Biological Control

Polymorphism of the Grasshopper *Schistocerca pallens* (Thunberg) (Orthoptera: Acrididae) and its Natural Pathogen *Metarhizium flavoviride* Gams & Rozsypal (Hyphomycetes), Revealed by RAPD Analysis

Eni B. Silveira, Salah M. Al-Janabi, Bonifácio P. Magalhães, Luiz J.C.B. Carvalho and Myrian S. Tigano
Embrapa - Recursos Genéticos e Biotecnologia, Caixa postal 02372, 70849-970, Brasília, DF.

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Polimorfismo do Gafanhoto *Schistocerca pallens* (Thunberg) (Orthoptera: Acrididae) e seu Patógeno Natural *Metarhizium flavoviride* Gams & Rozsypal (Hyphomycetes), Revelado pela Análise de RAPD.

RESUMO - Foi investigada a variabilidade genética através de análise de RAPD entre 27 indivíduos de duas populações distintas (Rio Grande do Norte e Distrito Federal) do gafanhoto-praga *Schistocerca pallens* (Thunberg) e de oito isolados do seu patógeno natural, o fungo *Metarhizium flavoviride* Gams & Rozsypal. Para os gafanhotos, 79 caracteres binários gerados por dez primers foram selecionados para análise. A estimativa média da diversidade de nucleotídeos nas populações de *S. pallens* do Distrito Federal e do Rio Grande do Norte , feita através do programa RAPDDIP, indicou alta variabilidade genética intrapopulacional, com valores de 2,2 e 2,3% respectivamente. No entanto, o valor estimado de divergência de nucleotídeos de 0.004 revelou que praticamente não existe diferença entre essas populações estudadas. Com relação aos isolados de *M. flavoviride*, foram selecionados para análise 388 caracteres binários gerados por 31 primers. A análise fenética destes dados revelou uma alta homogeneidade (similaridade ≥ 79,5%) entre os isolados, principalmente entre os brasileiros (similaridade ≥ 98,3%), os quais foram obtidos de regiões geograficamente próximas e do mesmo hospedeiro, *S. pallens*.

PALAVRAS-CHAVE: Insecta, variabilidade genética, fungo entomopatogênico, controle biológico, marcadores moleculares.

ABSTRACT - The genetic variability of 27 individuals from two distinct populations (Northeast and Central Brazil) of the grasshopper *Schistocerca pallens* (Thunberg) and eight isolates of its natural pathogen, the fungus *Metarhizium flavoviride* Gams & Rozsypal, was investigated using RAPD analysis. For the grasshoppers, ten different 10-mer oligonucleotide primers of arbitrary sequence were selected for analysis, resulting in 79 scorable binary characters. The program RAPDDIP applied to *S. pallens* revealed nucleotide diversity of 2.3 and 2.2% for the populations from Rio Grande do Norte (Northeast Brazil) and Federal District (Central Brazil), respectively. These values indicate the presence of high genetic variability within these populations. Conversely,

the value for nucleotide divergence (0.004) showed almost no distinction between the two populations. In the case of *M. flavoviride*, thirty-one 10-mer oligonucleotide primers of arbitrary sequence were selected for analysis, producing 388 scorable binary characters. A dendrogram obtained for *M. flavoviride*, using the program NTSYS, revealed high homogeneity (similarity ≥79.5%) among the 8 isolates analyzed. The Brazilian isolates, all from the same geographical area and host (*S. pallens*), were even more homogeneous (≥98.3%).

KEY-WORDS: Insecta, genetic variability, entomopathogenic fungus, biological control, molecular markers.

The grasshopper, *Schistocerca pallens* (Thunberg), is a serious pest of several crops in Northeast Brazil, including beans, maize, cotton, sugarcane, cashew, and native as well as cultivated pastures (Moreira *et al.* 1996). With few exceptions, this insect can be found all over the country (Duranton *et al.* 1987), including Central Brazil (DF), where it occurs at low population densities (F. G. Schmidt, personal communication). The only currently adopted pest control strategy is based on chemical insecticides. A promising alternative would be the use of biopesticides, in particular those based on pathogenic fungi (Morley-Davies *et al*. 1995, Miranda *et al*. 1996, Moreira *et al*. 1996). Therefore a search for a natural enemy of *S. pallens* is needed.

