SHORT COMMUNICATION

SIMPLE, RAPID AND ACCURATE PCR-BASED DETECTION OF PANTOEA ANANATIS IN MAIZE, SORGHUM AND DIGITARIA sp.

J.E.F. Figueiredo¹ and L.D. Paccola-Meirelles²

¹ Molecular Biochemistry Laboratory, Embrapa Milho e Sorgo, Rod MG 424, Km 65, Cx. Postal 151, CEP 35701-970, Sete Lagoas, MG, Brazil
² Departamento de Biologia Geral, Universidade Estadual de Londrina, Rod PR 445 Km 380, Cx. Postal 6001, CEP 86051-980, Londrina, PR, Brazil

SUMMARY

Detection of Pantoea ananatis in the early growing season is important for disease prediction and management. We developed a simple molecular marker based on PCR for conclusive diagnosis of P. ananatis in maize, sorghum and Digitaria sp. A pair of primers was used for amplifying only one of the seven internal transcribed spacer (ITS) regions of P. ananatis 16S-23S rRNA genes. Sixty-one strains of P. ananatis from diverse ecological origins; total DNA of pool of maize white spot (MWS) lesions and MWS-like lesions from different sources and P. allii isolated from Allium cepa and the identity of all amplicons was confirmed by DNA sequencing. The present results provide a rapid and reliable tool for the accurate identification of P. ananatis isolates and for direct PCR-based diagnosis of P. ananatis associated with maize, sorghum and Digitaria sp.

Key words: Maize white spot disease, plant pathogen detection, molecular diagnosis, PCR.

Pantoea ananatis (Pa) the causal agent of maize white spot (MWS) disease (Paccola-Meirelles et al., 2001), is becoming a serious problem for maize producers in many countries (Alippi and López, 2010; Krawczyk et al., 2010; Pérez-y-Terrón et al., 2009; Pomini et al., 2007). Pa is also associated with severe economic losses in a broad range of agricultural crops as well as forest tree species worldwide, including honeydew melon (Wells et al., 1987), cantaloupe (Bruton et al., 1991), onion (Gitaitis and Gay, 1997; Gitaitis et al., 2002), sun-dangrass (Azad et al., 2000), eucalyptus (Coutinho et al., 2001), rice (Cortesi and Pizzatti, 2007; Coother et al., 2004), netted melon (Kido et al., 2008) and sorghum (Cota et al., 2010). Pa can be found as an epiphyte, endophyte or pathogen during different life-cycle stages of host plants. Thus, latent Pa infections in the host leaves usually serve as inoculum for epidemic outbreaks (Coutinho and Venter, 2009), the same as Pa living as a saprophyte in plant debris. Detection of initial inoculum in the early growing season is important for disease prediction and management (Huang et al., 2011).

Traditionally, the tentative identification of Pantoea species has been based on different phenotypic characters and biochemical tests. These methods, however, are time consuming and laborious and require specialized taxonomic knowledge, so that the correct identification of bacterial isolates at the species level and the resolution of the taxonomic framework of the genus are difficult (Brady, 2005). Therefore, a rapid and sensitive diagnostic test for Pantoea species is desirable.

Species-specific primers targeting the rRNA genes of Pa recognize also Pantoea stewartii subsp. stewartii, a species closely associated with maize plants (Gitaitis et al., 2002; Walsect et al., 2002). We now describe a simple, rapid, sensitive and accurate PCR-based test for conclusive Pa identification in maize, sorghum and Digitaria sp. which can be performed by any laboratory technician and which does not require taxonomic expertise.

Bacterial isolates from sorghum, pools of MWS lesions from maize and of MWS-like lesions from sorghum and Digitaria sp. were collected from growing plants in the experimental fields of Embrapa Maize and Sorghum at Sete Lagoas (Minas Gerais, Brazil).

Pa isolates of epiphytic or saprophytic origin (maize culture residues), or obtained from MWS lesions of field-grown maize plants and from MWS-like lesions from sorghum and Digitaria sp. were deposited in the microorganism collection of the fungal laboratory of the State University of Londrina (Paraná, Brazil) and identified by morphological, biochemical and rDNA sequencing methods.

FTA cards (Whatman, USA) with preserved DNA
from bacterial cultures of the following reference species: *Pantoea agglomerans* (strains: PNG 06-3, PNG 09-1 and PNG 97-2), *P. ananatis* (strains: PNA 08-2, PNA 97-5 and PNA 99-13), *P. stewartii* (strains: ES 02-1 and ES 02-2) and *P. allii* (strains: Bsf 24, HH 24, BD 380 and BD 390T= LMG 24248T) were kindly provided by Prof. R.D. Gitaitis (University of Georgia, USA).

