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Peanut clump virus and *Polymyxa graminis* interactions with pearl millet (*Pennisetum glaucum* [L.] R. Br.) and sorghum (*Sorghum bicolor* [L.] Moench)[1]

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Abstract

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) and sorghum (*Sorghum bicolor* [L.] Moench) are common natural hosts of *Peanut clump virus* (PCV) and its vector, *Polymyxa graminis*. They increase the inoculum potential in infested soils. In selecting adapted cultivars, it is important to improve our understanding of the plant resistance mechanisms. The susceptibility of six pearl millet and six sorghum accessions to the virus and its vector was compared. The susceptibility to the virus was assessed under field conditions. The infection frequencies of roots and leaves, analysed by a penicillinase-based DAS-ELISA, confirmed the differences in the pearl millet and sorghum accessions. In pearl millet, the virus was detected in roots and leaves, whereas in sorghum the infection was common in roots but rare in leaves. This was linked with the seed transmission of PCV in millet and not in sorghum. These results confirm the preliminary observations of the movement of the virus within these plant species, underlining the potential role of the viral P15 gene, a suppressor of post-transcriptional gene inactivation. The susceptibility to *P. graminis* f. sp. *tropicalis* was assessed under controlled conditions using a zoospore inoculation assay. The infection was quantified by a real-time quantitative PCR assay targeting the ribosomial DNA and highlighting the differences between plant accessions and species. In addition to guidance on the selection of resistant cultivars, this study also sought to provide a toolbox for improving the understanding of the plant/vector/virus interactions.

Introduction

Peanut clump disease causes yield losses of up to 70% in groundnut (*Arachis hypogaea* L.) (GERMANI & DHERY, 1973). In West Africa, the causal agent is *Peanut clump virus* (PCV), a *Pecluvirus* characterized by rigid rod-shaped virions encapsidating a bipartite RNA genome (THOUVENEL *et al.*, 1976). It

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is transmitted by the plasmodiophorid *Polymyxa graminis* Ledingham (THOUVENEL & FAUQUET, 1981), an obligate telluric root endoparasite living in the soil as clusters of thick-walled resting spores. Two *formae speciales* of this species, *P. graminis* f. sp. *tropicalis* and *P. graminis* f. sp. *subtropicalis,* with specific ecological requirements and rDNA sequences, are associated with the transmission of the *Pecluvirus* (LEGRÈVE *et al.*, 2002). PCV and its vector are often found in asymptomatic association with pearl millet (*Pennisetum glaucum* [L.] R. Br.) and sorghum (*Sorghum bicolor* [L.] Moench). These cereals are traditionally cropped in West Africa in association or rotation with groundnut, thus contributing to an increase in inoculum potential. These species therefore play a major role in the epidemiology of peanut clump disease. The use of resistant varieties should be a strategy for controlling the multiplication of the inoculum. In order to study the susceptibility of pearl millet and sorghum accessions to PCV and *P. graminis*, two experiments were conducted. First, the susceptibility of six accessions of pearl millet and six accessions of sorghum to PCV under field conditions was assessed by Double Antibody Sandwich Enzyme-Linked ImmunoSorbent Assay (DAS-ELISA). Second, their susceptibility to *P. graminis* was evaluated after the inoculation of plants grown under controlled conditions by estimating the level of root infection by quantitative real-time PCR. The results should be useful in varietal selection and help improve the understanding of the plant/vector/virus interactions.

Materials and methods

Six accessions of pearl millet and six accessions of sorghum were selected on the basis of a preliminary screening conducted in the 2002 rainy season in which the susceptibility to PCV of 20 pearl millet accessions and ten sorghum accessions was assessed by quantifying the infection percentages of plants grown in a naturally PCV-infested field by a penicillinase-based form of DAS ELISA (Table 1). The area under the disease progression curve (AUDPC) that indicates the progress over time of the virus infection rates was calculated for each accession, and classes of susceptibility were formed (SHANER & FINNEY, 1977).

TABLE 1
Susceptibility of pearl millet and sorghum accessions to PCV assessed according to AUDPC value in the 2002 rainy season

Species / Accessions	AUDPC						Agronomical features
	Roots			Leaves			
	Mean	S.D.	Classes	Mean	S.D.	Classes	
Pearl millet							
Ankoutess	4988	1312	A	3697	797	A	Local variety
ICMV IS 89305	2070	929	C	2050	1213	C	Cross between CIVT GMS, ¾ HKP B-78, Souna
ICMV IS 92222	3359	1835	B	3775	687	A	Improved variety
ICMV IS 94206	3220	2095	B	2603	1641	C	Improved variety
Sadoré local	645	548	D	1758	900	D	Local variety
Zatib	1650	1370	C	2264	1106	C	Cross between Zanfarwa and Tchinin Bajini
Sorghum							
Irat 204	3147	1101	B	2670	885	C	Cross between Niger, Sénégal and Ethiopian varieties
Mota maradi	3258	1810	B	2780	989	B	Local variety
Nad 1	3172	828	B	3005	1267	B	Hybrid developed by INRAN and Purdue university
Sariaso 10	3308	2423	B	2188	492	C	Burkina Faso variety
Sepon 82	4081	1225	B	2519	995	B	Improved variety
Zinder local	3589	1641	B	2255	907	C	Local variety