Metarhizium flavoviride Gams & Rozsypal has been found to infect *S. pallens* in Northeast Brazil (Magalhães *et al.* 1997). One of its isolates (CG423) has shown high virulence against another grasshopper, *Rhammatocerus schistocercoides* (Rehn), from Central Brazil (Magalhães *et al.* 1997) and against *Stiphra robusta* Mello-Leitão (Orthoptera: Proscopiidae) from Northeast Brazil (M. Moreira & B. P. Magalhães, unpublished). These results indicate the possible use of this fungus against grasshopper pests. However, an important aspect in the development of a successful control strategy involves the understanding of the genetic variability of the host population and that of the fungal isolate. Host populations that are genetically distinct may present different levels of susceptibility, affecting the choice of isolate. Genetic characterization of isolates also allows accurate monitoring following application. This information can be used to assess fungal isolate factors such as efficiency, stability and persistency in the field.

PCR-based DNA markers, such as those generated by random amplified polymorphic DNA-RAPD (Williams *et al*. 1990), also known as arbitrarily primed PCR markers (Welsh & McClelland, 1990), have been extensively used to study polymorphism of several organisms, including grasshoppers within the genus *Melanoplus* (Chapco *et al*. 1992) and fungal isolates of *Metarhizium* spp. (Fegan *et al.* 1993, Bidochka *et al*. 1994, Tigano-Milani *et al.* 1995). This technique is based on PCR amplification using random primers that produce several DNA fragments from an individual genomic DNA. By comparing several individuals, the presence/absence of a specific amplified DNA fragment may produce useful genetic information to identify an individual by its DNA fingerprint or polymorphic fragments as a genetic character to study population dynamics. Based on that, in this study we have used RAPD markers with the objective to obtain preliminary data on the intra and interpopulational genetic variability of *S. pallens* from Northeast and Central Brazil. Another objective was to investigate the existence of molecular markers to separate isolates of *M. flavoviride* that show

potential to control grasshopper pests in Brazil.

Material and Methods

Insects. Twenty-seven female adults, 11 from Central Brazil and 16 from Northeast Brazil were used. The insects were stored in absolute ethanol at -20°C prior to DNA extraction.

Fungi. Eight isolates of *M. flavoviride* were used. Five of these isolates were obtained from *S. pallens* from Northeast Brazil and 3 from other countries and acridid hosts. One isolate of *M. album* Petch and one isolate of *M. anisopliae* (Metschn.) Sorokin were used as outgroups (Table 1). Mycelium was obtained from a submerged culture of spores in Saboraud dextrose broth, which was previously shaken at 150 rpm for 3 days at 25°C. Mycelium was harvested by filtration through filter paper (Whatman Nº 1), lyophilized and stored at -80°C.

Grasshopper DNA Extraction. Femoral muscles of grasshopper individuals were removed and incubated at 60°C for at least 1 h with 400ml of salt lysis buffer (0.4M NaCl, 10mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0), containing proteinase K (70mg) and 2% SDS. After adding 350ml of 6M NaCl to the suspension, the mixture was then vortexed for at least 1 min, followed by centrifugation at 14,000 rpm for 20min. DNA was precipitated from the supernatant by adding an equal volume of isopropanol, incubated at -20°C for 1 h and then centrifuged at 14.000 rpm for 20min. The pellet was washed with 70% ethanol, dried and resuspended in 300ml of sterile distilled water.

Fungus DNA Extraction. Approximately 3g of lyophilized mycelium for each *Metarhizium* spp. isolate was grounded to a powder in a mortar and pestle, using liquid nitrogen. Genomic DNA extraction was carried out as described by Raeder & Broda (1985).

Grasshopper RAPD Analysis. PCR amplifications were performed in a reaction final volume of 30ml, using 10ng of each tem-

Table 1. Isolates of *Metarhizium* spp. used in RAPD analysis.

