



Characterization of *Phytophthora infestans* Isolates from Two Potato Varieties in the Highlands Agro-Ecological Zone of Cameroon

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Authors' contributions

This work was carried out in collaboration among all authors. Author SLDL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AH, WNKT, MB and FCGG assisted in manipulation and managed the analyses of the study. Authors JFD and ZA managed the literature searches and corrected the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Late blight caused by Oomycete *Phytophthora infestans* remains a huge problem in potato production and one of the most severe crop diseases worldwide. In Cameroon, the populations characterization of this pathogen remain very little known.

Aims: This study aims to characterize isolates of *P. infestans* collected in twelve localities of Highlands zone of Cameroon.

Study Design: Twelve localities in three main potato production areas of the Highlands agro-ecological zone (HAZ) in Cameroon were selected for sample collection. The phenotypic

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parameters of sporangia were measured. The presence of mating types and pathogenicity tests on detached leaflets of two potato varieties (*MANATE* and *CIPIRA*) were assessed.

Results: A total of 36 isolates were collected. Height (08) Sporangia shape of *P. infestans* were obtain and 02 new different shape were pip form and oval to ellipsoid. Sporangia length/width ratio ranged from 1.62 to 2.18. Mating types A1 and A2 were present in the studied areas. Pathogenicity test on detached potato leaflets was positive with all the 36 isolates. The isolate HPBT02 from Tsela locality was more aggressive ($P < 0.05$). *MANATE* variety was more susceptible.

Conclusion: The study shows that mating types A1 and A2 of *P. infestans* exit in Cameroon and the morphology of sporangia varies according to the localities. Molecular characterization is needed.

Keywords: *Potato; Phytophthora infestans; isolates; phenotypic characterization; mating types; Cameroon.*

1. INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) De Bary is one of the most severe diseases of potatoes worldwide [1]. This pathogen which belongs to the class Oomycetes is capable to attack all vegetative and reproductive parts of plants. Despite researches carry out on potato late blight in Cameroon and elsewhere in the world, the disease remains a huge problem in potato production [2,3]. In Cameroon, this disease cause significant damage and 100% yield losses on potatoes and tomatoes specially in highlands agro-ecological zone where climate is favorable to the pathogen [2]. Control measures involve the use of many homologated chemical fungicides. Moreover, the Institute of Agricultural Research for Development (IRAD) in collaboration with the International Potato Centre (CIP) started to produce several improved potato varieties in Cameroon since 1980. Between these varieties, two (*CIPIRA* and *TUBIRA*) have been widely adopted by the Cameroonian public for their high yields and resistance to late blight. In addition, farmers cultivate some local and imported varieties. However, late blight management with these chemical fungicides and improved potato varieties showed a relative efficacy, which is generally limited due to the development of resistance and mutation of the *P. infestans* populations [4]. Efficiency of most commonly uses active ingredients like metalaxyl and maneb in late blight control has been demonstrated to be reduced in Cameroon since the years 2000 [2,5]. Besides, numerous studies in Cameroon have shown the importance of controlling this disease using biofungicides in order to reduce the effects of these chemicals, whose pathogen resistance forms have been revealed [6,7,8]. Also, the misuse of systemic fungicides that are toxic to humans and the environment [9] in

recent years has led to major changes in pathogen dynamism and resistance in *P. infestans* populations. The "old" A1 mating type population, which was simple and reproduced only asexually, has been replaced by a "new", more aggressive and diverse population containing two mating types A1 and A2, which allows the pathogen to reproduce sexually [10]. Many studies have confirmed the presence of this type of sexual compatibility in America [11,12], Europe [13] and North Africa [14-18]. Characterization of the different isolates of *P. infestans* at the local level therefore, remains essential for the creation of an effective management control strategy to this disease as reported by Cooke et al. [19]. The main objective of the present work is to contribute to the phenotypic characterization of *P. infestans* isolates in Cameroon. Three research questions will be investigated: (i) Are morphological and morphometric traits of *P. infestans* isolates vary within the Highlands agro-ecological zone (HAZ) of Cameroon? (ii) Are mating types A1 and A2 of *P. infestans* present in the HAZ of Cameroon? (iii) Are isolates of *P. infestans* populations in the HAZ of Cameroon have the same level of aggressiveness?