The susceptibility of the twelve selected accessions to PCV was re-assessed in 2003 by growing them in a naturally PCV-infested field at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Sadoré, Niger. In order to reduce variability because of the heterogeneous distribution of the inoculum potential, the twelve accessions were sown in incomplete blocks based on an alpha-design that consisted of four replications of four blocks of three accessions (DAGNÉLIE, 1981, PATTERSON & WILLIAMS, 1976; PIEPHO & SPILKE, 1999).

Every twelve days, 32 plants of each accession (eight per replication) were sampled in the field. The presence of PCV in the roots and leaves of each plant was tested by a penicillinase-based form of DAS-ELISA. The polyclonal antiserum was originally produced in 1988 against a Nigerian isolate (PCV-Ni). The test was performed as described by REDDY *et al.* (1998). The reaction was considered to be positive when the absorbance value of this sample was lower than the threshold value (T) calculated by:

$$T = (NC) - [(NC) - (PC)] / 2$$

where NC is the absorbance value of the negative control (healthy groundnut) and PC is the positive control (sugarcane leaves infected with homologous PCV).

The twelve accessions were assayed for their susceptibility to *P. graminis* in a bioassay conducted under controlled conditions. Ten seeds per accession were sown individually in glass culture tubes filled with sterile sand. After fifteen days growth at 25–30°C (night-day temperature) with a photoperiod of twelve hours, the plantlets were inoculated with zoospores released from pearl millet plants infected by *P. graminis* f.sp. *tropicalis* (isolate Ni-1) grown in an automatic immersion system (AIS). In this system, the culture of a *Polymyxa* isolate is maintained on host plants by growing two to three week-old healthy plantlets in the presence of heavily infected plants (LEGRÈVE *et al.*, 1998). A high quantity of zoosporangia is produced in the healthy plants within two weeks. After 18 hours without watering, zoospores were released from these plants by soaking the roots in a 20% Hoagland solution previously refreshed at 12°C. After one hour releasing at room temperature in the darkness, the zoospore concentration was assessed using a Thoma counting cell. Eight plant by accession were inoculated with One ml containing 80,000 zoospores and the other two plants were used as a negative control. The plants were placed in darkness for two hours, and then grown for three weeks in the conditions described above. *P. graminis* infection was assessed in the plant accessions using a quantitative real-time PCR assay. Total DNA was extracted from a 100 mg root sample of each plant using the Qbiogene FastDNA® kit as described by LEGRÈVE *et al.* (2003). The forward primer targeting a specific sequence of *P. graminis* f. sp. *tropicalis* PGTR2 (5'-GGGTTTTTTGTTGCGAAATGTC-3') was developed using the Eprimer3 program in the specific ITS 1 region of the ribosomal DNA of *P. graminis* f. sp. *tropicalis*. The reverse primer was PSP2REV (5'-AGGGCTCTCGAAAGCGCAA-3') targeting a specific sequence of the 5,8S *Polymyxa* spp. gene (LEGRÈVE, 2003). The specificity of PGTR2 to *P. graminis* f. sp. *tropicalis* was verified using the Blast program (ALTSCHUL *et al.*, 1997). The real-time quantitative PCR was performed using the iCycler IQ™ Real-Time Detection system of Biorad. For one sample tested, the reaction mix contained 0.8 µl of each primer, 14.4 µl of diethylpyrocarbonate (DEPC) treated water and 20 µl of iQ™ SYBR® Green Supermix (Biorad). Thirty six µl of the mix were prepared per reaction and four µl of the ten-fold-diluted DNA extract or control were added. The PCR reaction was performed under the following conditions: one cycle of three minutes at 95°C (denaturation step), 40 cycles of 15 seconds at 95°C, and 15 seconds at 60°C with a recording of the fluorescence after each elongation phase. Afterwards, the melt curve of the PCR products was generated by recording the fluorescence every ten seconds during heating from 55°C to 95°C, with an increment of 0.5°C every ten seconds. Two replicates were tested per sample. The relative quantification of *P. graminis* f. sp. *tropicalis* was done by comparing the C_t (Threshold cycle) 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. This standard DNA extract enabled the quantitative real-time PCR reactions to be compared. No fluorescence with DNA from a healthy plant was recorded. The efficiency of the quantitative real-time PCR assays was shown to be above 90%. The data were transformed (according to this formula:

$$y = \sqrt{Log\ (x+1)}$$

with x = the sample DNA relative quantity) to achieve the normal distribution needed to conduct the statistical analysis.

