Acquisition and Transmission of *Peanut clump virus* by *Polymyxa graminis* on Cereal Species

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ABSTRACT

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The objective of this study was to investigate the specificity of the interactions between *Polymyxa graminis*, *Peanut clump virus* (PCV), and cereals, particularly the acquisition and the transmission of the virus by three *P. graminis* formae speciales. A new strategy has been developed: it involves using sugarcane as the common host for both the virus and its vector in order to produce the viruliferous zoospores of *P. graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* that were then inoculated on cereal species. This experiment enabled the role of *P. graminis* f. sp.

tropicalis and *subtropicalis* zoospores in PCV transmission to be demonstrated. The efficiency of this transmission was shown to vary, depending on the *P. graminis* special forms. Interestingly, the high transmission of the PCV isolate from Burkina Faso by an isolate of *P. graminis* f. sp. *tropicalis* from Niger on pearl millet suggests that there is a coevolution mechanism in this pathosystem. The study also provides evidence that the host plant species in which *Polymyxa* zoospores are produced could affect the infectivity of the vector. Finally, using *Polymyxa* quantitation by quantitative reverse-transcription polymerase chain reaction and in situ observations of the virus, the study demonstrates the independence of the development of PCV and its vector in the host plants.

Polymyxa graminis Ledingham is a soilborne protist endoparasite of roots belonging to the order Plasmodiophorida, commonly referred to as plasmodiophorids (6), and belonging to the *Cercozoa* (4,7,64). Within *P. graminis*, five formae speciales with particular ecological properties, geographical distribution, and rDNA sequence relationships are described: *P. graminis* f. sp. *colombiana* on rice in Colombia; *P. graminis* f. sp. *subtropicalis* on barley, maize, sorghum, pearl millet, or wheat in the Indian subcontinent and West Africa; *P. graminis* f. sp. *temperata* on barley or wheat in Belgium, Canada, China, France, Germany, and the United Kingdom; *P. graminis* f. sp. *tepida* on barley, oat, or wheat in Belgium, Canada, and the United Kingdom; and *P. graminis* f. sp. *tropicalis* on maize, sorghum, or pearl millet in India and West Africa (34,37,61).

P. graminis is not considered to be a pathogen but it is economically important because it transmits at least 14 plant viruses causing diseases on major crops such as maize, peanut, sorghum, sugarcane, and small grain cereals (3,8,23,24,29,45,46,51,57). These viruses belong to four genera: *Benyvirus*, *Bymovirus*, *Furovirus*, and *Pecluvirus* (3,59).

P. graminis f. sp. *temperata* and *tepida* are both associated with cereal mosaic disease in temperate areas (61), and *P. graminis* f. sp. *subtropicalis* and *tropicalis* are both associated with peanut clump disease, a severe disease occurring in the tropics (14,15,38). Peanut clump disease is caused by two *Pecluvirus* spp., *Indian peanut clump virus* (IPCV) and *Peanut clump virus* (PCV) in the Indian subcontinent (48) and West Africa (58), respectively. These viruses have wide host ranges, including peanut but also important cereals such as barley, pearl millet, sorghum, or wheat (14,57). PCV is also the causal agent of sugarcane red leaf mottle disease (5).

The implication of *P. graminis* in virus transmission has been shown or suggested in various ways in different plant–virus–vector pathosystems. For *Oat mosaic virus* (29), PCV (56), *Rice necrosis mosaic virus* (24), and *Rice stripe necrosis virus* (23), the co-occurrence of the virus and *P. graminis* in infested fields is the only indication of transmission by *P. graminis*. For IPCV (46) and *Wheat spindle streak mosaic virus* (51,62), the use of soil or sporosori extracted from roots as inoculum in transmission experiments highlighted the predominant role of the soilborne vector in the disease. For *Barley mild mosaic virus* (9) and *Soil-borne wheat mosaic virus* (SBWMV) (22), the association of the virus and its vector was revealed visually using microscopic tools and immunolabeling. *Chinese wheat mosaic virus*, *Oat golden stripe virus*, *Soil-borne cereal mosaic virus*, *Sorghum chlorotic spot virus*, and *Wheat yellow mosaic virus* are believed to be *P. graminis* transmitted, based on the transmission of similar viruses, but no direct or indirect evidence has been found. To date, only the transmission of *Barley yellow mosaic virus* (BaYMV) (3) and SBWMV (45) has been reproduced under controlled conditions using *P. graminis* zoospores as inoculum.

The virus transmission process by *Polymyxa* spp. can be divided into three main steps: the in vivo acquisition of the virus by the vector, the transmission by the viruliferous vector to the plant, and the development of the virus in the plant. The mechanisms of internalization of the virus in *Polymyxa* spp. were studied by Adams et al. (2) based on in silico analysis of plasmodiophorid-transmitted virus sequences. This study, completed by Crutzen et al. (12), underlined the importance of transmembrane domains for the transmissions of beny-, bymo-, furo-, and pomoviruses by plasmodiophorids. The absence of any similar transmembrane regions in the proteins of the *Pecluvirus* spp. suggests that the virus-vector association is peculiar to this genus.

Other characterization studies highlighted the regions of the virus genomes involved in the transmission of the virus by the vector. Deletions observed on the coat protein (CP)-readthrough domains of *Beet necrotic yellow vein virus* (BNYVV) transmitted

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by *P. betae* and of *Potato mop top virus* transmitted by the plasmodiophorid *Spongospora subterranea* induced a loss of vector transmission (47,55). Among the *Pecluvirus* spp., it has been suggested that P39, encoded by RNA-2 and expressed by leaky scanning of P23 (30), is involved in the transmission by *P. graminis* (40), based on its similarity with P75, encoded by RNA-2 of BNYVV (50,54). P39 also undergoes deletion after multiple mechanical transfers (40). To date, however, it is not known if PCV isolates bearing deletions on P39 lose their *P. graminis* transmission ability.