2. MATERIALS AND METHODS

2.1 Samples Collection

Infected potatoes leaves, stems, fruits and tubers showing typical symptoms of late blight were collected from potato farms of three Divisions (Bamboutos, Menoua, and Mezam) of the Highlands agro-ecological zone (HAZ) of Cameroon. A total of 36 potatoes fields distributed in 12 localities (4 localities per Division) were sampled at a distance of 8 to 15 km from each other along the main roads according to Fontem et al. [2]. These localities

were: Abonghen 1 (642162 N et 670778 E), Abonghen 2 (642177 N et 670810 E), Bambui (634890 N et 666313 E) and Ntah (637642 N et 658081 E) for Mezam Division ; Ligan (620728 N et 615672 E), Melo (619354 N et 615559 E), Ndoh (617728 N et 614572 E) and Ngui (619432 N et 615637 E) for Menoua Division ; Tiamekie (632401 N et 622517 E), Tola (623307 N et 628303 E), Tsela (630267 N et 620544 R) and Bamaka (625185 N et 624901 E) For Bamboutos Division. The samples collected at each point of the locality were introduced in sealed envelopes, codified and placed in a cooler containing ice and take to the laboratory for pathogen isolation.

2.2 Cultivation, Isolation and Identification of the Pathogen

Four culture media (water-agar, Pea-agar, Potato Dextrose Agar and V8) supplemented with antibiotics (nystatin: 19 mg/ml, rifampin: 20 mg/ml and ampicillin : 200 mg/ml) were used to isolate and purify isolates of *Phytophthora infestans* [20]. Two isolation techniques were used. The first consisted in removing infected organs and then cutting them with a razor blade into small fragments of 2-4 cm² for leaves and 0.56-0.84 cm² for stems. These fragments were washed with tap water and then disinfected with 1% sodium hypochlorite solution for 2 min. They were then collected and incubated in moistened boxes at 18-20°C in the dark for 24 hours to promote sporulation of the fungus. The isolation of this pathogen consisted in transplanting sporangia taken under aseptic conditions from the surface of infected tissue using a sterile needle whose tip contains a 1-2 mm diameter fragment of the agar. The agar fragment should only touch sporangia attached to the end of the mycelium. This sample should preferably be taken from the newly developing lesion. The inoculated agar fragments were placed in 90 mm Petri dishes containing the four different culture media. Incubation was carried out in the dark at 20°C then isolates were purified after 3 to 4 days of incubation.

The second technique consisted in taking the mycelium developed at the front of the lesion on the detached leaflets and placing it under potato slices about 5 cm in diameter and 1.5 cm thick previously disinfected with 1% sodium hypochlorite for 2 min, washed with sterile distilled water and placed in boxes containing water-soaked cotton. Incubation was carried out

here at 4°C and in the dark until the mycelium has completely formed on potato slices [21].

Inoculum was collected directly from 10 to 14 days old pure cultures by adding 10 ml of sterile distilled water to each isolate and lightly scraping the surface with a sterile platinum loop to dislodge sporangia. Sporangia suspensions were filtered through a muslin cloth to remove mycelial fragments and diluted to 20 000 sporangia/ml using a hemocytometer [2]. The sporangia were cooled to 4°C for 2 h to induce spore release [21].

2.3 Morphological and Morphometric Characterization of Sporangia

To carry out the morphological characterization of the isolates collected in the field, the different isolates of *P. infestans* from infected potato leaves and stems were taken directly to the laboratory. Then, stem and leaf fragments of the infected parts were introduced into Petri dishes on water-soaked cotton. Once the mycelium and sporangia were developed, the phenotypic structures of the different sporangia from each isolate were observed using a microscope. The shapes of each sporangium were recorded according to the locality of origin. An optical microscope mounted with a micrometer objective was used to measure the size [length (L) and width (W)] of the sporangia at 100X magnification. Then, the length/width (L/W) ratio was calculated for each locality [22].

2.4 Checking for the Presence of Mating Types A1 and A2

To determine the presence of mating type, two isolates from different localities were randomly selected. Mycelium discs obtained with a 7 mm diameter punch and taken from 7-10-day old cultures of *P. infestans* were deposited in the center at 2-3 cm apart in Petri dish containing V8 medium. The plates were then incubated at 20°C in the dark for 14-21 days. The operation was repeated by comparing all isolates between localities and Divisions for the presence of different Mating types. After the two colonies have come in contact with each other, the developed mycelium was aseptically collected from the confrontation areas for microscopic observations. The presence of these two mating types A1 and A2 is therefore considered to occur when sexual reproduction is observed through the oospores under the microscope [23].

