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ORIGINAL PAPER

PCR-based determination of colonization patterns during potato tuber infection by single and multiple pathogens

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Abstract Potato tubers piled in storage are prone to infection by numerous pathogens. Each pathogen can cause damage alone, but severe losses often arise when more than one pathogen is involved. Currently, only a visual diagnosis is practiced on potato tubers before storing them, which does not allow any prediction of further disease spread. The aim of the present study was to determine differences in patterns of tissue colonization by several tuber decay pathogens and how late blight infection affects further tuber colonization by other important tuber pathogens. This study was conducted using artificial inoculation of potato tubers and PCR to provide an early and accurate diagnosis of disease development for major potato tuber rots, and to assess potential synergism/antagonism between *Phytophthora infestans* and other pathogens in stored tubers. In order to accurately follow the progress of each pathogen in tuber tissues, samples were collected over time from both the surface (peel, 0–2 mm depth) and internal tissues (flesh, depth > 2 mm) of the tubers at various distances from the inoculation site, at 3, 5, 7, 10, 12, 14, 17, and 19 days after inoculation. Successful detection of single or multiple pathogens was achieved using specific PCR-primers for each pathogen. Pathogens were always detected several centimeters ahead of the visible lesions. This tracking enabled us to determine the extent of colonization both on the tuber's surface and in internal tissues by each tested pathogen, either after single or multiple infections involving *P. infestans* as the primary pathogen. The presence of *P. infestans* was shown to enhance the development of *Pectobacterium atrosepticum* and to slow down that of *P. erythrospetica* and *Pythium ultimum*. No noticeable effect on further tuber colonization by *F. sambucinum*, *V. dahliae* or *V. albo-atrum* was observed in the presence of *P. infestans*. This approach involving more than one pathogen is more realistic than classical studies considering single pathogens, and may be helpful in monitoring the sanitary status of stored tubers. Our results make the outcome of certain combinations of pathogens in potato tubers more predictable and may result in more efficient preventive measures.

Keywords Pathogen dynamics · Potato tuber rot · Storage · Single and multiple infections · PCR

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Introduction

Potato (*Solanum tuberosum*) is one of the most important crops cultivated for human foods and trade worldwide (Potato News, 2004). However, potato growers face many difficulties during both the growing season and storage due to several pathogens and pests (Bradshaw & Mackay, 1994; Spielman et al., 1991). None of the integrated management programmes currently used completely eliminate pathogens, which persist and propagate during storage, leading to serious post-harvest losses. Tooley, Carras, and Lambert (1998) have reported that even when tubers are clean in appearance at harvest, they may still harbour one or more pathogens that can spread quickly under conducive storage conditions, thus decreasing income. Therefore, heavy use of chemical pesticides is necessary to maintain reasonable yield during the growing season (Platt & Mahuku, 2000).

Several potato pathogens, including *Phytophthora infestans* (cause of late blight), *P. erythroseptica* (cause of pink rot), *Pythium ultimum* (cause of leak or watery wound rot), *Fusarium sambucinum* (teleomorph: *Gibberella pulicaris*) (cause of dry rot), *Verticillium dahliae* and *V. albo-atrum* (cause of Verticillium vascular discolouration of the flesh), and bacteria such as *Pectobacterium atrosepticum* (Gardan, Gouy, Christen, & Samson, 2003) (cause of soft rot) (=*Erwinia carotovora* subsp. *atroseptica*) are known to cause serious damage to stored potatoes (Bohl & Nolte, 2003; Voss & Hall, 2003). Although each of these pathogens alone can cause disease and loss of income, the most serious damage often arises from complexes involving more than one pathogen. Daayf and Platt (1999) have reported that more than one pathogen can be involved in tuber rot in storage and it is well established that some pathogens such as *P. infestans* predispose tissue to secondary pathogens, resulting in much more severe losses. The most serious storage disease of potato tubers is the secondary soft rot caused by *P. carotovorum*.

To date, no existing information accurately describes how tuber rot develops over time in potato storage. Such data are lacking for tubers infected with either a single or combined pathogens. Gathering such information has been difficult due to time and resource constraints. However, the advent of molecular techniques now provides faster and relatively cheaper solutions to such impediments.

Without accurate data about the presence of individual pathogens and how they interact with others within tubers during storage, predicting the risk of storage rot and developing adequate prevention and management strategies will continue to be a challenge. An appropriate integrative approach, which takes into account some characteristics of the pathogens involved in tuber rot, is needed to establish storage conditions that can help minimize damage from storage diseases. To achieve this goal, it is hence necessary to develop an understanding of how these pathogens interact with each other and with the tuber. This can be achieved through the development of accurate detection tools for the presence of specific pathogens either alone or as part of a pathogen complex. To date, diagnosis has been usually made visually before placing potato tubers into storage.

Polymerase chain reaction (PCR) is a method that allows us to enzymatically amplify a DNA sequence of interest using specific oligonucletide primers (Edel, 1998; Mullis & Faloona, 1987). In plant pathology, PCR offers several advantages compared to more traditional methods of plant disease detection, such as specific ELISA assays or plating the pathogens onto selective media. PCR also possesses sensitivity and specificity, and is rapid and versatile (Henson & French, 1993). Moreover, with PCR it is possible to detect either single or multiple pathogens (Tooley, Bunyard, Carras, & Hatziloukas, 1997; Trout, Ristaino, Madritch, & Wangsomboondee, 1997).

The main objective of this study was to determine whether there are significant differences in the extent of tissue colonization by several tuber decay pathogens and the effect of late blight infection on patterns of further colonization by other important tuber pathogens. Such pathogens include those causing pink rot, soft rot, and leak. This is very important for the development of further preventive measures that would anticipate negative effects due to secondary invaders. Different pathogen combinations

and their impact on tuber colonization during storage were analyzed. Detection of each pathogen was conducted by PCR, both in space (tuber peel and flesh) and time (time after inoculation). Such data were gathered from tubers inoculated with one, two or three pathogens. Possible synergistic or antagonistic interactions that may occur among these pathogens were investigated. This information is important for any further studies that would consider disease modelling where the pathogens tested in this study are involved.

Materials and methods

Plant material

Tubers of cv. Russet Burbank were used in this study.

Pathogens

Oomycete isolates used in this study (*P. infestans* US-8 genotype; *P. erythroseptica*; and *P. ultimum*) were monospore cultures from our collection kept in 10% glycerol at –80°C or on appropriate media at 4°C. The bacterial isolate of *P. atrosepticum* as well as *F. sumbucinum*, *V. dahliae* and *V. albo-atrum* isolates were stored in 10% glycerol at –80°C until used. For *P. infestans* inoculum, cultures on V8-PDA (150 ml V8, 10 g PDA, 10 g Agar, 3 g $CaCO_3$, 850 ml H_2O) were first flooded with sterile distilled water (SDW). After draining, the Petri dishes were incubated overnight at room temperature. The sporangia were collected the following day in a Sato's reagent consisting of 40 ml of .25 M Hepes, 20 ml of 5 mM $MgSO_4$, 40 ml of 10 mM $CaCl_2$ for 1 l of SDW (Sato, 1994). The sporangial suspensions were then incubated at 12°C for 2 h to induce zoospore production. For *P. ultimum* inoculum, a plug of V8 agar culture was transferred to a flask containing V8 broth medium and incubated for 10 days at room temperature on an orbital shaker (100 rpm, Laboratory rotator Model G2, New Brunswick Scientific Co. Inc., N.J.). Mycelium was suspended in SDW and blended for 10 s, filtered through cheesecloth, and the suspension adjusted to 10^6 sporangia ml^{-1}. For *P. erythroseptica*, a plug (5 mm diam) taken from the edge of a 3 day-old culture was placed in 15 ml of clarified V8 broth in a 50 ml plastic sterile centrifuge tube. Tubes were placed in the dark at 18°C for 3 days to allow growth of the pathogen. The V8 broth was then decanted and mycelium washed twice with 10 ml of SDW. Cultures were immersed in 10 ml of sterile pond water and kept at 18°C under continuous fluorescent light. Pond water was changed after 24 h incubation and the new solutions were kept under the same conditions for a further 24 h. Cultures were then chilled at 9°C for 90 min and placed in the dark at room temperature (22°C) for 30 min to enhance zoospore release. Zoospores were counted under a microscope and the suspensions used for inoculation were adjusted to 10^6 zoospores ml^{-1}. As for *P. atrosepticum*, the inoculum was produced on nutrient agar and the bacterial suspensions were calibrated at 10^6 CFU ml^{-1}. For *F. sambucinum* and both *V. dahliae* and *V. albo-atrum*, the inoculum was produced on PDA for two weeks at 20°C. Inoculum suspensions were adjusted to 10^6 spores ml^{-1} before inoculation.