Virus transmission by *P. graminis* occurs frequently in the field but reproducing the acquisition and transmission processes under controlled conditions is complicated by the parasitic obligate nature of the vector and the difficulties in maintaining cultures of the vector and the virus together on the same host plant. Years of research on *Polymyxa* spp.-transmitted viruses has resulted in the establishment of a collection of *Polymyxa* isolates from various areas and in greater knowledge about their ecological requirements. No study has yet reproduced the complete virus transmission process by *P. graminis*, including the acquisition of the virus, its transmission, and the further development of the virus and the vector in the plant host. The objective of this study was to investigate the specificity of the interaction, particularly the acquisition and the transmission of PCV by three *P. graminis* formae speciales that could be naturally associated with PCV according to their natural distribution or their common host ranges. After the acquisition step of PCV in a common host of this virus and the three *P. graminis* formae speciales, the multiplication of the virus and the vector in barley, pearl millet, sorghum, and wheat was studied. The influence of PCV on the *Polymyxa* multiplication rate and the localization of PCV within plant root tissues after its transmission to the plant were also studied.

MATERIALS AND METHODS

***Polymyxa* spp. and PCV isolates.** The *P. graminis* isolates used in this experiment were isolate I₉ of *P. graminis* f. sp. *subtropicalis* from Boraj, India, isolated on sorghum and multiplied on pearl millet; isolate B₁ of *P. graminis* f. sp. *temperata* from Loupoigne, Belgium, isolated and multiplied on barley (37); and isolate Ni₁ of *P. graminis* f. sp. *tropicalis* from Sadoré, Niger, isolated and multiplied on pearl millet (16).

Isolate PCV-B of PCV from sugarcane collected in Burkina Faso was used in the study. This isolate was maintained on sugarcane by cuttings.

Plant materials. Five *Poaceae* spp. were used in the experiment: barley (*Hordeum vulgare*) 'RD-103' and 'Tiffany', pearl millet (*Pennisetum glaucum*) 'ICMH-451', sorghum (*Sorghum bicolor*) 'Irat-204', sugarcane (*Saccharum officinale*) 'CP89-2377', and wheat (*Triticum aestivum*) 'RR-21'. Seeds were disinfected for 2 min in 5% commercial sodium hypochlorite. After a pre-germination for 36 h at 20°C in an atmosphere saturated with water, they were grown in glass tubes filled with autoclaved quartz or in plastic tubes adapted to the automatic immersion system (AIS) (36). The plants were watered with Hoagland solution, as described by Legrève et al. (36).

PCV-infected sugarcane plants were multiplied by cuttings. The cuttings were planted in glass tubes with Hoagland solution for 15 days to promote the production of new rootlets.

For the experiments using *P. graminis* f. sp. *subtropicalis* and *tropicalis*, the plants were grown in a phytotron at 25 to 30°C with a 12-h photoperiod, and in the experiment using *P. graminis* f. sp. *temperata*, the plants were grown at 17 to 20°C with a 12-h photoperiod.

***Polymyxa* culture and zoospore production.** The aviruliferous status of the three isolates of *P. graminis* was controlled by verifying the absence of PCV in the dried roots used to initiate the

culture of the three isolates using reverse-transcription polymerase chain reaction (RT-PCR) (17).

For the production of *P. graminis* f. sp. *subtropicalis* and *tropicalis* cultures, sporosori suspensions were prepared from infected dried pearl millet roots infected by sporosori in Hoagland nutritive solution, as described by Legrève et al. (36). For each *Polymyxa* isolate, 5-day-old pearl millet plants grown in tubes adapted for the AIS were each inoculated using 10,000 sporosori. These plants were then placed in the AIS, in which roots were automatically immersed for 6 h every 12 h to induce *Polymyxa* multiplication (36). After 3 weeks of incubation in a phytotron at 25 to 30°C with a 12-h photoperiod, the plants were removed from the system, allowing the synchronized maturation of the zoosporangia. Then, 18 h later, they were placed in a diluted and refreshed (14°C) Hoagland solution (20%) for 90 min at room temperature to release the zoospores. Finally, the root exudates containing the zoospores released from the plants were collected; this constituted the aviruliferous zoospore suspensions.

The culture of *P. graminis* f. sp. *temperata* on barley was carried out using a sporosori suspension prepared from dried roots infected with isolate B₁ following the same procedure, except that barley was used as host plant and plants were grown at 17 to 20°C. Aviruliferous zoospores of *P. graminis* f. sp. *temperata* were produced following the same protocol as mentioned above, except that barley roots with zoosporangia were dipped in a solution refreshed at 7°C.

The concentration of zoospores in each suspension was determined with a hemacytometer, the Thoma counting chamber. The concentrations were adjusted by dilution of the suspensions in a one-fifth diluted Hoagland solution.

Viruliferous zoospores production. Aviruliferous zoospores (40,000 per plant) of the three *P. graminis* isolates were separately inoculated on PCV-infected sugarcane plantlets grown in three AIS. The AIS with sugarcane inoculated with the zoospores of *P. graminis* f. sp. *subtropicalis* or *tropicalis* was placed in a phytotron at 25 to 30°C with a 12-h photoperiod. The AIS with plants inoculated with *P. graminis* f. sp. *temperata* was placed in a phytotron at 17 to 20°C with a 12-h photoperiod. After 3 to 6 weeks of growth in these conditions, the presence of zoosporangia in the roots was confirmed by optical microscopy examination of the blue lactophenol-stained root fragments collected in the AIS. Zoospores were produced from these plants as described above. Their viruliferous status was controlled by verifying the presence of PCV by RT-PCR (17).