2.5 Evaluation of the Aggressiveness of Isolates of *P. infestans* on Detached Potato Leaflets

To achieve this activity, healthy leaflets were collected from two potato varieties (resistant variety CIPIRA from Agricultural research Institute for Development and sensitive variety MANATE from local farmers) in farmer's fields. These leaflets were washed with 2% of bleach solution of sterile distilled water during 5 min and dried before being placed upside down in 90 mm diameter Petri dishes containing moistened filter paper to form a wet environment, suitable for *P. infestans* growth. A contact fungicide Bravo 720 with 720 g chlorotalonil per liter as an active ingredient were applied to the detached leaflets using a syringe. Once the leaflets were dried (1 h), they were then inoculated with a single drop (50 μ l, 2×10^4 sporangia/ml) of sporangia suspension applied to the center of the leaflet using a pipette. Control treatments contained either one drop of sterilized distilled water containing 0.05% Tween 20 as the negative control or one drop of sporangial suspension for the positive control. Petri dishes were stored in 20°C in the dark. After 7 days incubation, the length (L) and width (W) of the lesion were measured for each leaflet and the area of the lesion (A in cm²) was calculated using the following formula: $A = [\pi (L + l) 2] / 4$ [24].

2.6 Statistical Analyses

Data collected on morphometric and aggressiveness of isolates were analyzed using the R software version 3.6.2 which uses the standard analysis of variance (ANOVA) method. For the Tukey HSD test, the significance level

was assessed at the 5% threshold. The Excel 2013 spreadsheet was used for the establishment of plots, histograms and tables.

3. RESULTS

3.1 Isolation and Identification of *P. infestans* Isolates

Direct and indirect techniques for obtaining isolates from the mycelium developed above potato slices allow us to obtain 36 isolates that were stored as sporangial suspensions in sterile test tubes. Each isolate was obtained from one of the 36 plots. The macroscopic and microscopic (Fig. 1) characters of these pure strains revealed that the *P. infestans* isolates in HAZ are aerial aseptate mycelium, which are sometimes dense and whitish in color (Fig. 1A) in different culture medium. These mycelium present terminal sporangia (Fig. 1B) which release after a thermal shock the zoospores (Fig. 1C) characteristic of *P. infestans*.

3.2 Morphological Characteristics of Sporangia

The shape of the sporangia varied from isolate of one Division to another. A total of 8 shapes (Fig. 2) were observed; these are: oval to ellipsoid; ellipsoid; pip form; elliptic; subglobose; globose; ovoid; lemoniform. These sporangia are deciduous and have papillae and short pedicels typical of *P. infestans*. These shapes revealed that the populations of *P. infestans* in HAZ in Cameroun are highly diverse. The new shape of sporangia from different isolates as pip form and oval to ellipsoid can be observe.



Fig. 1. Macroscopic and microscopic characters of a pure 14-day-old HPMN02 isolates of *P. infestans* from Ngui locality on V8 medium (A), sporangia (B) and zoospores (C) seen with a photonic microscope (magnification X 100)

