogenic Chromista *Phytophthora* [22] and *Pythium* [23] and trigger plant responses to elicitor treatment [20]. These results indicate a tendency toward host specificity among *P. nicotianae* isolates tested in this study (Table 4) and in accordance with previous results [15].

3.2. Molecular analyses

RAPD–PCR was also used to establish the occurrence of DNA of *P. nicotianae* in the hybrid isolates. RAPD analysis could be used to compare genotype background and to estimate genotypic variation among strains [24]. The electrophoresis of the RAPD products showed a polymorphism between isolates based on the size and the number of the fragments amplified. The size of amplified DNA fragments generated with the six primers ranged from 0.2 to 2 kb. The six primers detected 19 polymorph amplified fragments, most (79.5%) of the RAPD bands detected were polymorphic in the *P. nicotianae* isolates from pepper and showed inter- and intra-isolate variability. But, the polymorphism showed 51% and 17%, respectively, for tomato and tobacco isolates of *Phytophthora*. For the total number of the RAPD, the percentage of markers common on the isolates of a same host varied 2.56% for *P. nicotianae* isolates from tomato to 38.46% for isolates from pepper.

Genetic similarity among all strains was based on the amplified fragments generated with the six primers, which was shown in a phenogram. A strong dissimilarity (93%) has been observed between pepper isolates of *Phytophthora*. On the other hand, the genetic similarity, however, is low (<40%) among most strains isolated from tomato (12% of dissimilarity) and tobacco (40%) (Fig. 1).

The genetic distances between two individuals are equivalent to their total number of observed band differences. The genetic distances between the isolates of *P. nicotianae* isolated from pepper and those isolated from tomato were weaker (7–62.9%) that those determined at the isolates between pepper and tobacco (33–100%) or at the isolates of tomato and tobacco (46–74%). Numerous methods are available for analyzing molecular data for use in genetic variation of fungi. All methods have inherent assumptions, but through the use of appropriate methods, genetic variation can be made more definitive. The UPGMA is based on the assumption that mutation rates among the different lineages are constant. Since it is currently used for analyses of genetic variation of fungi [11], it was employed in our results. All being tested strains were differentiated into four clusters (Fig. 1). The four phylogenetic groups have each a bootstrap similarity of 65%. This tree also shows a bigger similarity between isolates of *P. nicotianae* isolated from tomato and pepper, in relation to those of tobacco. Such a result agrees with others authors have shown a bigger morphological resemblance between tomato and pepper isolates than with isolates of *P. nicotianae* isolated from tobacco. This suggests that, *P. nicotianae* isolated from pepper might be a hybrid, possibly involving *P. nicotianae* that infect tomato as one parent.

As in a previous study, DNA of *P. nicotianae* was detected in three of four hybrids by hybridization of RAPD–PCR products with species-specific DNA from *P. nicotianae* [24].

Little is known about interspecific hybridization in the genus *Phytophthora.* If exchange of genetic material occurs