Experimental device to assess PCV transmission rates by three *P. graminis* formae speciales. The rates of PCV transmission by *P. graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* on cereal species were assessed after inoculation with standardized quantities of viruliferous zoospores on healthy 2-week-old plantlets grown in individual glass tubes for 3 weeks at 25 to 30°C for *P. graminis* f. sp. *subtropicalis* and *tropicalis* and for 13 weeks at 17 to 20°C for *P. graminis* f. sp. *temperata*. To optimize the multiplication of the three *P. graminis* isolates, the growing conditions were adapted for each forma specialis. For *P. graminis* f. sp. *subtropicalis* and *tropicalis* (25 to 30°C with a 12-h photoperiod), the viruliferous zoospores (40,000 viruliferous zoospores/plant) were inoculated on 25 plants of each cereal species (barley [RD-103], pearl millet, sorghum, and wheat). As negative controls, Hoagland solution, 40,000 aviruliferous zoospores of *P. graminis* f. sp. *subtropicalis* and *P. graminis* f. sp. *tropicalis* from pearl millet, and exudates from PCV-infected (*P. graminis*-free) sugarcane plants were each inoculated on 10 plants of barley, pearl millet, sorghum, and wheat. The viruliferous zoospores of *P. graminis* f. sp. *temperata* (17 to 20°C with a 12-h photoperiod) were inoculated on 20 plantlets of barley (Tiffany), pearl millet, and wheat. Here, 24,000 viruliferous zoospores were inoculated per plant because a higher quantity of zoospores was not produced for *P. graminis* f. sp. *temperata* on sugarcane,

probably because of a lower compatibility between *P. graminis* f. sp. *temperata* and sugarcane than between *P. graminis* f. sp. *tropicalis* or *subtropicalis* and sugarcane. As negative controls, Hoagland solution, 40,000 aviruliferous zoospores of *P. graminis* f. sp. *temperata* from barley, and exudates from the PCV-infected (*P. graminis*-free) sugarcane plants were each inoculated on 10 plants of barley, pearl millet, and wheat.

Cereals inoculated with *P. graminis* f. sp. *subtropicalis* and *tropicalis* viruliferous zoospores were incubated for 3 weeks. Those inoculated with *P. graminis* f. sp. *temperata* zoospores were incubated for 13 weeks. The difference of incubation time is based on knowledge of the different *Polymyxa* special forms life cycles: the development of *P. graminis* f. sp. *temperata* is slower than the development of *P. graminis* f. sp. *tropicalis* and *subtropicalis*, even at optimal temperature (36). After incubation, the plants were then harvested and kept at -70°C . Then, 30 to 100 mg of tissue was sampled on each root system. For most plants, this quantity represented the whole root system. For plants with root systems heavier than 100 mg, composite samples (100 mg) of 15 samples taken randomly in all parts of roots were constituted.

Detection of PCV and *P. graminis*. The presence of PCV and *P. graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* in roots was detected by RT-PCR after total RNA extraction using a phenol chloroform-based method (33) for 30 to 100 mg of the root samples. The same root tissue sample was used to detect both virus and vector.

P. graminis was detected by RT-PCR using Pspbd1 (5'-TAG CAATGGATATCTTGGTTCCC-3') and Psp2rev (5'-AGGGCTCT CGAAAGCGCAA-3') (34), both specifically targeting *Polymyxa* ribosomal DNA.

cDNA synthesis from RNA extract was performed by reverse transcription in two steps. First, 10.5 μl of reaction mix containing 1 μl of reverse primer, 8.5 μl of diethylpyrocarbonate (DEPC)-treated water, and 1 μl of RNA extract were mixed and incubated at 65°C for 10 min. Second, a reaction mix made up of 4 μl of M-MLV RT buffer (Promega Corporation, Madison, WI), 2 μl of dNTP (20 nmol), 0.25 μl of M-MLV Reverse Transcriptase (200 U/ μl) (Promega Corporation), and 3.25 μl of DEPC-treated water was added to the first reaction mix. The total volume (20 μl) was incubated at 42°C for 60 min.

The cDNA was amplified by RT-PCR. The reaction component for each sample was 26.25 μl of water, 5 μl of MgCl_2 (25 mM), 10 μl of Green Gotaq Flexi buffer (Promega Corporation), 1.5 μl of dNTPs (20 nmol), 1 μl of each primer (20 pmol), 0.25 μl of Gotaq polymerase (5 U/ μl) (Promega Corporation), and 5 μl of cDNA. The cycling times and temperature were 94°C for 2 min (1 cycle); then 35 cycles at 94°C for 2 min, 61°C for 30 s, and 72°C for 30 s; and a terminal step of 72°C for 7 min (1 cycle).

The detection of PCV in zoospore suspensions and in sugarcane was done by RT-PCR using J8 and J9 primers as described by Dieryck et al. (17). The detection of PCV in RNA extracts from cereal plant was done using TaqMan technology as described by Dieryck et al. (17).