Results

DYNAMICS OF PCV INCIDENCE IN PEARL MILLET AND SORGHUM

Only twelve days after sowing, PCV was detected in the roots of 5–20 % of the plants for eleven accessions out of the twelve tested. Fifty days after sowing, PCV infection in pearl millet (Figure 1) was highest for the accession Ankoutess, with 87.5% infection of the roots and 70% infection of the leaves. PCV was detected in 69% and 67% of the roots of accessions ICMV IS 92222 and ICMV IS 94206 and in about 50% of the roots of ICMV IS 89305, Sadoré local and Zatib. The leaf infection followed the root infection, with a slight delay. In sorghum (Figure 2), PCV was detected in the roots of all accessions twelve days after sowing in a range similar to that observed for the pearl millet accessions. The root infection increased to only 31% for the accession Sariaso 10, to 46% for Irat 204 and up to between 60 and 80% for the other four accessions (Mota Maradi, Nad 1, Sepon 82 and Zinder local). The incidence of the virus in sorghum leaves was lower than that observed in pearl millet, with only 19–28% infection.

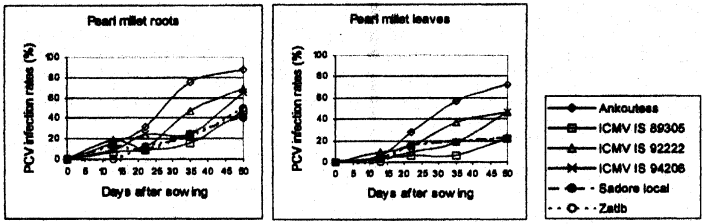

Figure 1. Evolution of PCV infection in roots and leaves of six pearl millet accessions grown in a PCV-infested field at Sadoré, Niger in the 2003 rainy season. Each point is based on the analysis of 32 plants.

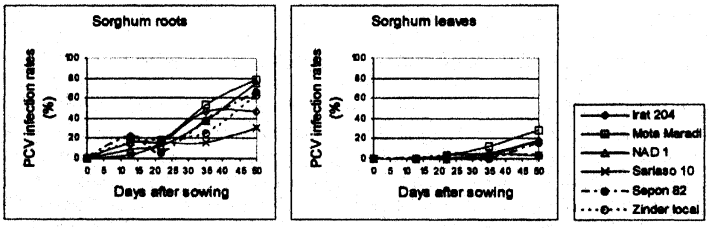

Figure 2. Evolution of PCV infection in roots and leaves of six sorghum accessions grown in a PCV-infested field at Sadoré, Niger in the 2003 rainy season. Each point is based on the analysis of 32 plants.

The AUDPC in roots and leaves enabled different classes of susceptibility to be distinguished, using the General Linear Model (GLM) (with $\alpha = 0.05$) (Table 2). Based on the AUDPC analysis, for pearl millet roots three accessions showed a lower virus incidence (ICMV IS 89305, Sadoré local and Zatib) than the other accessions (Ankoutess, ICMV IS 92222, ICMV IS 94206). For leaves, the AUDPC analysis produced four classes. The accession ICMV IS 89305 was the least susceptible for leaves and the accession Ankoutess appeared to be the most susceptible for roots and for leaves. For sorghum, two classes were formed. The Mota Maradi accession was the only one considered to be more susceptible for both roots and leaves, while the other accessions were considered to be less susceptible.

TABLE 2

The susceptibility of six pearl millet and six sorghum accessions to *P. graminis* and to PCV. (A = susceptible; D = less susceptible). The statistical analysis was conducted separately for the virus and the vector. The *P. graminis* DNA quantity was transformed ($y = \sqrt{Log\,(x+1)}$ with x = relative quantity of *P. graminis* DNA)

Species	*P. graminis*		PCV					
			Roots			Leaves		
Varieties	Trans. DNA quantity	Classes	AUDPC	S.D.	Classes	AUDPC	S.D.	Classes
Pearl millet								
Ankoutess	2.47	B	2120	582	A	1664	404	A - B
ICMV IS 89305	2.46	B	702	629	B	334	526	D
ICMV IS 92222	1.94	C	1394	681	A	1091	907	B
ICMV IS 94206	1.93	C	1159	612	A	731	537	C
Sadoré local	2.24	B	814	395	B	613	345	C
Zatib	2.82	A	819	436	B	598	448	C
Sorghum								
Irat 204	2.22	A	1194	812	B	344	429	B
Mota Maradi	2.2	A	1606	584	A	733	695	A
Nad 1	2.28	A	1267	548	B	425	618	B
Sariaso 10	1.56	B	695	534	B	150	242	B
Sepon 82	1.41	B	1184	392	B	359	265	B
Zinder local	1.46	B	1095	801	B	163	174	B