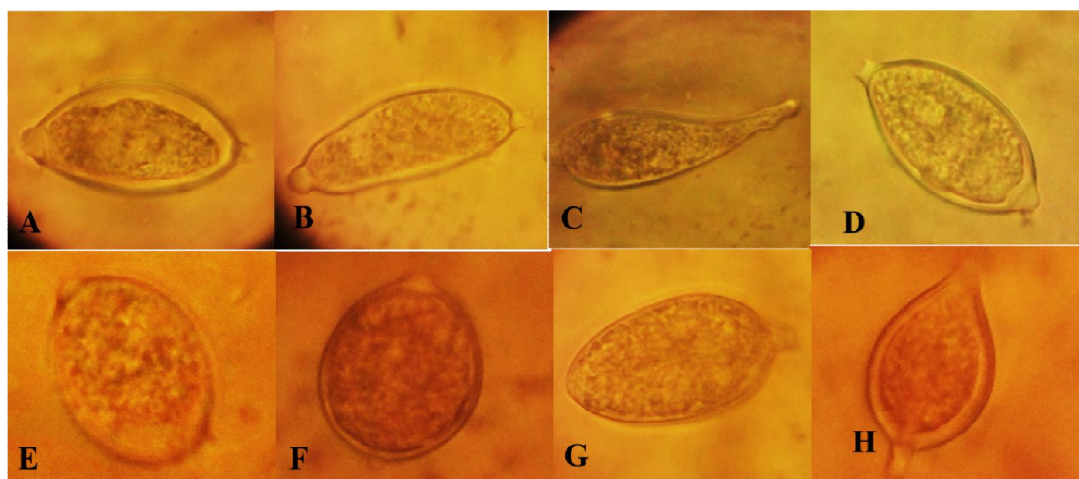


Fig. 2. Sporangia shapes of *Phytophthora infestans* isolates from 12 different localities in the Highlands zone of Cameroon (magnification X 100)

A: Oval to ellipsoid, B: Ellipsoid, C: Pip-shaped, D: Elliptic, E: Subglobose, F: Globose, G: Ovoid, H: Lemoniform

3.3 Morphometric Characteristics of Sporangia

Sporangia lengths (L) and widths (W) of *P. infestans* isolates ranged from 0.63 to 0.96 μm and from 0.36 to 0.50 μm respectively. The length/width ratio of all *Phytophthora* isolates studied ranged from 1.58 to 2.18 with a mean ratio which of 1.62; 1.81 and 1.83 in Mezam, Menoua and Bamboutos Divisions respectively (Table 1). There was no significant difference ($P>0.05$) between sporangia sizes between localities and Divisions of origin of isolates.

3.4 Presence of Mating Types A1 and A2 among *P. infestans* Isolates

Daily observation of mycelium from different isolates in contact with each other reveals that the populations of *P. infestans* in HAZ is two Mating types (A1 and A2). There are sexual reproduction and asexual reproduction. The sexual mode of reproduction is siphonogamous oogamy, with the antheridia on the oogonia being amphigynous (Fig. 3A). After fertilization, the central part of the oogonia becomes zygote, called oospore (Fig. 3A). These organs will germinate later to produce new generations. Moreover, after further confrontations of isolates between them, microscopic observations reveal the presence of two identical Mating types through asexual reproduction. The sporangia

either will directly emit a germ tube or will encyst before emitting a germ tube (Fig. 3B).

3.5 Aggressiveness of *P. infestans* Isolates on Detached Potato Leaflets

All the 36 isolates of *P. infestans* obtained were pathogenic on detached potato leaflets with a clear development of typical lesions of late blight (small light to dark green water-soaked spots on young lesions and blackish/brown lesions with whitish sporangia on mature lesions). The level of aggressiveness of three randomly chosen isolates (HPBT02, HPMN02 and HPMeA03) from Bamboutos, Menoua and Mezam Divisions respectively showed that the areas of lesions were 5.21; 6.01 and 8.02 cm^2 for isolates HPBT02, HPMN02 and HPMeA03 respectively in the positive control in CIPIRA variety, while they were zero for the three isolates in the negative control. The areas of lesions of leaflets inoculated with Bravo 720 were significantly lower than in the controls. In fact, they were 1.6; 2.06 and 4.3 cm^2 for HPBT02, HPMN02 and HPMeA03 isolates respectively. The same results were obtained with leaflets of the MANATE variety. Isolate HPBT02 from the Tsela locality and Bamboutos Division showed the highest significant ($P<0.05$) aggressiveness (12.53 cm^2), unlike the HPMN02 and HPMeA03 isolates which showed no significant difference both between treatments and varieties (Table 2).