Quantitation of *P. graminis*. *P. graminis* f. sp. *tropicalis* was quantified in plants inoculated with viruliferous zoospores using a specific real-time quantitative RT-PCR (RT-qPCR) assay using Sybrgreen technology developed by Dieryck et al. (16). Before quantitation by RT-qPCR, total RNA extracted was quantified using Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE) to standardize samples made from the extracts of different quantities of roots. The quantities of *P. graminis* in cereal species inoculated with aviruliferous zoospores from *P. graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* were compared using a broad-spectrum *P. graminis* RT-qPCR assay. For each cereal species inoculated with a particular *P. graminis* forma specialis, the mean quantity of *P. graminis* was assessed from a composite sample constituted by 1 μl of RNA extract of each *Polymyxa* spp.-

infected plant. The pair of primers used was Pspbd1 and Psp2rev, as described above, for classic RT-PCR. The RT-qPCR was performed using the iCycler IQ Real-Time Detection system (Bio-Rad, Nazareth, Belgium). For each sample tested, the reaction mix contained 0.8 μl of each primer, 14.4 μl of DEPC-treated water, and 20 μl of iQ SYBR Green Supermix (Bio-Rad). Then, 36 μl of mix was prepared per reaction and 4 μl of the cDNA or control was added. The PCR reaction was performed as follows: 1 cycle of 3 min at 95°C and 40 cycles each composed of 15 s at 95°C and 15 s at 61°C , with the fluorescence recorded after each elongation phase. Subsequently, the melt curve of the PCR products was generated by recording the fluorescence every 10 s while they were heated from 55 to 95°C , with an increment of 0.5°C every 10 s. Two replicates were tested per sample. The relative quantitation of *P. graminis* was done by comparing the threshold cycle (C_t) value of the sample and the C_t values obtained for four serial dilutions of a standard DNA extract prepared from roots infected by the *P. graminis* f. sp. *tropicalis* isolate Ni-1. No fluorescence with DNA from healthy plants was recorded. The efficiency of the RT-qPCR assays was shown to exceed 90%.

PCV in situ hybridization in sorghum root sections using riboprobes. Three digoxigenin (DIG)-labeled single-stranded cDNA probes were developed. The first fragment is located at the 3' untranslated region of RNA-1 and RNA-2. It was amplified using the J8 to J9 pair of primers, as described by Dieryck et al. (17) and allowed the sense (J9R) and anti-sense (J9F) probes to be designed. The second amplified fragment corresponded to the P14 encoding gene, nucleotide position 3512 to 3880 on RNA-2 of PCV-B, and allowed the third sense-probe (P14R) to be produced. The cDNA was transcribed in RNA and, during the same reaction, the latter labeled with DIG using the DIG RNA labeling Kit (Roche, Germany). Sorghum root sections grown on PCV-infested soil were pretreated by dipping in histoclear and ethanol in turn. They were then dipped in successive baths of ethanol, phosphate-buffered saline (PBS), Pronase (Sigma-Aldrich, St. Louis), glycine, PBS, formaldehyde, PBS, acetic anhydride, and PBS ethanol. The hybridization for each slide was carried out using the formamide method. For development, the sections were successively covered with: Tris-HCl/NaCl, blocking reagent (Boehringer, Germany), bovine serum albumin (Sigma-Aldrich), Triton X-100, anti-DIG-AP (Boehringer, Germany), nitroblue tetrazolium salt in dimethylformamide, and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide. The reaction was stopped by incubation in an ethanol-water series. The slides were counterstained with calcofluor. Once dried, the slides were mounted in Euparal (BDH, U.K.) and dried overnight. The lyophilized rabbit serum was resuspended in rivanol. Antibody specificity for the CP was checked by Western blotting on the fresh sap of PCV-infected plants. The sections were placed on a rack and dipped in xylene and ethanol in turn. They were then dipped in successive baths of an ethanol, saline/ethanol and saline series, PBS, milk powder, and PBS Tween-20. FITC conjugate solution (Sigma-Aldrich) was dropped on the slide and covered with Parafilm. "Safranin-Fast green" histological coloration was applied to every type of section (43). The slides were observed under transmitted light and under 4',6-diamidino-2-phenylindole (DAPI) and FITC fluorescence for hybridization assays and immunoreaction assays, respectively. The microscope DAS Mikroskop Leica DMR/HCS (Leica Microsystem Wetzlar GmbH, Wetzlar, Germany) was equipped with a DAPI filter with an illumination of 340 to 380 nm and an observation range of ≈ 425 nm, and with a FITC filter with an illumination between 470 to 490 nm and an observation range of ≈ 515 nm. The slides colored with Safranin-Fast green were observed under an Axiolab Zeiss microscope (Jena, Germany).

Data analysis. The quantities of *P. graminis* in cereal species after inoculation of viruliferous zoospores were compared using a Wilcoxon rank-sum test. The PCV and *P. graminis* infection rates

of each cereal species were analyzed using Z tests for two proportions.

RESULTS

PCV acquisition by *P. graminis* spp. using PCV-infected sugarcane. After 3 weeks of growth, clear symptoms of red leaf mottle appeared on sugarcane plants grown from all cuttings used in the experiment, and PCV was detected by RT-PCR in the leaves and roots samples of each plant. Aviruliferous isolates of *P. graminis* f. sp. *subtropicalis* (isolate I₀), *temperata* (isolate B₁), and *tropicalis* (isolate Ni₁) were then inoculated separately on PCV-infected sugarcane plants in order to produce viruliferous zoospores (Fig. 1). After 3 weeks of co-culture, PCV and *P. graminis* were detected by specific RT-PCR in the zoospore suspensions released from the roots of PCV-infected sugarcane plants. In contrast, the virus was not detected in the solution of root exudates from PCV-infected sugarcane plants that had not been infected by *P. graminis*, used as a negative control. This demonstrates that the aviruliferous isolates of *P. graminis* from *P. graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* became viruliferous at least 3 weeks after being inoculated on PCV-infected sugarcane plants.

Transmission of PCV by viruliferous zoospores. Standardized quantities of viruliferous zoospores produced on sugarcane infected by PCV and *P. graminis* f. sp. *tropicalis*, by PCV and *P. graminis* f. sp. *subtropicalis*, or by PCV and *P. graminis* f. sp. *temperata* were each inoculated on individual cereals. The same zoospore suspension was used to inoculate all plants with a specific PCV-*P. graminis* forma specialis association. After the incubation period, the presence of PCV and *P. graminis* in roots of each plant was assessed by specific molecular tools. The transmission of PCV by *P. graminis* f. sp. *tropicalis* had occurred on the four tested cereals but the percentage of plants infected by the virus was significantly higher on pearl millet (44%) than on barley (8%), sorghum (4%), or wheat (4%) (Table 1) (Z tests, $P < 0.0019$).