Isolate[1]	Species	Host	Geographical Origin[2]
CG288	*M. flavoviride*	*Schistocerca pallens* (Ort., Acrididae)	PB/Brazil
CG291 (ARSEF324)	*M. flavoviride*	*Austracris guttulosa* (Walker 1870) (Ort.,Acrididae)	Australia
CG343 (ARSEF2575)	*M. anisopliae*	*Curculio caryae* (Horn) (Col., Curculionidae)	USA
CG366 (IMI330189)	*M. flavoviride*	*Ornithacris cavroisi* (Finot) (Ort., Acrididae)	Nigeria
CG423	*M. flavoviride*	*Schistocerca pallens*	RN/Brazil
CG429	*M. flavoviride*	*Schistocerca pallens*	PB/Brazil
CG430	*M. flavoviride*	*Schistocerca pallens*	RN/Brazil
CG431	*M. flavoviride*	*Schistocerca pallens*	RN/Brazil
CG442 (ARSEF3391)	*M. flavoviride*	*Zonocerus elegans* (Thunberg) (Ort., Acrididae)	Tanzania
CG515 (ARSEF1941)	*M. album*	*Nephotettix virescens* (Dist.) (Hom., Cicadellidae)	Philippines

[1] CG, Embrapa - Genetical Resourses and Biotechnology Collection, Brasília, DF, Brazil; ARSEF, Agricultural Research Service Entomopathogenic Fungus Collection, USDA-ARS, Ithaca, NY, USA; IMI, International Mycological Institute, Egham, UK.
[2] PB, Paraíba State; RN, Rio Grande do Norte State.

plate DNA. The amplifications were done using the following reaction mixture: 0.2mM of each dNTP, 0.4mM of primer, 2U of Taq DNA Polymerase (CENBIOT-RS) and 1x polymerase buffer containing 1.5mM $MgCl_2$. Thirty-two 10-primers were screened using genomic DNA from two grasshopper individuals. Ten 10-mer primers (Operon Technologies) were then selected for this study based on their ability to produce easily distinguishable polymorphisms (OPC-04, 06, 10; OPE-01, 03, 04, 06, 08, 11, 15). The temperature profile for all reactions was 94°C for 3 min, followed by 40 cycles of 93°C for 1min, 35°C for 1min, 72°C for 2min, with a final extension at 72°C for 5min, using a 9600 cycler (Perkin-Elmer) system. The amplification products were stored at 15°C prior to the analysis on a 2% Tris/Borate/EDTA (TBE) agarose gel and visualized by staining with ethidium bromide (Sambrook *et al.* 1989).

Fungus RAPD Analysis. PCR amplifications were performed in a reaction final volume of 15ml. Two different concentrations of each template DNA (5 and 15ng) were used to eliminate sporadic amplifications products from the data. Fifty-eight 10-mer primers (Operon Technologies) were tested using DNA from isolates of *Metarhizium* spp. Of these 55, 31 were selected for further study (OPA-11; OPD-01, 02, 04, 05, 07, 08, 11, 12, 13, 15, 18, 20; OPE-02, 03, 09, 13, 14, 15, 16, 17, 18, 19; OPN-13; POR-01, 09, 12, 14, 15, 19; OPX-07). Reaction mixture, temperature profile, and visualization of the products were done as described above.

Statistical Analysis. Only well-resolved amplified products were scored from photographs of ethidium bromide stained gels. The presence or absence of a specific band of defined size was recorded. Independent characters were analyzed using NTSYS-pc v.1.8 (Rohlf 1993) by calculating the Jaccard similarity coefficient. The generated pairwise matrix was used to group individuals by the Unweighted Pair Group Method, Arithmetic Average (UPGMA; Sneath & Sokal 1973). For *S. pallens*, further analyses were performed using the RAPDDIP program (Clark & Lanigan 1993) in order to access the genetic diversity within and between geographical populations. Nucleotide diversity represents a percentage of the nucleotide substitution per primer annealing site in the genome, serving as an index to evaluate the genetic diversity within a population. Nucleotide divergence represents a pairwise comparison of the nucleotide diversity values, serving as an index to evaluate de genetic distance among different populations. Nucleotide diversity within population and nucleotide divergence between populations (Nei 1987) were estimated using the following assumptions: polymorphic bands segregate in a Mendelian fashion, same-size fragments in different individuals are allelic and have not arisen through independent mutation, and single nucleotide substitution rather than insertion and deletions are the principal cause of lack of amplification of a particular DNA fragment at a particular primer annealing site.

Results and Discussion

Polymorphism of *S. pallens*. The 10 primers used for analysis produced an average of 15 fragments per primer. Seventy-nine DNA fragments of molecular size varying from 180 to 3000 pb were used as distinct characters for statistical analysis. An example of the results is shown (Fig. 1).