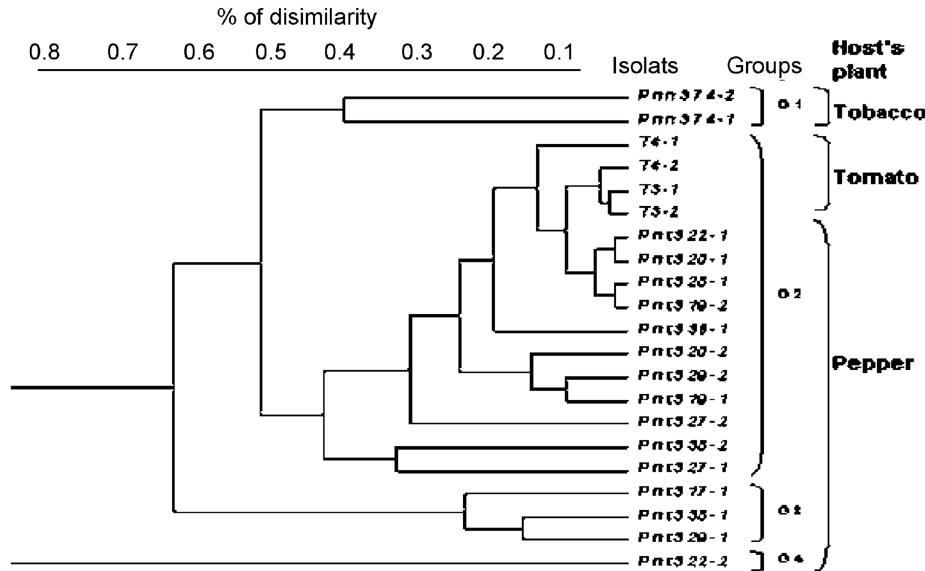

Fig. 1. Dendrogram (UPGMA) showing the relationships among different isolates of *Phytophthora nicotianae* based on randomly amplified polymorphic DNA (RAPD) analysis with six decamer primers.

in the hybrid oospores, interspecific crosses may be an important source of variability.

Recently, Brasier et al. [8] analyzed several *Phytophthora* isolates from an *Alnus* sp. All isolates possessed ornamented oogonia, a feature typical for *Phytophthora cambivora*. However, the alder *Phytophthora* isolates differed from *P. cambivora* by their homothallism, colony morphology, lower cardinal temperatures for growth, and unusually high chromosome numbers and dimorphic ITS arrays. It was concluded that the UK variants represented a heterogeneous group of interspecific hybrids between a *P. cambivora*-like species and an unknown species closely related to *Phytophthora fragariae*. Brasier also suggested that *Phytophthora citrophthora* originated as a hybrid of *P. nicotianae* on citrus and *P. capsici* on cocoa [9]. In the laboratory, hybrids were created between *P. nicotianae* and *P. capsici* by zoospore fusion [25]. Some of the resulting hybrid isolates had an extended host range. Hence, it was hypothesized that interspecific hybridization in nature may result in extended host ranges. However, a reduced host range was found by Goodwin and Fry [26] in interspecific hybrids between *Phytophthora infestans* and *Phytophthora mirabilis*. Both of these results may be possible depending on the type of host–pathogen interaction involved. Our hybrid isolates, from pepper, may be an example of reduced host range, because one of the parental species was reported on this host previously [3,27,28].

The analysis of the amplification profiles permitted us to provide markers common to most isolates of *P. nicotianae* independent of plant host of origin. Although some unique bands were present in certain groups of isolates in *P. nicotianae*, the absence of bands of common length did not mean that there were no sequence similarities. Among the 39 RAPD generated fragments, seven (17.9%) are present in most isolates.

The markers OPD-06-1100, OPD-06-800, OPD-06-500 and OPD-10-1200 showed a frequency of 0.93, 1, 1 respectively to pepper, tomato and tobacco isolates. Fifteen (38.5%) RAPD bands characteristic patterns for the two isolates of *P. nicotianae* isolated from pepper and tomato. The two bands OPK-08-400 and OPK-08-300 were strongly present in the tomato and pepper isolates of *Phytophthora* with a frequency 1 and 0.93, respectively. Whereas there were not any band common to the pepper and tobacco isolates. Sixteen bands generated (41%) discriminated the isolates of *P. nicotianae* isolated from pepper such as OPD-01-1300 and OPD-10-1500 are present at a frequency of 0.53 and 0.46, respectively. Indeed, these last two bands were considered as specific markers for the most pathogenic isolates on pepper (Fig. 2). The OPK-08-1950 locus is specifically detected at the isolates of *P. nicotianae* isolated from tomato. Whereas there is not a specific band to the tobacco isolates of *Phytophthora* (Fig. 3). The results showed RAPD technique is a powerful tool in the analysis of the genetic diversity in *P. nicotianae*.

In the present paper, these pathogenicity and molecular data suggest that the pepper isolates of *P. nicotianae*, which showed a pronounced adaptation on pepper plants, might be a somatic species hybrids of *P. capsici* (from pepper) and *P. nicotianae* (from tomato) as parent species.