The percentages of PCV transmission on the four cereals were lower than the infection percentages by *P. graminis* f. sp. *tropicalis*. The zoospore suspension of this isolate enabled all the pearl millet plants and all but one of the sorghum plants to be infected. The barley (68%) (Z tests, $P < 0.0011$) and wheat (60%) (Z tests, $P < 0.0050$) plants were significantly less infected by the vector than pearl millet and sorghum (Table 1). Although all plants infected by PCV were also infected by the vector, there was no correlation between the percentages of cereal infection by PCV and by *P. graminis* f. sp. *tropicalis*.

The transmission of PCV by *P. graminis* f. sp. *subtropicalis* also occurred but to a lesser extent than by *P. graminis* f. sp. *tropicalis*: PCV was detected in barley (4%) and wheat (8%) but not in pearl millet and sorghum (Table 1). The infection percentages of the four cereals by the vector *P. graminis* f. sp. *subtropicalis* were higher than those by PCV. Statistical analyses of the infection of the four cereals by the vector produced three clusters: the pearl millet plants were the most frequently infected plants (100%), followed by sorghum (80%) and wheat (80%), and finally by barley (68%). Surprisingly, PCV was not detected in the pearl millet and sorghum plants, although the infection rates of these two cereals by *P. graminis* were very high (100 and 80%, respectively).

The ability of *P. graminis* f. sp. *temperata* to transmit PCV was tested using the same procedure, except that the sugarcane plants were grown at 17 to 20°C to allow *P. graminis* f. sp. *temperata* to develop. The sorghum plants were not grown at this temperature and could not be tested in this part of the experiment. Although PCV was detected in the zoospore suspension used as inoculum, the virus was not detected in the barley, pearl millet, or wheat plants inoculated with the zoospore suspension of *P. graminis* f. sp. *temperata* (Table 1). However, infection by the vector still occurred in 55% of the pearl millet plants, 45% of the barley plants, and 35% of the wheat plants.

Therefore, the effective PCV transmission rates, calculated by the ratio between the infection percentages of PCV and *P. graminis*,

Fig. 1. Scheme of the experiment: the source of *Peanut clump virus* (PCV) was sugarcane cuttings from PCV-infected fields; the source of *Polymyxa graminis* was sporosores. Healthy pearl millet plants were inoculated with *P. graminis* f. sp. *subtropicalis* or *tropicalis* and barley plants were inoculated with *P. graminis* f. sp. *temperata*. Aviruliferous zoospores suspension was produced from these plants and inoculated on PCV-infected sugarcane plants in order to produce viruliferous zoospores that were then inoculated on healthy cereals.

differed from one *Polymyxa* isolate to another, with higher values for *P. graminis* f. sp. *tropicalis* than for *P. graminis* f. sp. *subtropicalis*.

***Polymyxa* multiplication in plants.** The transmission experiment showed that the *Polymyxa* infection percentages were relatively high for all the tested species after inoculation with the viruliferous or aviruliferous zoospores, except for the viruliferous

zoospores of *P. graminis* f. sp. *temperata*. In this case, 24,000 zoospores were used as inoculum in place of 40,000 zoospores. The three *P. graminis* isolates, however, differed in their ability to multiply on the four tested species. The ability of the three *P. graminis* isolates to multiply on the four tested species after aviruliferous zoospore inoculation was quantified by RT-qPCR (Fig. 2).

TABLE 1. Percentages of plants infected by *Polymyxa graminis* f. sp. *subtropicalis*, *temperata*, and *tropicalis* and *Peanut clump virus* (PCV), percentages of plant infected by PCV using these three *Polymyxa* formae speciales as inoculum, and PCV effective transmission rates

Inoculum prepared from	PCV detection ^a	Inoculated species (number) ^b	<i>P. graminis</i> infection (%)	PCV infection (%)	PCV transmission (%) ^c
<i>Zoospores of P. graminis</i> f. sp. <i>tropicalis</i> released from					
PCV-infected sugarcane	+	Barley (25)	60	8	13
	+	Pearl millet (25)	100	44	44
	+	Sorghum (25)	96	4	4
	+	Wheat (25)	68	4	6
PCV-free pearl millet	–	Barley (10)	80	0	0
	–	Pearl millet (10)	100	0	0
	–	Sorghum (10)	100	0	0
	–	Wheat (10)	100	0	0
<i>subtropicalis</i> released from					
PCV-infected sugarcane	+	Barley (25)	68	4	6
	+	Pearl millet (25)	100	0	0
	+	Sorghum (25)	80	0	0
	+	Wheat (25)	80	8	10
PCV-free pearl millet	–	Barley (10)	50	0	0
	–	Pearl millet (10)	100	0	0
	–	Sorghum (10)	100	0	0
	–	Wheat (10)	100	0	0
<i>temperata</i> released from					
PCV-infected sugarcane	+	Barley (20)	45	0	0
	+	Pearl millet (20)	55	0	0
	+	Wheat (20)	35	0	0
PCV-free barley	–	Barley (10)	100	0	0
	–	Pearl millet (10)	90	0	0
	–	Wheat (10)	80	0	0
Hoagland solution		Barley (10)	0	0	0
		Pearl millet (10)	0	0	0
		Sorghum (10)	0	0	0
		Wheat (10)	0	0	0
Hoagland w/soaked roots ^d		Barley (10)	0	0	0
		Pearl millet (10)	0	0	0
		Sorghum (10)	0	0	0
		Wheat (10)	0	0	0

^a PCV detection in the inoculum.