Using these data, a pairwise comparison matrix was generated using the Jaccard similarity coefficient (Fig. 2). The average similarity among individuals within the Northeast and the Central Brazil populations was 54.6 and 60.1%, respectively. However, cluster analysis by UPGMA did not resolve distinct phenetic groups correlating with geographic region (data not shown). Recently, Clark & Lanigan (1993) developed a computer program, RAPDDIP, that estimates genetic parameters derived directly from population information. Using this analysis, values of 2.3 and 2.2% for nucleotide diversity were obtained for the Northeast and Central Brazil

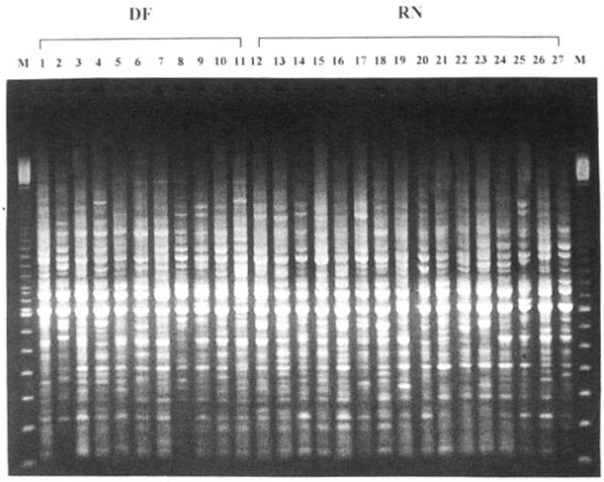

Figure 1. Agarose gel showing DNA amplification products using primer OPE-06 for 27 individuals of *S. pallens.* Abbreviations refer to Brazilian States: DF, Federal District (Central Brazil); RN, Rio Grande do Norte (Northeast).

populations respectively (10% is the maximum possible value according to Clark & Lanigan's method). A nucleotide divergence score of 0.004 (standard error of 0.0007), indicates a high homogeneity between the two grasshopper populations. Thus, it was not possible to characterize individuals belonging to a particular population. Similar intra

1	1.000																									
2	0.577	1.000																								
3	0.444	0.600	1.000																							
4	0.491	0.558	0.604	1.000																						
5	0.491	0.630	0.527	0.574	1.000																					
6	0.534	0.586	0.571	0.534	0.655	1.000																				
7	0.471	0.444	0.434	0.434	0.608	0.600	1.000																			
8	0.448	0.458	0.519	0.456	0.525	0.621	0.580	1.000																		
9	0.643	0.576	0.534	0.552	0.477	0.563	0.474	0.541	1.000																	
10	0.576	0.508	0.492	0.559	0.508	0.569	0.571	0.600	0.625	1.000																
11	0.552	0.559	0.478	0.515	0.571	0.562	0.524	0.521	0.727	0.671	1.000															
12	0.484	0.541	0.500	0.444	0.556	0.619	0.571	0.684	0.530	0.576	0.611	1.000														
13	0.322	0.421	0.453	0.418	0.491	0.561	0.481	0.660	0.452	0.517	0.471	0.569	1.000													
14	0.475	0.508	0.545	0.509	0.632	0.672	0.585	0.717	0.590	0.571	0.542	0.737	0.623	1.000												
15	0.500	0.478	0.557	0.524	0.609	0.672	0.541	0.578	0.574	0.677	0.613	0.627	0.574	0.677	1.000											
16	0.455	0.519	0.453	0.444	0.623	0.630	0.531	0.509	0.533	0.596	0.613	0.618	0.608	0.623	0.774	1.000										
17	0.410	0.517	0.647	0.518	0.484	0.548	0.441	0.579	0.532	0.556	0.571	0.661	0.574	0.632	0.585	0.566	1.000									
18	0.466	0.517	0.474	0.518	0.596	0.600	0.547	0.607	0.548	0.556	0.528	0.590	0.635	0.704	0.594	0.604	0.614	1.000								
19	0.443	0.452	0.509	0.526	0.649	0.581	0.574	0.643	0.500	0.538	0.514	0.587	0.611	0.696	0.641	0.630	0.649	0.755	1.000							
20	0.492	0.525	0.508	0.667	0.590	0.578	0.544	0.532	0.613	0.561	0.620	0.609	0.452	0.607	0.565	0.500	0.565	0.644	0.597	1.000						
21	0.524	0.523	0.508	0.574	0.585	0.574	0.596	0.578	0.588	0.618	0.704	0.603	0.477	0.552	0.583	0.583	0.537	0.522	0.591	0.688	1.000					
22	0.482	0.536	0.473	0.566	0.596	0.593	0.588	0.579	0.548	0.610	0.641	0.590	0.491	0.586	0.639	0.615	0.534	0.552	0.596	0.649	0.818	1.000				
23	0.483	0.508	0.517	0.508	0.574	0.538	0.492	0.567	0.554	0.609	0.652	0.619	0.508	0.617	0.597	0.600	0.600	0.524	0.581	0.578	0.551	0.586	1.000			
24	0.541	0.500	0.508	0.550	0.563	0.576	0.589	0.556	0.705	0.582	0.639	0.606	0.500	0.629	0.657	0.596	0.515	0.571	0.545	0.694	0.708	0.623	0.552	1.000		
25	0.475	0.525	0.459	0.525	0.617	0.578	0.526	0.583	0.563	0.585	0.620	0.635	0.579	0.633	0.662	0.636	0.516	0.524	0.500	0.619	0.636	0.702	0.578	0.750	1.000	
26	0.462	0.463	0.446	0.532	0.569	0.559	0.517	0.493	0.582	0.612	0.714	0.500	0.532	0.493	0.614	0.632	0.569	0.569	0.576	0.574	0.614	0.683	0.606	0.594	0.621	1.000
27	0.492	0.508	0.525	0.593	0.530	0.522	0.452	0.547	0.615	0.646	0.653	0.529	0.516	0.594	0.672	0.567	0.554	0.530	0.585	0.631	0.623	0.655	0.641	0.580	0.606	0.657	1.000