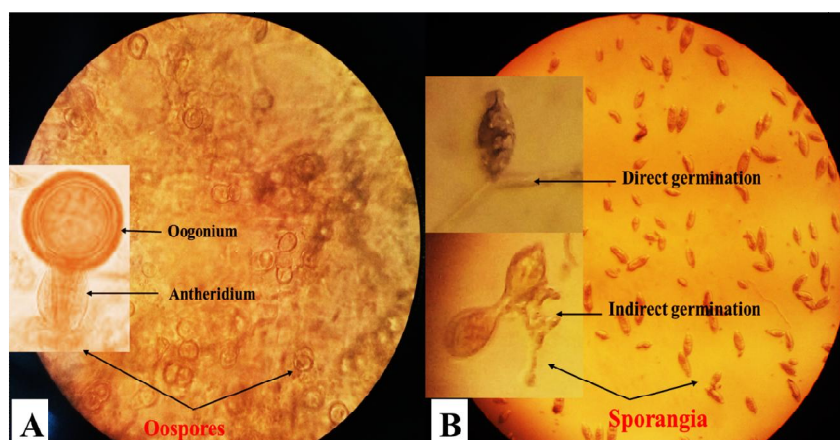


Fig. 3. Pictures showing the presence of sexual types A1 and A2 on contact zone of 14 days old colonies of *P. infestans* from potato late blight in different localities of the highlands agroecological zone in Cameroon; A and B are sexual and asexual reproduction respectively

Table 1. Morphometric characteristics of sporangia of *Phytophthora infestans* isolates from one locality to another

Divisions	Localities	Length of sporangia (L) in μm^*	Width of sporangia (W) in μm^*	Ratio (L/W)
Bamboutos	Tsela	0.96 ± 0.05 a	0.50 ± 0.00 a	1.93 ± 0.11 a
	Tiamekie	0.83 ± 0.11 a	0.46 ± 0.15 a	1.87 ± 0.42 a
	Bamaka	0.73 ± 0.11 a	0.40 ± 0.00 a	1.83 ± 0.14 a
	Tola	0.76 ± 0.15 a	0.46 ± 0.05 a	1.63 ± 0.15 a
Means		0.82 ± 0.12 a	0.45 ± 0.79 a	1.81 ± 0.23 a
Menoua	Lingan	0.93 ± 0.05 a	0.43 ± 0.05 a	2.18 ± 0.35 a
	Ngui	0.63 ± 0.05 a	0.40 ± 0.00 a	1.58 ± 0.14 a
	Melo	0.70 ± 0.17 a	0.40 ± 0.00 a	1.75 ± 0.43 a
	Ndoh	0.66 ± 0.11 a	0.36 ± 0.05 a	1.83 ± 0.28 a
Means		0.73 ± 0.15 a	0.40 ± 0.04 a	1.83 ± 0.35 a
Mezam	Abonghen 1	0.70 ± 0.26 a	0.40 ± 0.00 a	1.75 ± 0.66 a
	Abonghen 2	0.73 ± 0.11 a	0.43 ± 0.11 a	1.73 ± 0.23 a
	Bambui	0.73 ± 0.20 a	0.40 ± 0.00 a	1.83 ± 0.52 a
	Ntah	0.66 ± 0.11 a	0.40 ± 0.10 a	1.70 ± 0.27 a
Means		0.68 ± 0.19 a	0.43 ± 0.07 a	1.62 ± 0.52 a

*Means followed by the same letter in the same column are not significantly different at the 5% threshold according to the turkey test

Table 2. Surface area of late blight lesions (cm^2) on detached leaflets of two potato varieties 7 days after incubation

Varieties	Treatments	area of lesion in cm^2 *		
		Isolates of <i>P. infestans</i>		
		HPBT02	HPMN02	HPMeA03
CIPIRA	Negative control	0 a	0 a	0 a
	Positive control	8.02 ± 1.14 c	6.01 ± 0.56 b	5.21 ± 1.13 b
	Bravo 720 WP	4.3 ± 1.93 b	2.06 ± 0.46 a	1.6 ± 0.25 a
MANATE	Negative control	0 a	0 a	0 a
	Positive control	12.53 ± 3.56 c	8.42 ± 1.67 c	8.82 ± 1.32 c
	Bravo 720 WP	4.67 ± 0.3 b	1.51 ± 0.35 a	2.15 ± 1.06 a

**MEANS followed by the same letter in the same column are not significantly different at the 5% threshold according to the turkey test. Positive and negative controls are one drop of $50 \mu\text{l}$, 2×10^4 sporangia/ml and 0 sporangia/ml respectively