^b Numbers in parentheses are the numbers of inoculated plants.

^c PCV effective transmission rate = PCV infection/*Polymyxa* infection.

^d Hoagland solution in which PCV-infected sugarcane roots were soaked.

Fig. 2. Relative quantities of *Polymyxa graminis* infection assessed by quantitative reverse-transcription polymerase chain reaction in composite samples of roots of barley, pearl millet, sorghum, and wheat in *P. graminis*-infected plants after inoculation with aviruliferous zoospores. Numbers of composite root systems are in parentheses.

The infection levels of *P. graminis* f. sp. *subtropicalis* and *tropicalis* were higher in pearl millet and sorghum than in wheat and barley, whereas the multiplication of *P. graminis* f. sp. *temperata* was higher in barley than in pearl millet and wheat.

These results indicate that the *P. graminis* infection percentages were not correlated with the quantities of *P. graminis* individuals in the roots (Table 1; Fig. 2).

Influence of PCV and the host producing the zoospores on the multiplication of *P. graminis*. The influence of PCV and the host from which zoospores were produced on the multiplication of the vector in infected plants was analyzed by comparing the infection rates of the three *P. graminis* isolates, based on the viruliferous or aviruliferous status of the zoospores. With regard to *P. graminis* f. sp. *tropicalis*, the proportions of infection of barley and wheat by viruliferous zoospores were significantly lower than the proportions of infection by aviruliferous zoospores

(Z tests, $P = 0.0301$ and 0.0208 , respectively). This was not the case for pearl millet or sorghum. For *P. graminis* f. sp. *subtropicalis*, no significant difference was observed between the *P. graminis* infection percentages on the four cereals based on the viruliferous status of the zoospores used for inoculation. For both *P. graminis* f. sp. *tropicalis* and *subtropicalis*, pearl millet was always the most infected and barley the least infected. The proportions of *P. graminis* f. sp. *temperata* infection on barley, pearl millet, and wheat were lower for viruliferous zoospores than for aviruliferous zoospores. Different zoospore concentrations were used as inoculum (24,000 versus 40,000, respectively) but, in both cases, the quantities of inoculated zoospores were very high and in the same order of magnitude (Table 1).

The observed differences in infection percentages between cereals inoculated with viruliferous or aviruliferous zoospores could also be due to the presence of PCV and to the host plant

Fig. 3. Relative quantities of *Polymyxa graminis* f. sp. *tropicalis*, assessed by quantitative reverse-transcription polymerase chain reaction, in 25 individual root systems of pearl millet plants inoculated with viruliferous zoospores. Light and dark gray bars refer to PCV-infected plants and PCV-free plants, respectively.

Fig. 4. Localization of *Peanut clump virus* (PCV) in sections of PCV-infected sorghum roots. PCV labeling by immunoreaction (in green, arrows) in **A**, the cortex, phloem, and lignifying parenchyma and **B**, in the cortex, endodermis, vascular parenchyma, and phloem. **C**, Detection of viral RNA (in dark blue, arrows) in the phloem vessels using J9R riboprobe by in situ hybridization. **D**, Control section treated by in situ hybridization without probe.

producing the zoospores. To clarify this, the impact of the presence of PCV on the multiplication of the vector in plants was evaluated by comparing the multiplication levels of *P. graminis* f. sp. *tropicalis* in infected and noninfected pearl millet plants, using RT-qPCR. The Wilcoxon rank-sum test assessed that the quantities of *P. graminis* individuals in pearl millet plants infected by PCV were not significantly different from the quantities in the plants not infected by the virus ($P = 0,6867$) (Fig. 3).

Localization of PCV in sorghum root tissues after *Polymyxa* transmission. In order to gain a better understanding of the development of PCV in roots after the transfer of the viruliferous zoospore content in the root hair or the epidermis root cells, the distribution of the virus was assessed in the sorghum roots using in situ hybridization and immunoreaction (Fig. 4; Table 2). The roots showed viral infection of the epidermis, exodermis, endodermis, vascular parenchyma, pith, and phloem vessels, and heavy infection of cortical cells, but the xylem was hardly infected. The intensity of the signal using in situ hybridization was high in the phloem and, in particular, in the companion cells. This was observed in the entire root system. The detection with both J9F and J9R probes targeting the sense and anti-sense RNA showed that the virus replicated in companion cells. The CP was also present in phloem vessels and companion cells. From the endodermis, the virus could infect the vascular parenchyma and the pith when it was present. The results obtained by in situ immunoreaction corresponded with those obtained by in situ hybridization on successive sections (Table 2).

DISCUSSION

The present study demonstrates, for the first time, the acquisition and the transmission of PCV using *P. graminis* zoospores.

TABLE 2. Relative *Peanut clump virus* (PCV) infection observed in sorghum root tissues using in situ immunoreaction and hybridization^a

Tissue	In situ immunoreaction	In situ hybridization
Epidermis	+	+
Exodermis	+	+
Cortex	++	++
Endodermis	+	+
Young xylem vessels	—	—
Phloem	+	++
Vascular parenchyma	+	+
Pith	+	+

^a Symbols: — = hardly infected, + = infected, and ++ = heavily and frequently infected.

These steps of the virus infection process involve numerous interactions between the vector and the virus, the vector and the host plant, and the virus and the host plant. The main objective of this study was to determine the specificity of these interactions.