Inoculation procedure, incubation and harvest of tissue samples

Individual tubers were surface-sterilized using a 10% commercial bleach solution. Sterilized tubers were then wounded using the tip of a sterile scalpel and inoculated with 20 µl of inoculum consisting of either one or two pathogens. When two pathogens were involved, *P. infestans* was first introduced one day prior to inoculation with a second pathogen. Inoculated tubers and healthy controls were placed in plastic bags containing wet paper towels and kept in a thermohygro-incubator (Inter City Environment Inst., USA) simulating storage conditions with temperature maintained at 15°C (day/night) to mimic the early stages of transferring tubers into long term storage. At 3, 5, 7, 9, 12, 14, 17, and 19 days after inoculation (d.a.i.), controls and inoculated potato tubers were examined for symptom development. Samples from five tubers from each treatment were taken from both the peel (2 mm thick external tissues including the periderm) and the internal tissues (remaining tuber flesh) at different distances from

the inoculation site. The peel samples were taken in 1 cm increments up to 6 cm from each inoculation site. Samples from internal tissues were taken in 0.5 cm increments up to a depth of 3 cm. All the samples harvested were kept in Eppendorf tubes at 4°C until used for DNA extraction. Prior to sampling, visual estimation of colonization surface upon symptom progress was made by measuring the diameter of the visible lesions at each sampling time.

In vitro interaction between tested pathogens

Interactions between pathogens were evaluated by challenging pathogens with each other, in pairs, on V8, PDA or rye media (120 g of boiled rye filtered through cheesecloth added to 15 g agar and 1 g of dextrose for 1 l of SDW). All the experiments were conducted at 20 ± 2°C. Colony size was measured by recording the diameter at 3, 7, 12, and 15 days after the transfer.

DNA extraction

DNA was extracted from potato peel (external tissues, depth 0–2 mm) and flesh (internal tissue, depth > 2 mm) samples of both inoculated and control tubers following the rapid NaOH lysis protocol described by Wang, Qi, and Cutler (1993). Each sample was ground in 75 µl of .5 M sodium hydroxide using a pestle and suspended in 500 µl of .1 mM Tris-HCl pH 8.0 containing .4 mM of EDTA. Tubes were then centrifuged at 14,000 rpm for 6 min and 300 µl of the supernatant were transferred to new tubes, representing DNA solutions, and kept at –20°C until used for PCR.

Polymerase Chain Reaction (PCR)

For each pathogen, PCR was performed using pathogen specific primers (de Boer & Ward, 1995; Kageyama, Ohyama, & Hyakumachi, 1997; Liu, Chatteljee, & Chattedee, 1994; Tooley et al., 1998; White, Bruns, Lee, & Taylor, 1990; Zotobowska & Pospieszny, 1998) (Table 1). All the primers were first checked using DNA samples extracted from pathogens grown on culture media. Tests were also conducted in order to eliminate the possible cross-detection of the same pathogen with different primers and to ensure the specificity of these primers. All PCR assays were performed using a Techne Flexigene Thermo-Cycler (Flexigene, ON, Canada) using adequate programmes for each primer. When necessary, modifications were made to adjust each protocol in order to achieve a better detection of different pathogens under our laboratory conditions. The PCR products were visualized after amplification on 1.5–2% agarose gel electrophoresis, depending on the size and containing 1% ethidium bromide using a UV-transilluminator (Gel Doc 2000, Biorad Inc., USA) equipped with a video copy processor P90 U (Mitsubishi Inc., Japan).

To relate PCR detection data to the visual colonization of the tissues, static and dynamic

Table 1 Sequences of the internal transcribed spacer (ITS) primers used for the detection of potato tuber rot pathogens

Pathogens	Sequences of forward and reverse primers	Code	Product size (bp)	Ref.
P. infestans	5′-GCATCGATGAAGAACGCAGC-3′ 5′-CGATTCAAATGCCAAGCTAAAG-3′	ITS3 PINF2	456	1
P. erythroseptica	5′-TCCTCCGCTTATTGATATGC-3′ 5′-CTGTTCCGGCGTAAGCTGG -3′	ITS4 PERY2	136	1, 2
P. ultimum	5′-ACGAAGGTTGGTCTGTTG-3′ 5′-TCTCTACGCAACTAAATGC-3′	K1 K3	500	3
P. atrosepticum	5′-CCGCGAAAGTGGATTCAAAAGG-3′ 5′-TGCTTGCGCTTAGACC -3′	ECA1 ECA2	200	4, 5
F. sambucinum	5′-ACATACCTTTATGTTGCCTCG-3′ 5′-GGAGTGTCAGACGACAGCT-3′	FSF1 FSR1	315	6
V. albo-atrum and V. dahliae	5′-CTCATAACCCTTTGTGAACC-3′ 5′- CCGAGGTCAACCGTTGCCG -3′	UVD1 UVD2	452	7, 8

1: Tooley et al. 1998; 2: White et al. 1990; 3: Kageyama et al. 1997; 4: Liu et al., 1994; 5: Zotobowska and Pospieszny, 1998; 6: Mishra, Fox, & Culham, 2003; 7: Nazar, Hu, Schmidt, Culham, & Robb, 1991; 8: Robb, Hu, Platt, & Nazar, 1994

approaches were taken into account. In the static approach, data were reported in centimeters away from the inoculation site, whereas in the dynamic approach, progress in detection related to the distance from the front of the expanding lesion ahead of which the pathogen was detected by PCR.

Data analysis

All the experimental schemes were randomized complete blocks with at least 5 replications per treatment. Each experiment was repeated independently three times. Since no significant treatment *x* experiment interaction was recorded, data from the three replications were pooled in order to present data as an average ± SE. PCR detection of different pathogens from potato tuber samples was conducted at least twice for each tested sample. Over time detection of pathogen colonization and lesion area were followed for each treatment *x* replicate and the area under the disease progress curve (AUDPC) was calculated as follows: $\mathrm{AUDPC} = \sum_{i=1}^{n-1}[((y_i + y_{i+1})/2)\ (t_{i+1} - t_i)]$, where y_i is the surface area of the expanding lesion at the time t_i. Following the AUDPC principle, the area under colonization progress curve (AUCPC) was calculated using the same formula where y_i represents the distance ahead of the inoculation site where the pathogen has been detected by PCR at the time t_i. Raw data collected at different times after inoculation as well as AUDPC and AUCPC data were submitted to ANOVA analysis using Statistica software (Statsoft, 1999, USA) and the significance of differences between treatments was recorded at $P < 0.05$. In the case of data collected over time, analyses were conducted by considering the time as an intra-groups factor of repeated ratings on the same time series. In case of significant effects, pair-wise mean comparisons were conducted according to the Newman–Keuls test at $P < 0.05$. Linear regression was conducted using the same software between the disease development of each pathogen based on visual assessment of the expanding lesion and the rate of colonization based on PCR detection over time of the same pathogen in tuber tissues. Slopes were compared using ANOVA and R^2, and SEEs of each regression analyses were used to assess its significance ($P < 0.05$).

For interactions between pathogens in vitro, the growth was calculated for each pathogen using the formula described previously based on the two-week growth of each colony and submitted to ANOVA analysis. Data were analyzed separately for each used medium, since there was interaction of pathogen *x* media. Data were also subjected to co-variance analysis considering the medium as a co-variant factor since the interactions of pathogen *x* media were minimal. Significant differences were recorded at $P < 0.05$.