SUSCEPTIBILITY TO *P. GRAMINIS* IN PEARL MILLET AND SORGHUM

Different levels of susceptibility to *P. graminis* among the accessions (Figure 3) were obtained by assessing the relative concentration of *P. graminis* DNA in plants inoculated with a standard concentration of zoospores. The level of root infection was globally greater for pearl millet than for sorghum. On the basis of the statistical analysis, three classes of susceptibility to *P. graminis* were distinguished for pearl millet and two for sorghum (Table 2). For pearl millet, the highest level of root infection by *P. graminis* was observed on the accession Zatib, whereas the infection of ICMV IS 94206 and ICMV IS 92222 was the lowest. Sorghum accessions Irat 204, Mota Maradi and Nad 1 were more severely infected than the other three accessions. The infection on the less infected Sepon 82, Zinder local and Sariaso 10 accessions was lower than the less infected pearl millet accessions.

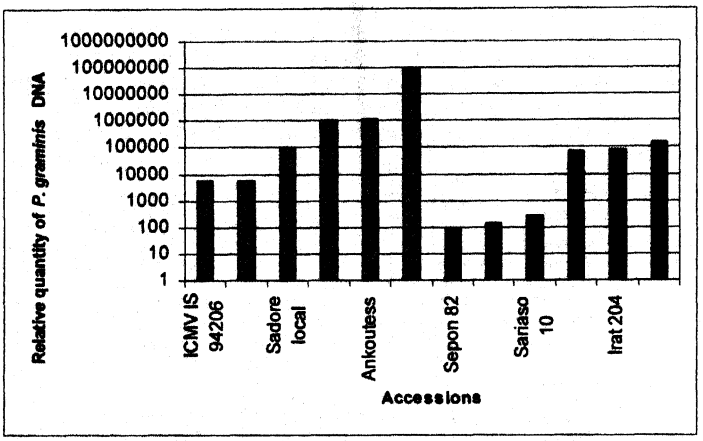

Figure 3. *P. graminis* relative DNA quantity of six pearl millet (grey bars) and six sorghum (black bars) accessions.

Discussion

The bioassay conducted under field conditions in 2003 permitted distinct PCV susceptibility classes among pearl millet and sorghum accessions to be detected, as previously shown in 2002. Accessions showed a similar behaviour in 2002 and 2003. The low susceptibility of the accessions ICMV IS 89305, Sadoré local and Zatib should be exploited in field. The level of PCV infection on the leaves of pearl millet increased in the same proportion as in roots, with only a slight delay, showing that PCV translocation is efficient in pearl millet. For sorghum, the levels of leaf infection were very low in comparison with root infection, showing that virus translocation from roots to shoots is restricted in this species. Further observation may explain why seed transmission occurs in pearl millet but is absent in sorghum (REDDY *et al.*, 1998). In addition, the pearl millet accession with the highest seed transmission rate, Ankoutess, also showed the highest level of PCV leaf infection (G. OTTO, unpublished data).

The quantitative real-time PCR assay developed for the relative quantification of *P. graminis* infection revealed differences of susceptibility in pearl millet and sorghum accessions. The highest levels of infection were found in pearl millet. Differences by a factor of 10,000 in pearl millet and by a factor of 100 in sorghum clearly suggest that the rates of multiplication of *P. graminis* among the accessions are different. They suggest that the choice of

accessions may greatly influence the level of infection potential in field. The classes of susceptibility to PCV and *P. graminis* are not correlated in pearl millet. This indicates that there are different resistance mechanisms to PCV and *P. graminis*. In sorghum, some correlation was found between the levels of PCV and *P. graminis* infection capacity to multiply *P. graminis* in its roots and its susceptibility to PCV infection, as for the accessions Mota Maradi, Sariaso 10, Sepon 82 and Zinder local. However, the susceptibility to PCV and *P. graminis* were not studied under equivalent conditions; it would be interesting to confirm these results under similar conditions. This study demonstrated that the mechanism of resistance to peanut clump disease can occur at different stages and depends on both the vector and the virus. More knowledge about vector multiplication, the dynamic of virus infection, the virus movement through the plant, the virus seed transmission and the potential role of a viral P15 involved in the suppression of post-transcriptional gene silencing is needed in order to develop an integrated approach to the control of the disease.

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