The study demonstrated that *P. graminis* f. sp. *subtropicalis* and *tropicalis* zoospores were able to transmit PCV on cereals but this was not the case with *P. graminis* f. sp. *temperata*. To achieve this result, a strategy using sugarcane plants as the common host for the PCV and its vector to produce viruliferous zoospores was used. The ease of the vegetative propagation of sugarcane and the systemicity of PCV infection on this species enabled new root systems infected by PCV but free from other pathogens or parasites to be produced. Systemic infections obtained at 25 to 30°C as well as at 17 to 20°C enabled work to be done under both temperate and tropical conditions. The use of PCV-infected sugarcane cuttings and aviruliferous isolates of *P. graminis* also avoided the bias encountered in several previous studies on the soilborne transmission of viruses using *Polymyxa*-infested soil or dry roots as inoculum; bias had been caused, for example, by the possible interference of other soil microorganisms or the presence of various soilborne viruses and vector populations in the soil (58,60) or in the same plant (10,11,60,63). To date, the direct transmission of viruses by *Polymyxa* zoospores has been demonstrated for only 3 of the 18 *Polymyxa* spp.-transmitted viruses (Table 3): BNYVV (52,53), BaYMV (3), and SBWMV (45). The present study demonstrated the role of *P. graminis* f. sp. *tropicalis* and *subtropicalis* zoospores in the transmission of PCV. The viruliferous status of zoospores of the three tested special forms produced on PCV-infected sugarcane plant, including *P. graminis* f. sp. *temperata*, showed that the in vivo acquisition of PCV by all tested special forms of *P. graminis* occurred. The PCV transmission rates and ranges differed among the *P. graminis* special forms, highlighting the specificity of the interaction between the virus and its vector. PCV was transmitted to barley, pearl millet, sorghum, and wheat by *P. graminis* f. sp. *tropicalis* zoospores, whereas it was transmitted only to barley and wheat by *P. graminis* f. sp. *subtropicalis* zoospores. No transmission of PCV was observed using the viruliferous zoospores of *P. graminis* f. sp. *temperata*. The low temperatures required by this form species are not optimal for PCV (26,56) and could explain the lack of PCV transmission by this *P. graminis* isolate. The greatest transmission occurred in the case of PCV transmission by *P. graminis* f. sp. *tropicalis* on pearl millet, indicating specificity between the vector and the virus, as suggested by Tamada and Kusume (54) and raised by Adams (1) and Vaianopoulos et al. (61). The absence of PCV transmission by *P. graminis* f. sp. *temperata* might

TABLE 3. List of *Polymyxa* spp.-transmitted viruses and of publications referring to specific transmission assays

<i>Polymyxa</i>		Virus			Transmission assays using	
Species	Formae speciales	Name	Acronym	Genus	Sporosori or soil	Zoospores
<i>P. betae</i>	...	<i>Beet necrotic yellow vein virus</i>	BNYVV	<i>Benyvirus</i>	53	53
<i>P. betae</i>	...	<i>Beet soil-borne mosaic virus</i>	BSBMV	<i>Benyvirus</i>	65	...
<i>P. betae</i>	...	<i>Beet soil-borne virus</i>	BSBV	<i>Pomovirus</i>	28, 32	...
<i>P. betae</i>	...	<i>Beet virus Q</i>	BVQ	<i>Pomovirus</i>	...	...
<i>P. graminis</i>	...	<i>Barley mild mosaic virus</i>	BaMMV	<i>Bymovirus</i>	...	...
<i>P. graminis</i>	...	<i>Oat mosaic virus</i>	OMV	<i>Bymovirus</i>	...	...
<i>P. graminis</i>	...	<i>Rice necrosis mosaic virus</i>	RNMV	<i>Bymovirus</i>	...	...
<i>P. graminis</i>	...	<i>Wheat spindle streak mosaic virus</i>	WSSMV	<i>Bymovirus</i>	51	...
<i>P. graminis</i>	...	<i>Wheat yellow mosaic virus</i>	WYMV	<i>Bymovirus</i>	8	...
<i>P. graminis</i>	...	<i>Chinese wheat mosaic virus</i>	CWMV	<i>Furovirus</i>	...	...
<i>P. graminis</i>	...	<i>Oat golden stripe virus</i>	OGSV	<i>Furovirus</i>	...	...
<i>P. graminis</i>	<i>temperata, tepida</i>	<i>Soil-borne cereal mosaic virus</i>	SBCMV	<i>Furovirus</i>	60	...
<i>P. graminis</i>	...	<i>Soil-borne wheat mosaic virus</i>	SBWMV	<i>Furovirus</i>	45	45
<i>P. graminis</i>	...	<i>Sorghum chlorotic spot virus</i>	SrCMV	<i>Furovirus</i>	...	...
<i>P. graminis</i>	<i>colombiana</i>	<i>Rice stripe necrosis virus</i>	RSNV	<i>Benyvirus</i>	...	...
<i>P. graminis</i>	<i>subtropicalis, tropicalis</i>	<i>Indian peanut clump virus</i>	IPCV	<i>Pecluvirus</i>	46	...
<i>P. graminis</i>	<i>subtropicalis, tropicalis</i>	<i>Peanut clump virus</i>	PCV	<i>Pecluvirus</i>	57	Present study
<i>P. graminis</i>	<i>temperata, tepida</i>	<i>Barley yellow mosaic virus</i>	BaYMV	<i>Bymovirus</i>	3, 60	3