Genomic DNAs of *P. vagans* (strain: 105T= LMG 24199T), *P. allii* (strain: BD 390T= LMG 24248T), *P. anthophila* (strain: 689T= LMG 25983T), *P. eucalypti* (strain: 76T= LMG 24197T), *P. deleyi* (strain: BD767T= LMG 24280T), *P. rodasii* (strain: 518T= LMG 26293T), *P. rwandensis* (strain: 571T= LMG 26275T) and *P. wallesi* (strain: 682T= LMG 26277T) were kindly provided by Prof. T.A. Coutinho (University of Pretoria, South Africa).

Genomic DNA was extracted from Brazilian bacterial isolates according to Sambrook *et al.* (1989), and total DNA from 0.5 g of a pool of surface-sterilized lesions was extracted by the hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Samples in FTA cards were prepared according to the manufacturer’s instructions (Whatman, USA) for direct use in PCR reactions.

The species-specific forward primer ANAF: 5’-CGT-GAAACTACCCGTGTCTGTTGC-3’, designed by sequence alignments of the ITS region of 16S-23S rRNA genes of *Pantoea* spp. from GenBank, anneals to one of the seven copies of rDNA of *Pa* in a region containing a genomic rearrangement. The reverse universal primer EC5: 5’-TGCCAGGGCATCCACCGTACGCT-3’ (Gürtler and Stanish, 1996) was modified by addition of eight nucleotides at the 3’ end (in bold). The species-specificity of the primers was checked against all DNA sequences deposited in GenBank by the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to estimate PCR products with ANAF and the modified EC5 primers revealed two possible amplicons for the same ITS region of the *Pa* genome (360 bp for strain LMG20103 (De Maayer *et al.*, 2010) or 388 bp for strain AJ13355 (accession No. AP012032.1). In *sìlico*, two amplicons (3487 bp and 4643 bp) were also expected to be amplified with DNA of the non-plant-associated bacterium *Clostridium difficile*, a commensal bacterium of the human intestine (Harvey *et al.*, 2011).

As predicted by Primer-BLAST analysis, 72 PCR products amplified with ANAF and the modified EC5 primers showed a single DNA fragment per sample with approximately the expected size (361 or 389 bp) for *Pa* isolates from maize, sorghum and *Digitaria* sp. and four *Pa* reference strains (Table 1). A single amplicon per sample with 361 bp or 389 bp was also obtained from MWS lesions from maize, MWS-like lesions from sorghum and *Digitaria* sp. and four strains of *P. allii* (Table 1).

*P. allii* (formerly *Pa*) is a new species of *Pantoea* isolated from onion (Brady *et al.*, 2011). In the last decade, members of the genus *Pantoea* isolated from onion (*Allium cepa*) with center rot disease in the USA and onion seeds in South Africa were routinely identified as *Pa* based on phenotypic data (Gitaitis and Gay, 1997; Walcott *et al.*, 2002) and sequence analysis of the 16S rRNA gene (Goszczyńska *et al.*, 2006). Recently, MLSA (multilocus sequence analysis) and AFLP (amplified fragment length polymorphism) analyses have shown that the US/South Africa group of *Pantoea* isolated from onion consists of two different although closely related species: i.e. *Pa* proper and a new species named by *P. allii* (Brady *et al.*, 2011). So far, *P. allii* has been reported only from the USA and South Africa, (Brady *et al.*, 2011). The ability of *P. allii* to use adonitol is a useful phenotypic feature to distinguish it from *Pa* (Brady *et al.*, 2011). Due to insufficient information about the host range and the geographic distribution of this new species and considering the great genetic similarity with *Pa*, the adonitol fermentation test was applied to all bacterial isolates from maize, sorghum and *Digitaria* sp. used in this study and previously identified as *Pa* by biochemical tests and sequencing of 16S and ITS region of 16S-23S rRNA genes. The adonitol test was positive (yellow color) for *Klebsiella pneumoniae* used as positive control.
and negative (red color) for all maize, sorghum and *Digitaria* sp. isolates, reinforcing their identity as *Pa*.

In onion plants, ANAF and EC5 modified primers can be used as a first step to distinguish *P. allii* and *Pa* from other *Pantoea* species, whereas simple biochemical tests (Brady et al., 2011) can differentiate *P. allii* from *Pa*. *Pantoea vagans* is another new *Pantoea* species that causes brown stalk rot in maize (Brady et al., 2009). Although *P. vagans* has been isolated simultaneously with *Pa* from maize in South Africa (Brady et al., 2009), in Brazil *P. vagans* or other *Pantoea* species has not been isolated from MWS lesions, as demonstrated by morphological, biochemical and molecular analysis. In this study, no amplicons were obtained using primers ANAF

### Table 1. Size of PCR amplicons obtained with primers ANAF and EC5 modified for detection of *P. allii* and *P. ananatis* in different sources and ecogeographical regions.