Results

Symptoms and extent of colonization of potato tubers

Phytophthora infestans

Symptoms of late blight produced on the tubers were represented by small, slightly sunken and dark spots (Fig. 1). Over time, the spots became more shrunken and darker in colour. Spots were about 2–8 cm in surface diameter and 1.5–3 cm deep into the flesh, 5 and 14 d.a.i., respectively (Fig. 2). The progress of *P. infestans* throughout the tuber flesh was much slower than on the surface tissues (Fig. 3). A PCR product of 456 bp indicating the presence of *P. infestans*, was obtained from all tissues displaying visual symptoms of late blight (Table 1). The pathogen was also detected successfully in non-symptomatic tissues of inoculated tubers, 2–3 cm and 1–1.5 cm ahead of visible lesions on the tuber surface and in the internal tissues, respectively (Fig. 3; Table 2).

Phytophthora erythrospetica

Pink rot symptoms were characterized by tubers with oozing liquid where the tissue had become flaccid and rubbery. A sharp line separating healthy and diseased tissue was eventually observed in the internal tissues (Fig. 1). Pink to dark discolouration was observed in the diseased tissues that later became mushy and granular.

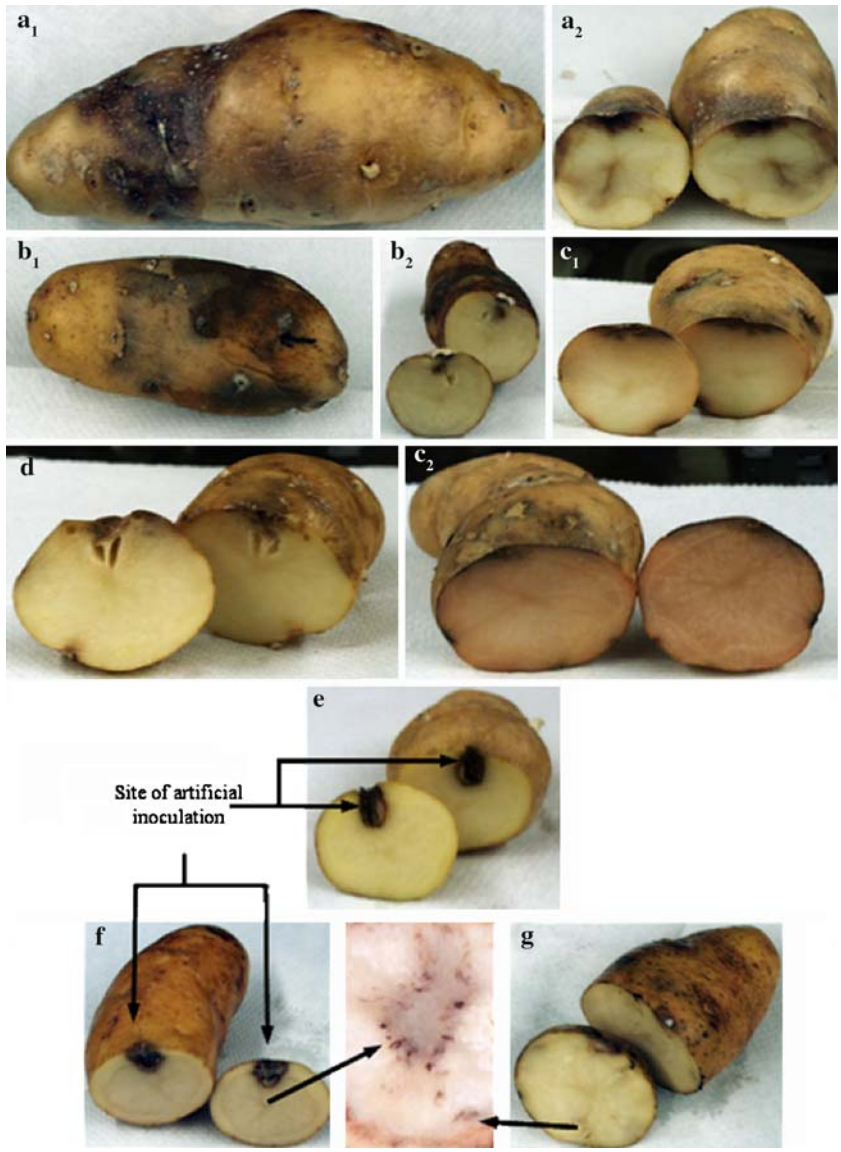


Fig. 1 Symptoms observed on tubers of cv. Russet Burbank inoculated with various tuber rot pathogens and incubated at 15°C. Inoculations were made by depositing 20 µl of calibrated inocula on a pre-wounded site. ($a_{1, 2}$) *P. infestans* 19 days after artificial inoculation (d.a.i.); ($b_{1, 2}$) *P. ultimum* 10 d.a.i.; ($c_{1, 2}$) *P. erythroseptica* 12 and 17 d.a.i., respectively; (d) *P. atrosepticum* 7 d.a.i.; (e) *F. sambucinum* 10 d.a.i.; *V. dahliae* (f) and *V. albo-atrum* (g) 10 d.a.i., with a highlight of vascular browning around 19 d.a.i

Lesions were 3–8 cm in diameter in the tuber surface and 1–5 cm deep in the internal tissues 5 and 14 d.a.i., respectively (Fig. 2). The progression of *P. erythrospetica* was fast both on the surface and in the internal tissues of the tuber (Fig. 3). PCR performed on positive samples from inoculated tubers generated a DNA fragment of 136 bp indicating the presence of *P. erythroseptica* (Table 1). Detection of this PCR product occurred in samples collected 2–4 cm ahead of the visible lesion on the external tissues, i.e., periderm, and 1–2 cm in the internal tissues of the tuber (Fig. 3; Table 2).

Pythium ultimum

Symptoms on potato tubers inoculated with *P. ultimum* were similar to those produced by *P. erythroseptica*, but without the pink-dark discolouration (Fig. 1). The progress of this pathogen was much quicker on the surface than in the internal tissues (Fig. 3). The lesions were 1.5–12 cm in diameter over a period of 3–12 d.a.i., respectively (Fig. 2). The PCR product of 500 bp, indicative of *P. ultimum* presence, was obtained in samples harvested from tubers (Table 1). The pathogen detection was recorded

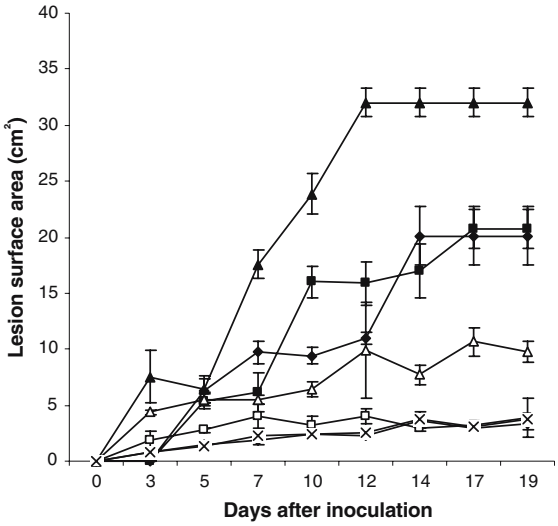


Fig. 2 Visual assessment of the progress of symptoms in the external tissues (depth 0–2 mm) of inoculated tubers of cv. Russet Burbank incubated at 15°C. *P. infestans* (■); *P. ultimum* (▲); *F. sambucinum* (Δ); *P. erythroseptica* (♦); *P. atrosepticum* (□); *V. albo-atrum* (◇); *V. dahliae* (×). The bars represent standard errors of the means of five replicates

in the tuber flesh 2–3 cm deep from the inoculation site 1–3 d.a.i. (Fig. 3; Table 2). However, the pathogen detection by PCR in the external tissues was similar to the expansion of the visual lesion, due to the rapid spread of the pathogen on the tuber surface (Fig. 3). As a result, the pathogen was quickly detected far from the inoculation site (4–5 cm at 10 d.a.i.; Fig. 3; Table 2).

Pectobacterium atrosepticum

This pathogen induced tuber softening with a watery decay, and a strong smell, eventually producing cavities in the flesh of inoculated tubers (Fig. 1). The lesion size increased quickly, from 2 to 2.5 cm in diameter on the tuber surface and 1 to 1.5 cm deep in the internal tissues at 5 and 10 d.a.i., respectively (Fig. 2). The pathogen was detected by PCR (200 bp, Table 1) 1–3 cm ahead of the inoculation site on the tuber surface and .5–1 cm ahead in the internal tissues (Fig. 3; Table 2).