4. DISCUSSION

Surveys carried out in 12 localities of the main potato producing Divisions of the Highlands agro-ecological zone of Cameroon have allowed to obtain a low number of purified isolates. This shows that obtaining pure strains of *P. infestans* on culture media remains a big constraint in plant pathology. Moreover, Abdelhadi [14] and Beninal [25] stressed this difficulty when characterizing the Moroccan and Algerian populations of *P. infestans* respectively. Indeed, this could be explained by the low competitiveness of *P. infestans* vis-à-vis other fungi. The results of morphological and morphometric characteristics of *P. infestans* isolates showed a significant variation in morphological characters. These morphological characters are similar to those described by Gallegly and Hong [26]. Several shapes (08) and new shape of sporangia of *P. infestans* isolates from different localities in HAZ were identified, thus indicating a high diversity of *P. infestans* populations in Cameroon. Kerroum [18] observed and identified 03 sporangia shapes of *P. infestans* on potato crops in Algeria. It can also be noted that the environment and growing media would influence these different forms. Indeed, Coulibaly et al. [27] had already reported this when working on the characterization of isolates of *Phytophthora* spp. from cocoa orchards in Ivory Coast. The mean of the length and width ratio (L/W) of sporangia ranged from 1.58 to 2.18, which would certainly be related to their different shapes. This corroborates Coulibaly et al. [27] results that showed that the L/W ratio of sporangia from all *Phytophthora* isolates studied ranged from 1.23 to 1.94 with a mean ratio of 1.72. Sexual compatibility tests revealed the coexistence of the two mating types A1 and A2 within *P. infestans* populations in the HAZ of Cameroon. The oospore produced in some contact zone of different isolate reveal the presence of mating type. Miller [28] showed that sexual reproduction is only feasible after a confrontation between two mycelia of different sexual types A1 and A2. The presence of these mating types in Divisions of HAZ of Cameroon would justify the high diversity of *P. infestans* populations and a new shape of sporangia due to recombination. Indeed, recent works have revealed the presence of the new mating type A2 in Africa [29,30,31]. This heterogeneous distribution would result from the different origins of potato seeds used in the growing areas. These seeds would be infected by strains of different mating types. Indeed, other factors could explain the presence of mating type A2

such as its virulence towards the varieties used. The presence of the two mating types, either in the same plot or in different plots, would lead to the formation of oospores in the plant tissue or in the soil. These sexual propagules would be another source of inoculum and genetic variability [32]. Pathogenicity tests were positive in all 36 isolates obtained, thus corroborating with the work of Kerroum [18]. Niks et al. [33] showed that the races of *P. infestans* isolates are very complex and can bypass several resistance genes (4 to 5 genes). All potato leaflets, inoculated with sporangial suspensions developed typical late blight lesions from 3 days after incubation. Results also show that aggressiveness HPBT02 isolate from the Tsela locality, Bamboutos Division was the most aggressive on both all treatments and varieties. Farmers probably relate this high aggressivity to the environment and the host varieties of the pathogen but also to the high frequency and diversity of fungicides use in this Division. The area of the lesions varies significantly between leaflets previously cleared with fungicide and controls. Maneb is an efficient fungicidal active ingredient used worldwide for late blight and others vegetables diseases control. Goufo, et al. and Djeugap, et al. [6,8] Also, potato leaflets of the susceptible variety (MANATE) developed lesion sizes larger than those of the tolerant variety (CIPIRA). This could be explained by the ability of the resistance gene in the tolerant variety to resist the aggressiveness of the pathogen.

5. CONCLUSION

The objective of this study was to carry out a phenotypic characterization of *P. infestans* isolates in 12 localities of three potato production Divisions in the Highlands agro-ecological zone (HAZ) of Cameroon. It was found that 36 isolates were obtained from the different localities. Sporangia shapes of *P. infestans* isolates vary from ellipsoid to globose, elliptic, ovoid and citriform. Sporangia sizes were also highly heterogeneous, but can be grouped into two large sizes, averaging 2.01 and 1.47 depending on the length/width ratio. Mating types A1 and A2 were present in the HAZ and in the three main potatoes producing Divisions. Pathogenicity tests were positive with all the isolates obtained. Areas of lesions varied significantly when potato leaflets were previously cleared with fungicide compared to controls. It is planned to carry out the genotypic characterization of *P. infestans*

populations in Cameroon in order to have a general overview of the diversity of this polyphagous pathogen in the country.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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