<table>
<thead>
<tr>
<th>Strains and isolates</th>
<th>Bacterial species</th>
<th>Host/Source</th>
<th>Country</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA 97-1</td>
<td><em>P. ananatis</em></td>
<td><em>Allium cepa</em></td>
<td>US</td>
<td>389</td>
</tr>
<tr>
<td>PNA 99-13</td>
<td><em>P. ananatis</em></td>
<td><em>Allium cepa</em> bulbs</td>
<td>US</td>
<td>389</td>
</tr>
<tr>
<td>PNA 200-5</td>
<td><em>P. ananatis</em></td>
<td><em>Allium cepa</em> leaf</td>
<td>US</td>
<td>389</td>
</tr>
<tr>
<td>PNA 08-2</td>
<td><em>P. ananatis</em></td>
<td><em>Allium cepa</em> bulbs</td>
<td>US</td>
<td>389</td>
</tr>
<tr>
<td>LMG 24248&lt;sup&gt;T&lt;/sup&gt; (BD390&lt;sup&gt;T&lt;/sup&gt;)</td>
<td><em>P. allii</em> (formerly <em>P. ananatis</em>)</td>
<td><em>Allium cepa</em> Britex seed</td>
<td>ZA</td>
<td>361</td>
</tr>
<tr>
<td>Bsf 24</td>
<td><em>P. allii</em></td>
<td><em>Allium cepa</em> seeds from bolting plants</td>
<td>US</td>
<td>389</td>
</tr>
<tr>
<td>HH 24</td>
<td><em>P. allii</em></td>
<td><em>Allium cepa</em> seeds from bolting plants</td>
<td>US</td>
<td>361</td>
</tr>
<tr>
<td>BD 380</td>
<td><em>P. allii</em></td>
<td><em>Allium cepa</em> Britex seed</td>
<td>ZA</td>
<td>361</td>
</tr>
<tr>
<td>PR234 to PR237, PR240 to PR246</td>
<td><em>P. ananatis</em></td>
<td><em>Zea mays</em> Epiphytic</td>
<td>BR</td>
<td>389</td>
</tr>
<tr>
<td>PR238, PR239</td>
<td><em>P. ananatis</em></td>
<td><em>Zea mays</em> Epiphytic</td>
<td>BR</td>
<td>361</td>
</tr>
<tr>
<td>PR258 to PR260, PR262, PR264</td>
<td><em>P. ananatis</em></td>
<td><em>Zea mays</em> Crop debris</td>
<td>BR</td>
<td>389</td>
</tr>
<tr>
<td>PR257, PR261</td>
<td><em>P. ananatis</em></td>
<td><em>Zea mays</em> Crop debris</td>
<td>BR</td>
<td>361</td>
</tr>
<tr>
<td>MS281, MS282, MS284, MG286, PR248, PR249, PR251 to PR254, PR271, PR272, PR276, PR279, PR280 PR247, PR250, PR255, PR256, PR270, PR273 to PR275 PR277, PR278, PR283</td>
<td><em>P. ananatis</em></td>
<td><em>Zea mays</em> MWS lesions</td>
<td>BR</td>
<td>389</td>
</tr>
<tr>
<td>PA10121, MG287, GO290</td>
<td><em>P. ananatis</em></td>
<td><em>Sorghum bicolor</em> MWS-like lesions</td>
<td>BR</td>
<td>389</td>
</tr>
<tr>
<td>GO288, GO289, MG285, MG292 to MG297</td>
<td><em>P. ananatis</em></td>
<td><em>Sorghum bicolor</em> MWS-like lesions</td>
<td>BR</td>
<td>389</td>
</tr>
<tr>
<td>PR265, PR266, MG302</td>
<td><em>P. ananatis</em></td>
<td><em>Digitaria sp.</em> MWS-like lesions</td>
<td>BR</td>
<td>389</td>
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</tbody>
</table>

US= United States of America; ZA= South Africa; BR= Brazil. GO, MG, MS, and PR are the Brazilian States of Goiás, Minas Gerais, Mato Grosso do Sul and Paraná, respectively. PA= strain from Minas Gerais State. Pool of MWS and MWS-like lesions were collected in maize and sorghum fields in the Minas Gerais State.
and EC5-modified and DNA samples of \textit{P. vagans}, even when the annealing temperature of primers was reduced to 50°C. This result confirmed our previous analysis performed using the Primer-BLAST program. Besides, an exhaustive analysis of complete genome of \textit{P. vagans}, strain C9-1 (Smits et al., 2010), confirmed the lack of similarity between the nucleotide sequence of the primer ANAF and the genome of \textit{P. vagans}.

The present study demonstrates that ANAF and EC5-modified primers represent a powerful tool for the rapid and dependable identification of \textit{Pa} isolates from maize, sorghum and \textit{Digitaria} sp., thus for a reliable diagnosis.

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REFERENCES


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