Fusarium sambucinum

Dry rot symptoms observed after inoculation with *F. sambucinum* consisted of sunken areas brown to dark in colour (Fig. 1). The surface of the tuber affected was large, deep-set and wrinkled with white protuberances. Lesion size varied from 1 to 8 cm in diameter, 3 and 12 days after inoculation, respectively (Fig. 2). The pathogen was detected by PCR (315 bp, Table 1) 1.5–3 cm ahead of the inoculation site on the surface and .5–1.5 cm ahead in the internal tissues (Tables 2 and 3).

Verticillium albo-atrum and V. dahliae

Tubers inoculated with *Verticillium* spp. developed a light brown discolouration circular in shape in the vascular ring (Fig. 1). Reddish pink to tan discolouration accompanied by irregular blotches was also observed on the surface of inoculated tubers especially around the eyes (Fig. 1). Lesion size varied from 1 to 2.5 cm in diameter, 3 and 12 d.a.i., respectively (Fig. 2). The pathogens were detected by PCR (452 bp, Table 1) 1–2 cm ahead of the inoculation site in the tuber peel and .5–1.5 cm deep in the internal tissues (Tables 2 and 3).

Comparison of the accuracy of detection of tuber rot pathogens using PCR and visual assessment

Colonization of the tubers by pathogens was estimated either by visual assessment of the lesions or by PCR. The assessment using PCR was more accurate, by always detecting the pathogen a few centimetres ahead of the visual expanding lesions, as deduced from the linear regression of the colonization of the tubers using PCR (predicted progress) vs. the lesion expansion (actual progress; Fig. 4). If both methods used for assessing the colonization of tuber tissues by these pathogens were each giving the same information, slopes of the regressions would not be significantly different from 1.0 and the *Y*-intercept would not significantly differ from zero. This was not observed

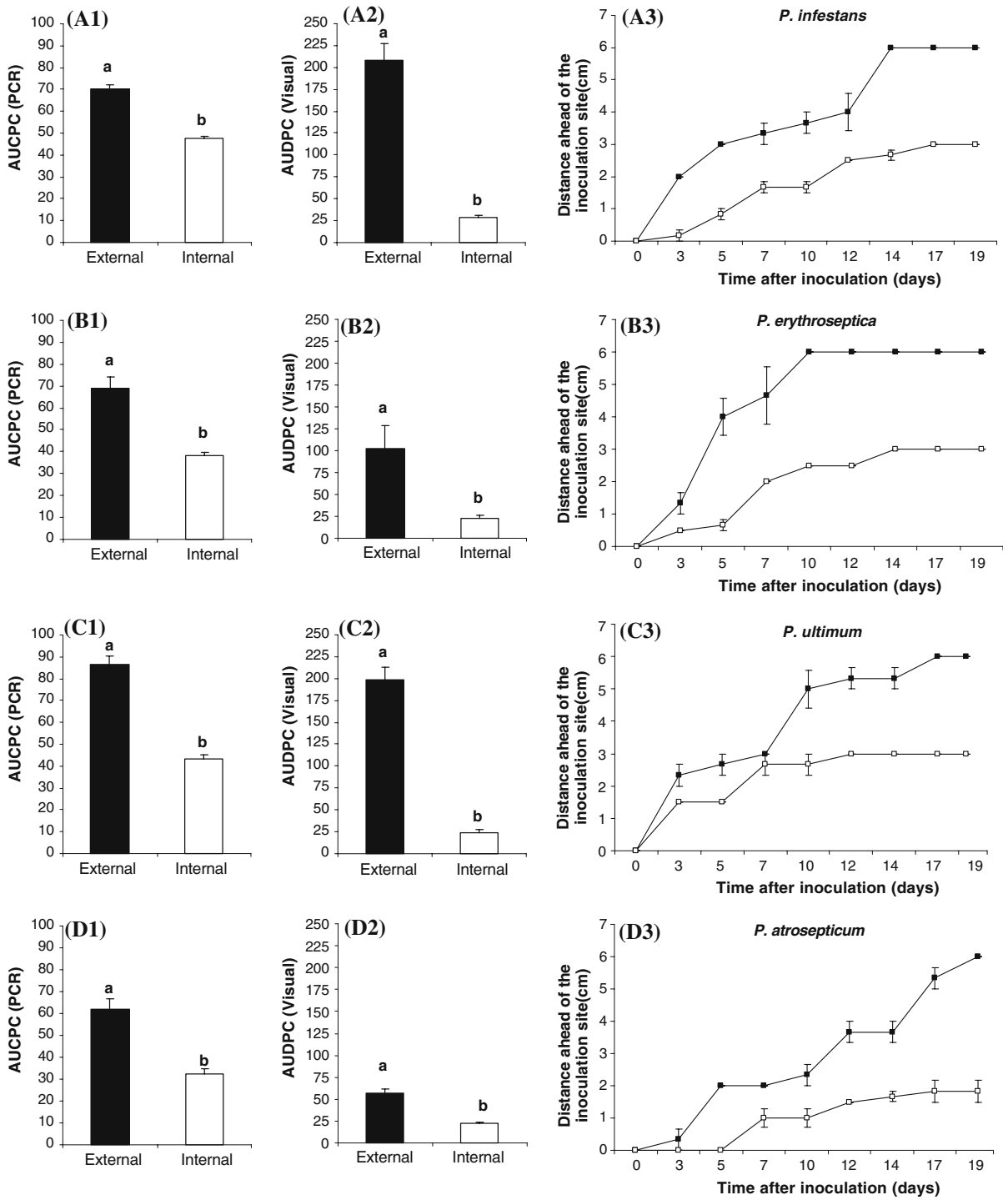


Fig. 3 Colonization of potato tuber tissues by different pathogens *(P. infestans* (A); *P. erythroseptica* (B); *P. ultimum* (C); and *P. atrosepticum* (D)). 1: AUCPC based on PCR detection over time in external or internal tissues of the tuber; 2: AUDPC based on visual assessments of the expanding lesions; 3: distance ahead of the inoculation site where the pathogen has been detected (■: external tissues; □: internal tissues). AUD(C)PC histograms with the same letter do not differ significantly according to Newman–Keuls test ($P < 0.05$). The bars represent standard errors of the means of five replicates

Table 2 Detection of several storage decay pathogens by PCR, in healthy-looking tissues beyond the expanding visible lesion, on tubers from cv Russet Burbank artificially inoculated and incubated at 15°C for 3 weeks

	Pathogen expansion in the healthy-looking tissues	
	External tissues (depth 0–2 mm)	Internal tissues (depth > 2 mm)
P. infestans	2–3 cm	1–1.5 cm
P. erythroseptica	2–4	1–2
P. ultimum	4–5	2–3
P. atrosepticum	1–3	.5–1
F. sambucinum	1.5–3	1.5–2
V. dahliae	1–2	.5–1.5
V. albo-atrum	1–2	.5–1

Data represent minimal and maximum distances between the front of the expanding lesion and where pathogens were detected

Table 3 Tuber colonization by *F. sambucinum*, *V. albo-atrum* and *V. dahliae* based on PCR and visible lesions

Pathogens	PCR detection in external tissues (AUCPC)	Visual assessment of the lesion (AUDPC)
F. sambucinum	73.8 ± 4.7 a	39.6 ± 1.1 b
V. albo-atrum	72.3 ± 2.5 a	42.4 ± 1.5 a
V. dahliae	68.8 ± 2.8 b	19.8 ± .9 c

Areas were calculated from three-week curves with a three-day increment in each. Data (mean of five replicates) are followed by standard errors, and do not differ significantly within the same column when followed by the same letter, according to Newman–Keuls test ($P < .05$)

for any of the regressions recorded (Fig. 4). Instead, slopes were significantly different than 1.0 and/or a *Y*-intercept different from zero. Differences in terms of regression slopes have shown also that the ability of each pathogen to colonize tuber tissues is unique and can be predicted based on PCR detection provided that all other environmental conditions are satisfied to allow the expression of the disease. Higher slopes were observed for fast-growing pathogens such as *P. ultimum* and *P. erythroseptica* while lower slopes were recorded for slow-growing pathogens such as *F. sambucinum* (Fig. 4).