Figure 2. Genetic similarity coefficient matrix for 27 individuals of *S. pallens*, based on 79 PCR fragment patterns obtained from 10 RAPD primers. Values were calculated by Jaccard coefficient. Individuals number 1 to 11 are from Central Brazil (DF) and 12 to 27 are from the Northeast (RN).

and interpopulational genetic variability has been obtained with two other grasshopper species, *Melanoplus sanguinipes* (Fabricius) and *M. femur-rubrum* (De Geer), using RAPD analysis (Chapco *et al.* 1992).

The RAPD technique was successfully used to show polymorphism within *S. pallens* populations. However, it was not possible to fully differentiate between these populations. Further analysis, using a greater number of insects and markers, as well as different populations, will be necessary to confirm these preliminary findings. Knowledge of the genetic variability between populations from different regions may be useful in the establishment of new strategies for the microbial control of this pest. Should a low variability between *S. pallens* populations be confirmed, the efficacy of *M. flavoviride* against any given population would not be expected to differ significantly under similar climatic conditions.

Polymorphism of *M. flavoviride*. The 31 primers used for analysis produced an average of 12 fragments per primer. DNA fragments (388) of molecular size varying from 200 to 3000bp were used as distinct characters for statistical analysis. An example of the fingerprints obtained is shown (Fig. 3).

Cluster analysis, based on similarities calculated from RAPD data, showed a high degree of homogeneity among *M. flavoviride* isolates (Fig. 4). The eight *M. flavoviride* strains analyzed were all isolated from acridid grasshoppers and, despite their origins, they clustered with a very high similarity (≥79.5%). Brazilian isolates (CG288, CG423, CG429, CG430 and CG431) showed even higher similarity (≥98.3%). Besides being from close geographic areas (Northeast Brazil), these isolates were also originally found infecting the same host, *S. pallens*. The Nigerian isolate (CG366), which is being developed as a biopesticide against grasshoppers in Africa (Bateman, 1992), clustered with Brazilian isolates, showing a high level of similarity (93.5%). Isolates from Australia (CG291) and Tanzania (CG442) clustered together with 85.6% similarity, and were found to be ge-