Other molecular analyses can be used in order to study this parasitic adaptation when we will include a wider range of isolates and primers. Molecular markers (e.g., RFLPs, RAPDs, AFLPs, SSCPs, DNA fingerprinting) have been used to distinguish fungal taxa that are difficult to characterize by traditional morphological means (e.g., [29] and [30]). Such markers are frequently found to be fixed within species that are delimited by other characters and are invaluable for identification purposes.

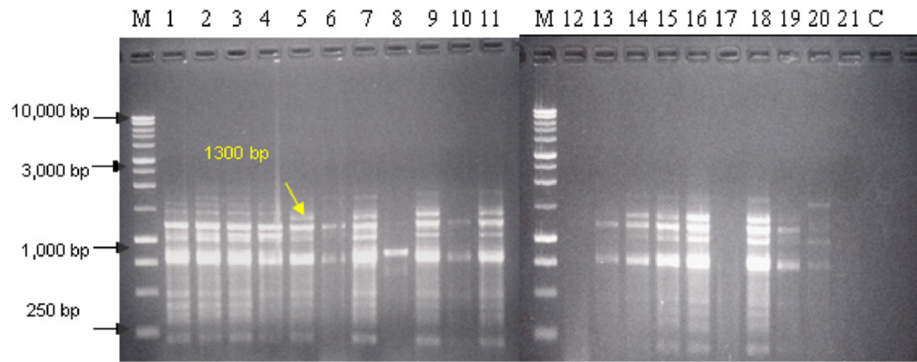

Fig. 2. RAPD–PCR patterns of *P. nicotianae* isolates with OPD-01. The arrow indicates a 1300 bp loci is specifically detected as specific markers for isolates of *P. nicotianae* most pathogenic on pepper. 1 = T3-1, 2 = T3-2, 3 = T4-1 were isolated from tomato. 5 = *Pnt336-1*, 6 = *Pnt317-1*, 7 = *Pnt338-1*, 8 = *Pnt338-2*, 9 = *Pnt329-1*, 10 = *Pnt329-2*, 11 = *Pnt328-1*, 12 = *Pnt327-1*, 13 = *Pnt327-2*, 14 = *Pnt319-1*, 15 = *Pnt322*, 17 = *Pnt320*, 18 = *Pnt320-2*, 19 = *Pnt320-2* were isolated from pepper. 20 = *Pnn374-2*, 21 = *Pnn374-1* were isolated from tabacco; C = control, M = markers.

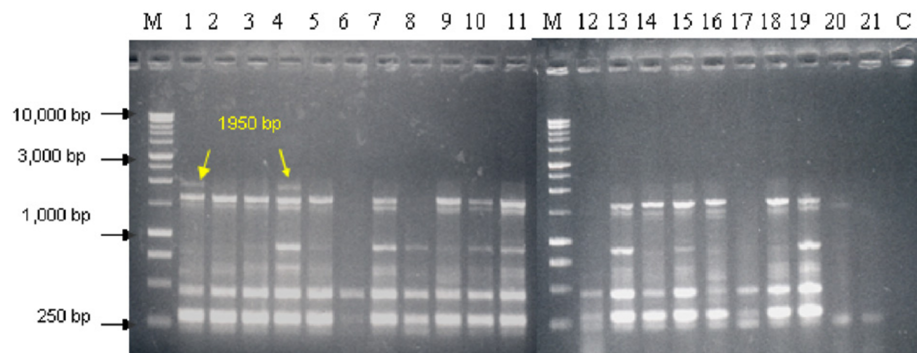

Fig. 3. RAPD–PCR patterns of *P. nicotianae* isolates with OPK-08. The arrow indicates a 1950 bp loci is specifically detected at the isolates of *P. nicotianae* isolated of tomato.

It constitutes a new and important field of investigation for understanding the mechanisms underlying self–non-self recognition in diverse hetero- and autotrophic organisms. By using plant LTPs, we will begin to understand the subtle molecular mechanism that determines competition and even plant–pathogen interactions.

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