Interactions of *P. infestans* with other rot pathogens on tubers and *in vitro*

Interactions between P. infestans *and* P. atrosepticum

Significant increase ($P < 0.05$) of the tuber colonization by *P. atrosepticum* was observed in the presence of *P. infestans* compared to when the bacterium was inoculated alone (Fig. 5). Such an effect of *P. infestans* on *P. atrosepticum* was much more important in the internal than in the external tissues (Fig. 5). Conversely, no significant impact ($P < 0.05$) of *P. atrosepticum* was observed on *P. infestans*, except a slight increase of colonization in the internal tissues probably due to cavities produced by *P. atrosepticum* (Fig. 5).

Based on the *in vitro* data using four culture media, there was no significant difference in terms of growth on PDA, Rye B or V8-agar of either *P. infestans* or *P. atrosepticum* when grown individually (Fig. 8). However, a significant decrease of the growth of both pathogens was observed when they were plated on water agar (Fig. 8). A significant decrease of the growth was also recorded for the two pathogens when they were grown together on the same plate compared to when they were grown individually, especially for *P. infestans* (Fig. 8). No such an effect was observable on water agar, which is an unfavourable medium for growth of both pathogens (Fig. 8).

Interactions between P. infestans *and* P. erythroseptica

Significant reduction ($P < 0.05$) of the tuber internal tissues from colonization by *P. erythroseptica* was observed in the presence of *P. infestans*, as compared to control tubers where *P. erythroseptica* was inoculated alone (Fig. 6). A similar effect was observed for *P. infestans* (Fig. 6). However, no significant reduction ($P < 0.05$) was observed on the external tissues for either *P. infestans* or *P. erythroseptica*, when tubers were further inoculated with *P. erythroseptica* as compared to the control inoculated with a single pathogen (Fig. 6). Lesion sizes were similar between tubers

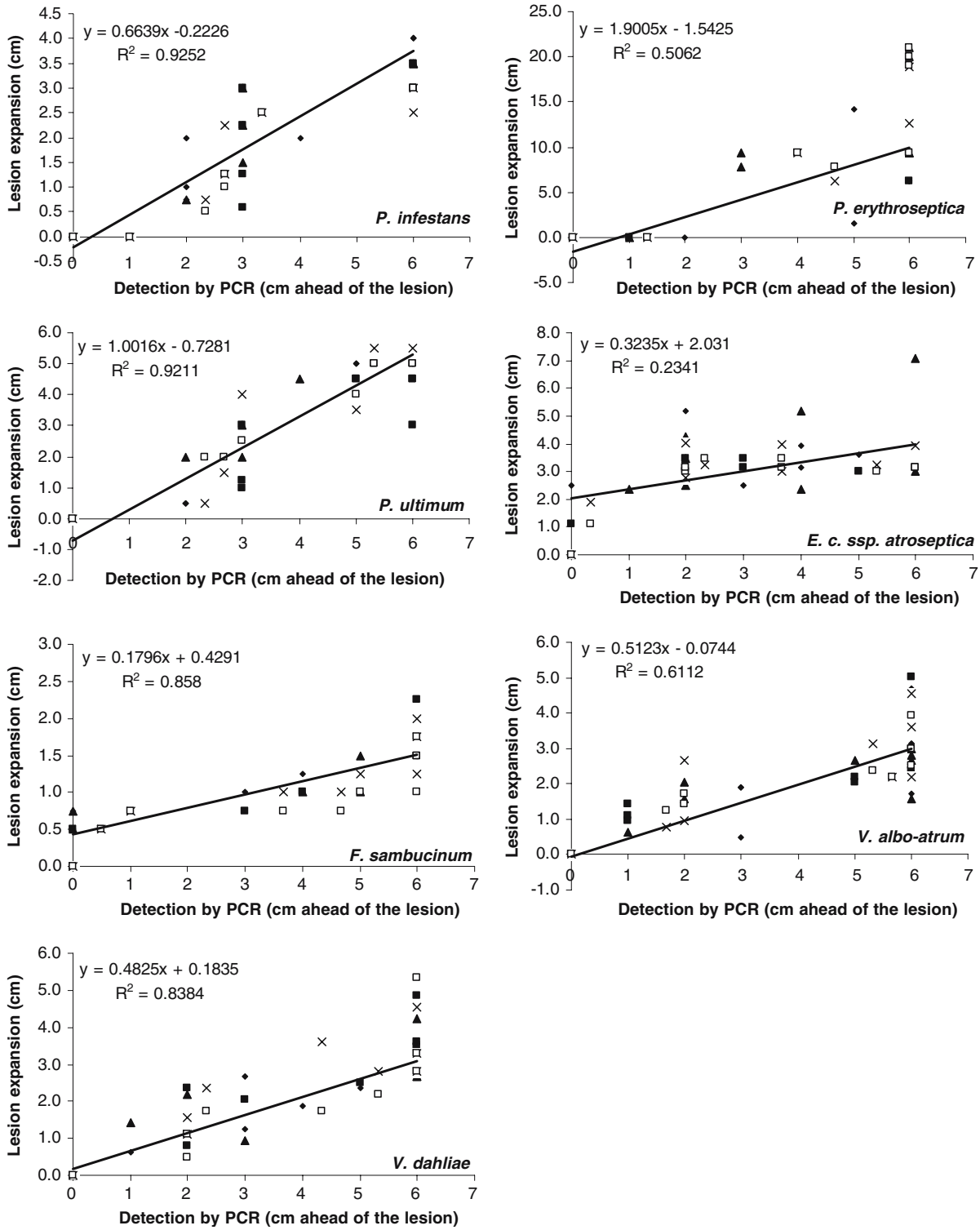


Fig. 4 Regression of the lesion expansion (actual progress of tuber rots) vs. detection of the pathogen colonization by PCR (predicted progress) in external tissues of potato tubers cv. Russet Burbank inoculated with various pathogens