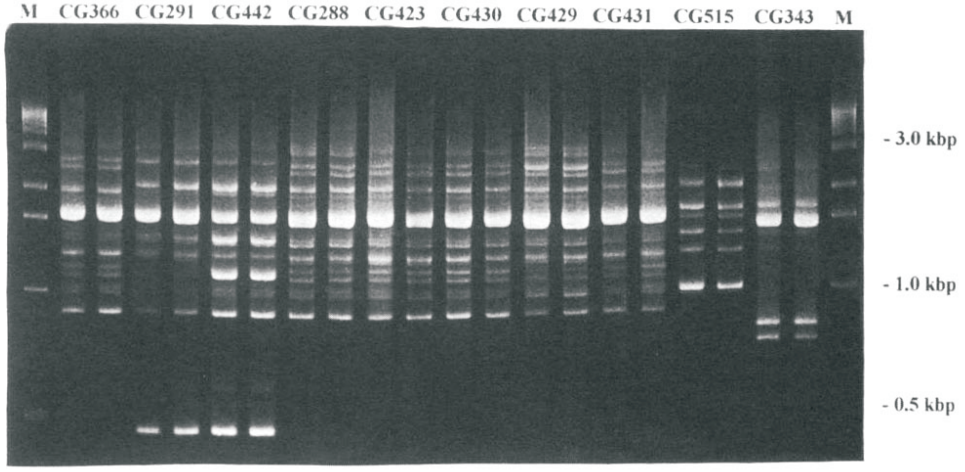

Figure 3. Agarose gel showing DNA amplification products using primer OPE 15 for isolates of *M. flavoviride, M. album* and *M. anisopliae.*

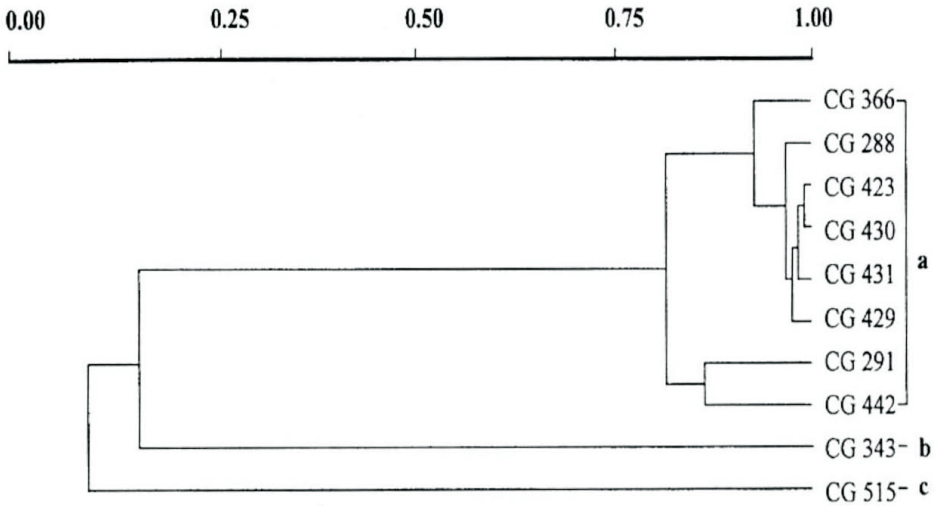

Figure 4. Dendrogram constructed from RAPD data, indicating relationships among isolates of *M.flavoviride* (a), *M. anisopliae* (b) and *M. album* (c). The UPGMA dendrogram was generated from a similarity matrix, calculated by Jaccard coefficient.

netically more distant (81.3% similarity) from the Brazilian and Nigerian isolates.

Low genetic variability of *M. flavoviride* strains obtained from acridid hosts has already been demonstrated by other RAPD analysis (Cobb & Clarkson 1993, Bidochka *et al.* 1994, Tigano-Milani *et al.* 1995, B.P. Magalhães, M. Faria, M.S. Tigano & B.W.S. Sobral, unpublished) and by rDNA sequence analysis (Curran *et al.* 1994). The representatives of outgroups used here, *M. anisopliae* (CG343) and *M. album* (CG515), were very distinct from the *M. flavoviride* isolates, with 16.7 and 10% similarity respectively, making the interspecific differences very clear.

As shown by the results, there was some clustering by geographic area and host. However, the low polymorphism observed among *M. flavoviride* isolates indicates the need to introduce an exogenous marker into the isolate selected as biological control agent. This will be a powerful tool for reliable identification and monitoring of the pathogen in the field.

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