be explained by the requirement of a minimum threshold of infection by the vector to see PCV infection. Clarification of the relationships between virus and vector is useful for evaluating the risk of dissemination of *Polymyxa*-transmitted viruses or new viruses imported by infected seed. *P. graminis* f. sp. *temperata* seems to represent only a limited risk of PCV transmission in temperate areas, which might be useful considering the risk of PCV dissemination in noncontaminated areas (18). The present study demonstrated that transmission efficiency depends on the *P. graminis* special forms. The variation of PCV transmission efficiency depending on the special forms of the vector is similar to the intraspecific effect observed in the transmission efficiency of viruses by some aphids, such as the transmission of *Barley yellow dwarf virus* by *Sitobion avenae* (13). Until now, this variation in transmission efficiency had not been demonstrated for plasmodiophorids. The high transmission of the PCV isolate from Burkina Faso by an isolate of *P. graminis* f. sp. *tropicalis* from Niger on pearl millet also suggests the existence of a co-evolution mechanism between the virus, the vector, and the host plant. Both the virus and the vector are obligate parasites of plants and the entry of the virus in the plant depends on the vector infection. Thus, the three actors of the pathosystem are closely linked. The PCV and the *P. graminis* f. sp. *tropicalis* isolates used in this experiment both originated from West Africa. Phylogenetic analysis revealed a higher diversity within *P. graminis* (35) and within *Pecluvirus* spp. (18,41) in West Africa than in the Indian subcontinent. These elements suggest a possible common center of origin for the virus and its vector in West Africa. The sub-Saharan area is also the center of origin of pearl millet (27,42). This is a propitious situation for co-evolution. This hypothesis is supported by the highest multiplication ability of *P. graminis* f. sp. *tropicalis* in pearl millet (37) and the symptomless infection of PCV on pearl millet (19,21). To confirm this co-evolution hypothesis, it should be interesting to test the transmission of PCV or IPCV isolates naturally vectored by other special forms; such as, for instance, *P. graminis* f. sp. *subtropicalis*.

In addition to the specificity of the interaction between the virus and its vector, there is also evidence of specificity in the interaction between PCV and host plants. No correlation between the infection percentages by PCV and those by *P. graminis* were observed. For example, pearl millet infection percentages by *P. graminis* f. sp. *tropicalis* and by PCV were high, whereas sorghum was highly infected by *P. graminis* f. sp. *tropicalis* but weakly infected by PCV. This suggests that the development of PCV and its vector are independent. Independence between PCV and *P. graminis* development was observed in the quantitation of *P. graminis* in the pearl millet roots in the presence or absence of PCV. Until now, the influence of soilborne viruses on the development of their vector has been little studied. The presence of *Beet soil-borne virus* in newly formed roots was shown to have a positive effect on the development of *P. betae* (44), whereas BNYVV induced a negative effect (49). This highlights the specificity of the interaction between vector and virus within each pathosystem.

In addition, the difference in the *P. graminis* infection percentages between plants inoculated with viruliferous and aviruliferous zoospores could be explained by either the influence of the host producing the zoospores or by the presence of PCV. Our study showed that, for *P. graminis* f. sp. *tropicalis*, the presence of PCV in plants had no influence on the multiplication of the vector. This suggests that the host plant species in which *P. graminis* zoospores are produced might affect the infectivity of the vector.

Our work on the localization of PCV in the sorghum roots also showed that the development of PCV in plants after the penetration of the virus is independent from its vector. Galamay et al. (25) observed that development of *P. graminis* sporosori is restricted to the epidermis and to the hypodermis tissues in sorghum roots. Similarly, Littlefield et al. (39) reported the absence of

sporosori in the sorghum tissues internal to the hypodermis. Doucet et al. (20) suggested that the endodermis is a natural barrier to the spread of *Polymyxa* spp. to the vascular cylinder. The present study showed that PCV was present in the cortex; both RNA strands were found, as well as the CP, indicating translation and replication. The CP was also present in the phloem vessels and companion cells. Its requirement for long-distance transportation was proposed by Herzog et al. (31). In addition, the spread of PCV in most of the sorghum root tissues highlighted the ability of sorghum to cause secondary infection. This increases the potential impact of sorghum in the epidemiology of the disease.

Apart from the independence between the development of the virus and its vector, differences in the multiplication level of *P. graminis* were observed, depending on the *P. graminis* special form used and the host species inoculated. This experiment is the first host range study of *P. graminis* f. sp. *subtropicalis* and *tropicalis* using equal quantities of zoospores as inoculum. Both *P. graminis* f. sp. *subtropicalis* and *tropicalis* presented the same multiplication profile in cereals. Pearl millet and sorghum could be considered as "preferred hosts" for these two special forms. With regard to *P. graminis* f. sp. *temperata*, multiplication was high in barley but low in pearl millet and wheat. Barley was also confirmed as the preferred host of *P. graminis* f. sp. *temperata*, as reported by Legrève et al. (35,37,38) and Vaianopoulos et al. (61). This study reveals infection of *P. graminis* f. sp. *temperata* on pearl millet and on sugarcane. This is also the first report of the sporangial multiplication and zoospores production of *P. graminis* f. sp. *temperata* on sugarcane. These results demonstrated the diversity in host specificity and aggressiveness among isolates and highlighted the specificity of the interaction between *Polymyxa* spp. and the host plant.

Finally, the *P. graminis* infection percentage and the ability of *P. graminis* to multiply in the roots appeared not to be correlated, suggesting that infection frequency and infection level by *Polymyxa* spp. are governed by distinct mechanisms. From an epidemiological point of view, *P. graminis* infection and multiplication are complementary. The ability of *P. graminis* to multiply in a plant is very important during the onset of the disease, leading to an increase of the *P. graminis* infectious potential of the soil. In contrast, the ability of *P. graminis* to infect host plants leads to the spread of the disease.

The model of PCV transmission using sugarcane as the common host for both the virus and its vector provides a new way of repeating the transmission of functional PCV by *P. graminis* special forms under controlled conditions. The present study shows the specificities of the interactions between the actors involved the peanut clump disease, as well as the differential development of PCV and its vector within host plants. Until now, screening assays have been conducted separately for the virus and its vector (14,16,37). In addition to improving our knowledge about the interactions, this model of virus transmission using viruliferous zoospores might be used for large-scale screenings of cereals to select cultivars resistant to both *P. graminis* and PCV. Using cultivars resistant to viruliferous zoospores is a way of limiting the incidence of peanut clump disease in infested areas.

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