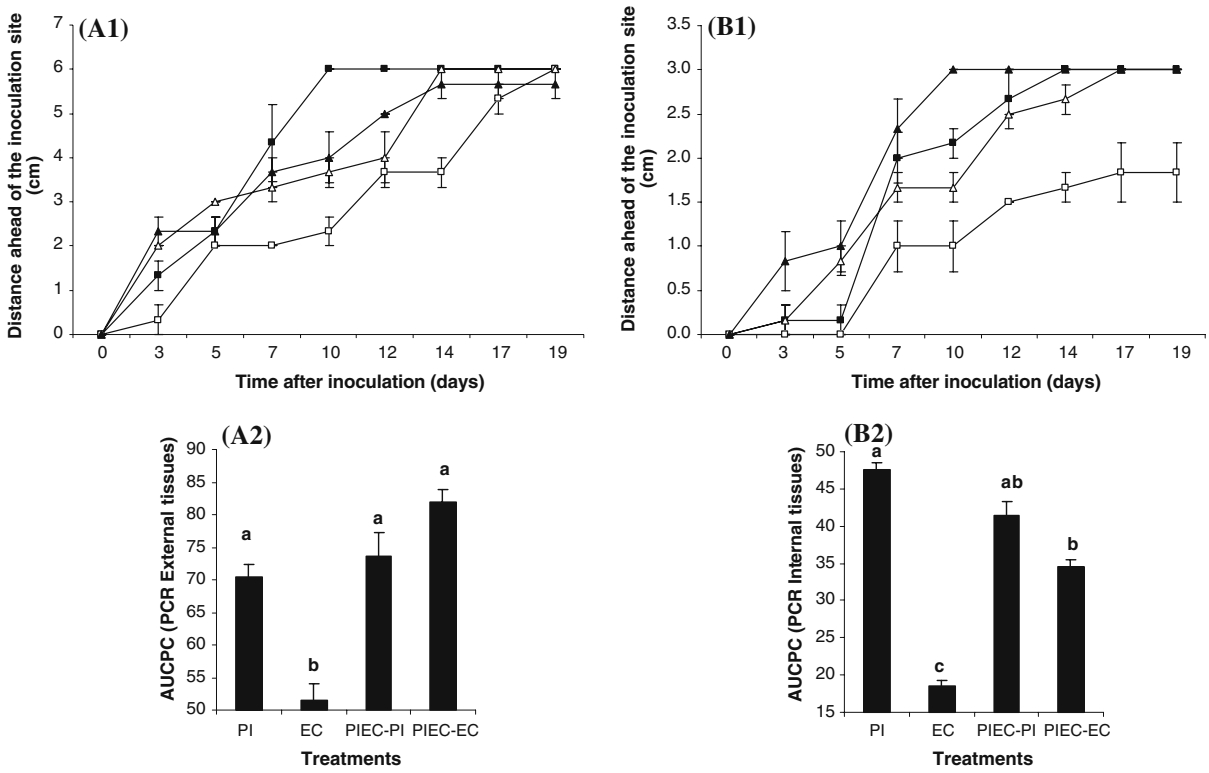


Fig. 5 Interaction between *P. infestans* and *P. atrosepticum* on potato tubers of cv. Russet Burbank. A1 and B1 represent the colonization by both pathogens of the tuber external and internal tissues. *P. infestans* alone (PI, Δ); *P. infestans* in presence of *P. atrosepticum* (PIEC-PI, ▲); *P. atrosepticum* alone (EC, □); *P. atrosepticum* in presence of *P. infestans* (PIEC-EC, ■). A2 and B2 represent the AUCPC calculated based on the detection by PCR at different times after inoculation of both pathogens either in the external or internal tissues of the tuber. Treatments with the same letter do not differ significantly according to Newman-Keuls test ($P < 0.05$). The bars represent standard errors of the means of five replicates

inoculated with only *P. infestans* or with both *P. infestans* and *P. erythroseptica* (Fig. 6).

Interactions between the two *Phytophthora* species were also studied on culture media and no significant differences were observed between their growth on PDA, Rye B or V8-agar. Although a similar growth pattern was observed for each pathogen grown individually or together on the same plates of water agar, a highly significant decrease of *P. infestans* growth was recorded in the presence of *P. erythroseptica* compared to when grown alone (Fig. 8). The same observations applied to *P. erythroseptica*.

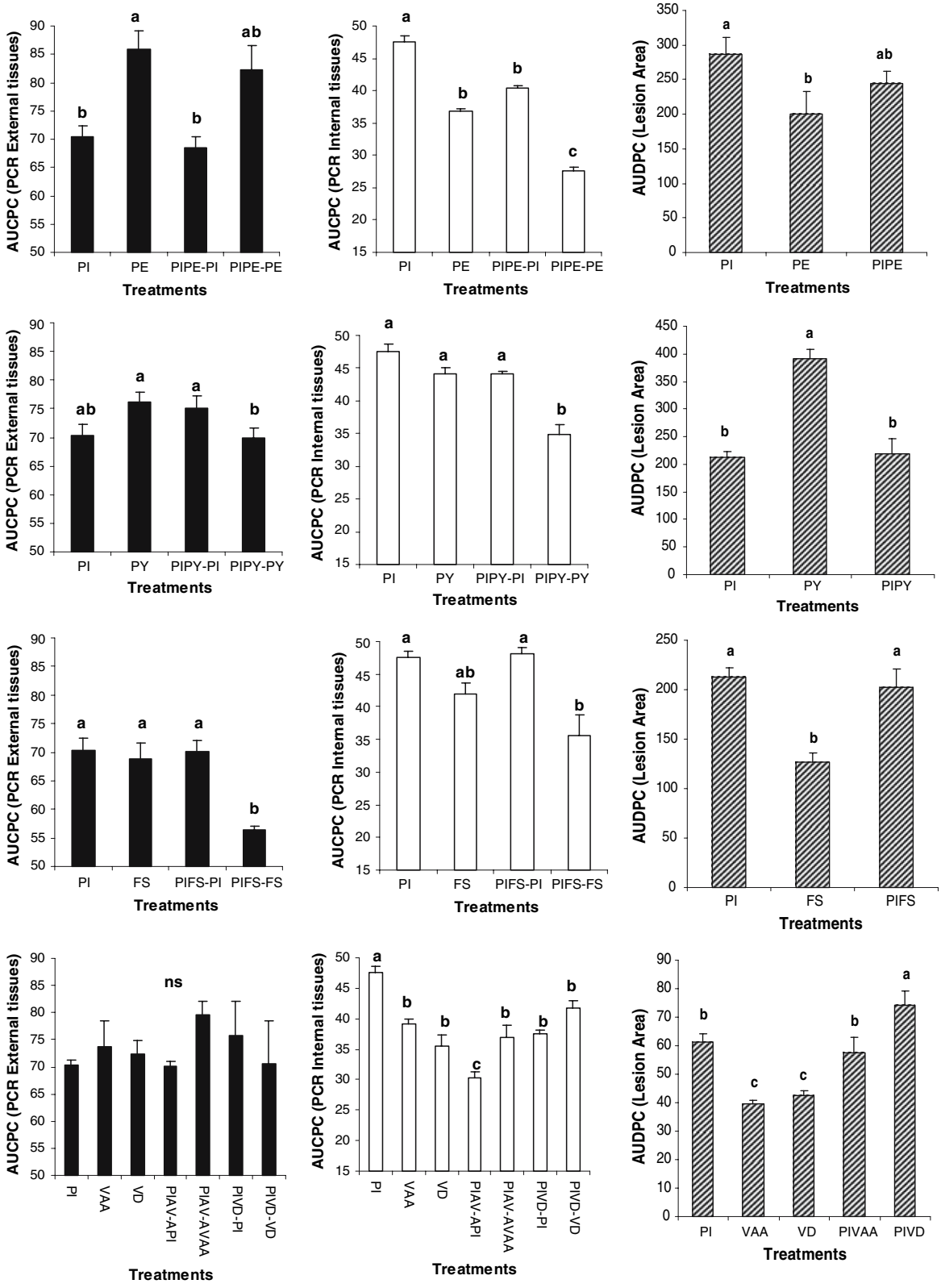
Interaction between P. infestans *and* P. ultimum

The presence of *P. infestans* on the tubers led to a significant reduction ($P < 0.05$) of the normal *P. ultimum* colonization (Fig. 6). Such an effect was more visible in internal than external tissues both visually and using PCR (Fig. 6). Conversely, the presence of *P. ultimum* led to a slight increase ($P < 0.05$) in the spread of *P. infestans* over the external tissues (Fig. 6).

Interactions *in vitro* between *P. infestans* and *P. ultimum* showed similar patterns as with *P. erythroseptica* on all tested media (Fig. 8).

Interaction between P. infestans *and* F. sambucinum

Based on the visual assessment, no significant ($P < 0.05$) difference was recorded between tubers inoculated with *P. infestans* alone and tubers that were inoculated with *P. infestans* and *F. sambucinum* (Fig. 6). However, as compared


AUCPC (PCR External tissues)
AUCPC (PCR Internal tissues)
AUDPC (Lesion Area)
Treatments
PI
PE
PIPE-PI
PIPE-PE
PIPE
PY
PIPY-PI
PIPY-PY
PIPY
FS
PIFS-PI
PIFS-FS
PIFS
VAA
VD
PIAV-API
PIAV-AVAA
PIVD-PI
PIVD-VD
PIVAA
PIVD
ns

◀ **Fig. 6** Potato tuber colonization based on pathogen detection by PCR assays in the external (■) and internal (□) tissues as well as lesion areas (▨). AUD(C)PC values were based on a three-week period progress curve data. *P. ultimum* (PY); *P. erythroseptica* (PE); *F. sambucinum* (FS); *V. albo-atrum* (VAA) and *V. dahliae* (VD) were inoculated either individually or after inoculation with *P. infestans* (PI) in samples PIXY-PI, where XY are the initials of the second pathogen. PIXY-PI represents data for PI detection in the presence of a second pathogen XY. PIXY-XY represents data for XY detection in the presence of PI. Histograms with the same letter do not differ significantly according to Newman–Keuls test ($P < 0.05$). The bars represent the standard error of the means of five replicates

to the tubers inoculated only with *F. sambucinum*, a significant increase of tuber rot was observed when both pathogens were inoculated (Fig. 6); the lesion size in the latter case was similar to that produced by *P. infestans* rather than *F. sambucinum*. Based on PCR detection, no significant ($P < 0.05$) variation in internal tissue colonization was detected for both pathogens when inoculated individually or in association with each other, whereas a significant reduction in colonization was detected in the external tissues for *F. sambucinum* in the presence of *P. infestans* (Fig. 6).

The patterns of *P. infestans* and *F. sambucinum* interactions in vitro (Fig. 8) were similar to what was observed with *P. erythroseptica* and *P. ultimum* on all tested media.

Interaction between P. infestans *and* V. albo-atrum *or* V. dahliae

Lesion expansion in the case of inoculations involving *P. infestans* and *V. albo-atrum* or *P. infestans* and *V. dahliae* was at least as large ($P < 0.05$) as that produced by *P. infestans* inoculated alone (Fig. 6). Based on PCR, no significant difference ($P < 0.05$) in terms of external tissue colonization was detected for all three pathogens either when they were inoculated individually or in the presence of *P. infestans* (Fig. 6). However, *P. infestans* colonization was reduced in the internal tissues in the presence of *V. albo-atrum* and *V. dahliae* while there was no significant effect of *P. infestans* on colonization by either *V. albo-atrum* or *V. dahliae* (Fig. 6).

Data obtained on the *in vitro* interactions between *P. infestans* and *V. albo-atrum* or *V. dahliae* were similar to those for *P. erythroseptica*, *P. ultimum* and *F. sambucinum*, with a significant ($P < 0.05$) mutual growth reduction of *V. albo-atrum* or *V. dahliae* and *P. infestans* when they were plated together compared to when they were plated separately. No significant ($P < 0.05$) effect of *P. infestans* on either *V. albo-atrum* or *V. dahliae* was observed on the water agar medium (Fig. 8).

Effect of *P. infestans* on subsequent infections by more than one pathogen

Figure 7 shows interactions of *P. infestans* on tubers with *P. ultimum* and *P. atrosepticum*, *P. ultimum* and *P. erythroseptica*, or *P. erythroseptica* and *P. atrosepticum*, based on the visual assessment of lesions and PCR detection of the pathogens in the inoculated tubers. Based on the visual assessment, expansion of the lesions was the same or slightly higher on tubers inoculated with multiple pathogens than the controls inoculated with only *P. infestans* (Fig. 7). Other pathogens inoculated individually showed slower lesion expansion than with *P. infestans* alone or with the three pathogens combined. An exception was, however, recorded with *P. ultimum* on the external tuber tissues. Based on PCR, the results confirmed the overall effect of *P. infestans* on *P. ultimum*, *P. erythroseptica* and *P. atrosepticum* previously observed with combinations of two pathogens (Figs. 5 and 6).

Discussion

Stored potato piles can be seriously at risk of deterioration by diseases and other disorders. From a management point of view, anticipating such problems is half way towards solving them. Much information is still needed to anticipate storage diseases and determine the necessary steps to prevent or reduce the chances of their occurrence. Late blight caused by *P. infestans* usually occurs during the growing season in spite of strict IPM procedures and can lead to further decline during storage even with potato tubers

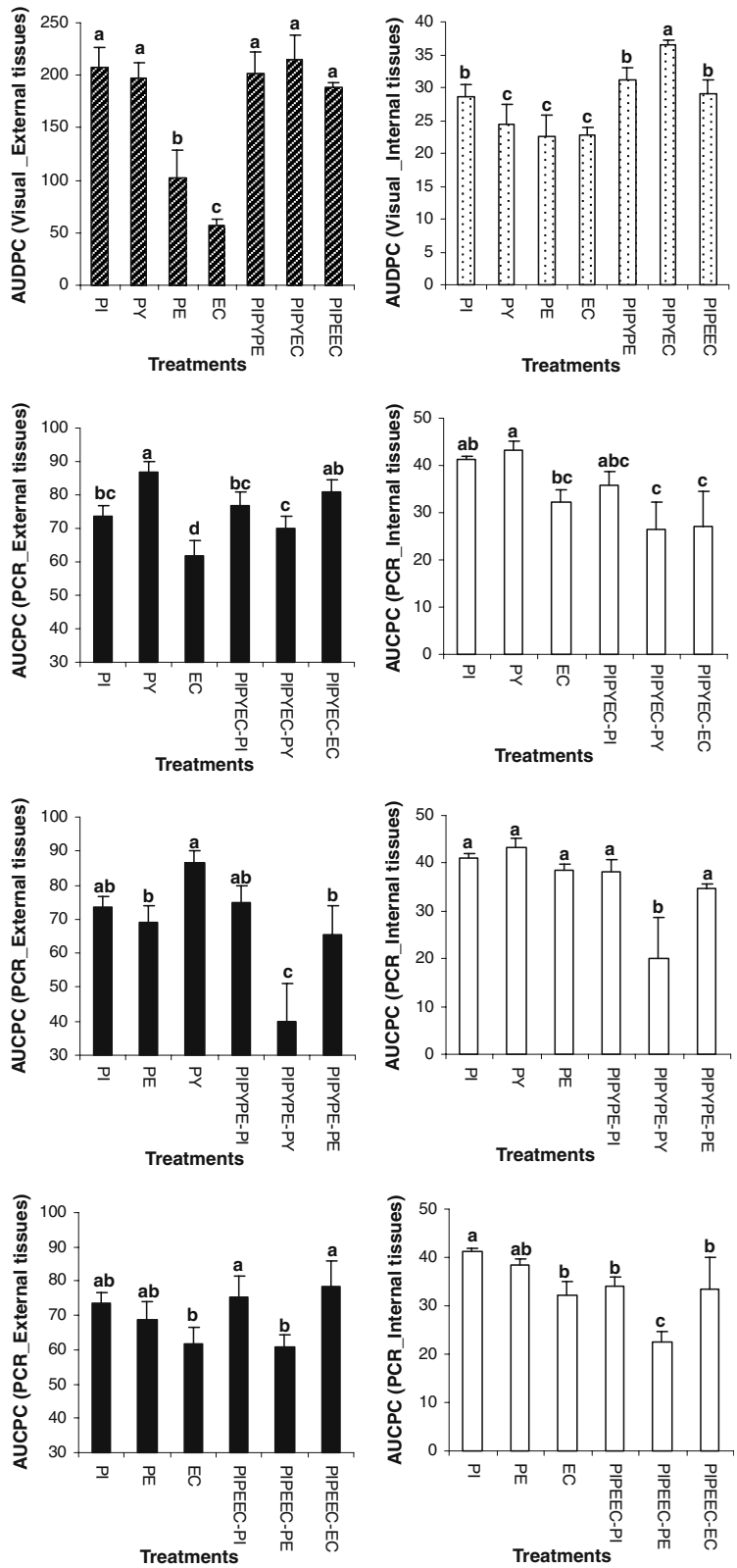


Fig. 7 Interaction between potato pathogens on tubers of cv. Russet Burbank in infections involving three pathogens. *P. ultimum* (PY), *P. erythroseptica* (PE), and *P. atrosepticum* (EC) were inoculated either individually or after inoculation with *P. infestans* (PI) and another pathogen (e.g. in the sample PIPYEC-PI, *P. infestans* was detected in tubers inoculated with *P. infestans*, and *P. ultimum*). Assessment of the expansion was conducted either visually (▨: external tissues, □: internal tissues) or using PCR (■: external tissues, □: internal tissues). Histograms with the same letter do not differ significantly according to Newman-Keuls test ($P < 0.05$). The bars represent the standard error of the means of five replicates

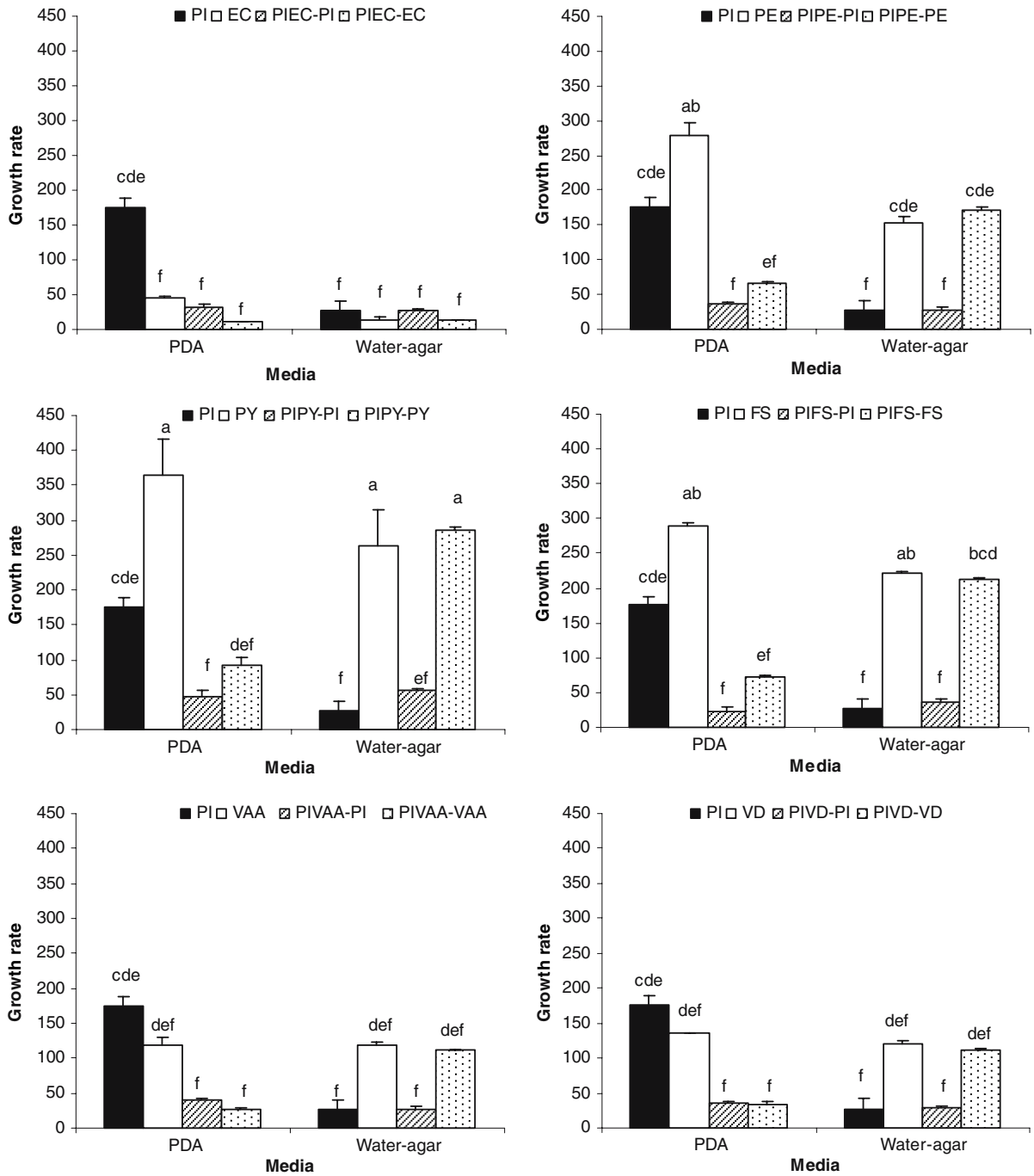


Fig. 8 In vitro interactions between *P. infestans* and other potato tuber pathogens on PDA and water agar media. Growth rates observed on V8-agar and on rye B media were not included here since they were similar to those observed on PDA. The growth rate was calculated based on individual growth curves over a two-week period. *P. ultimum* (PY), *P. erythroseptica* (PE), *P. atrosepticum* (EC), *F. sambucinum* (FS), *V. albo-atrum* (VAA), and *V. dahliae* (VD) were plated either individually or with *P. infestans* (PI). PIXY-PI, where XY are the initials of the pathogen, represents the growth of *P. infestans* on a plate having a second pathogen XY. Histograms with the same letter do not differ significantly according to Newman–Keuls test ($P < 0.05$). The bars represent the standard error of the means of five replicates

that initially look symptomless at harvest (Tooley et al., 1998). Potato storage decay is suspected to be due partly to in-storage travel of pathogens including *P. infestans* within tuber tissues and from tuber to tuber. However, no data are available on such pathogen spatio-temporal dynamics. In the present study, the first step was to follow the colonization by *P. infestans* of both external and internal tissues of the tuber. Such tracking was conducted using previously developed *P. infestans* specific primers (Tooley et al., 1998). PCR allowed the detection of *P. infestans* several days before symptoms were apparent. Results indicated that *P. infestans* first spreads rapidly in the internal tissues, accompanied by slower expansion over the tuber surface. Over time, equal tissue colonization eventually occurs in external and internal tissues.

The second step of the current study was to understand how the presence of *P. infestans* in potato tubers can impact on the development of other tuber diseases such as leak, pink, soft and dry rots as well as Verticillium vascular discolouration. PCR primers based on sequenced internal transcribed space (ITS) regions of rDNA have been proven useful in differentiating and detecting a wide range of fungi in plant tissues including *P. infestans* (Lee & Taylor, 1992; Tooley et al., 1998), *Pythium* (Chen, 1992), *Verticillium* (Nazar et al., 1991), *Fusarium* (O'Donnell, 1992) and others (Henson and French, 1993; White et al., 1990). In this study, PCR based on specific primers directed against each pathogen (de Boer and Ward, 1995; Kageyama et al., 1997; Liu et al., 1994; Mishra, et al., 2003; Nazar et al., 1991; Robb, 1994; Tooley et al., 1998; White et al., 1990; Zotobowska and Pospieszny, 1998) was successfully used to detect them either alone or in association with *P. infestans*. Pathogens were detected earlier than visual symptoms, very often a few centimetres ahead of the expanding lesion depending on the pathogen. A high similarity was observed between the frequency of detection and the disease progress suggesting that such tools can be used to monitor infections in their earliest stages in storage.

The data obtained also show different interactions between *P. infestans* and other potato tuber pathogens. The presence of *P. infestans* appears to significantly decrease the progress of *P. ultimum* and *P. erythroseptica*, both belonging to the Oomycetes (Fig. 6). Such antagonism might be due to direct competition among these pathogens for nutrients or through another mode of inhibition as shown for *Azobacter vinelandi* (Balis & Antonakou, 2000). It was reported that this pathogen has the ability to induce suppressiveness against some notorious soil borne root pathogens such as *Pythium*, *Phytophthora*, and *Rhizoctonia* species, through its intrinsic ability to produce siderophores (Balis & Antonakou, 2000). In contrast to *P. erythroseptica* and *P. ultimum*, the growth rate of *P. atrosepticum* was significantly enhanced in the presence of *P. infestans* especially in the tuber internal tissues (Fig. 5), confirming that this is a secondary pathogen of tubers already infected by *P. infestans* (Bohl & Nolte, 2003; Tooley et al., 1997). No noticeable effect on the colonization of the tubers by *F. sambucinum* or *V. albo-atrum* or *V. dahliae* was observed in the presence of *P. infestans* (Fig. 6). The progress of symptoms on the tuber tissues corroborated the interactions detected by PCR between the tested pathogens and *P. infestans*. However, the *in vitro* tests using different culture media suggested no direct effect of *P. infestans* on other pathogens (Fig. 8). Instead, a mutual growth 'escape' between *P. infestans* and other pathogens was observed when they were grown together, comparable to a control test with only two *P. infestans* colonies. Moreover, using a minimal medium such as water agar for growth of *P. infestans* favoured the other pathogens and confirmed the indirect effect of *P. infestans* on their growth. This does not concur with the antagonistic or synergistic effects that were observed in vivo within the tubers, where the initial interaction with *P. infestans* seemed to affect further development of other pathogens. These observations may suggest an indirect interaction between *P. infestans* with tuber tissue and other pathogens, although direct antagonism cannot be completely excluded.

In the light of the present findings, it might be concluded that specific PCR primers for different tuber rot pathogens are useful for the determination of their colonization of stored tubers over

time. Such data were successfully obtained when tubers were inoculated with either one or more pathogens. Colonization by pathogens was detected before the symptoms became visible, a few centimetres ahead of the expanding lesion. This can be helpful to monitor the sanitary status of stored tubers. Further studies are being carried out to better understand the nature and mechanisms of the interactions between the different pathogens and the tuber. This will help to set up management protocols for specific epidemiologic situations that may happen in storage, and make storage outbreaks due to some pathogen combinations more predictable, leading to an accurate anticipation of tuber rot problems in storage and to more successful preventive measures.

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