

Klebsiella endophytic bacteria control cassava bacterial blight in the eastern Amazon

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ABSTRACT

Cassava bacterial blight (CBB), caused by *Xanthomonas phaseoli* pv. *manihotis*, is one of the most important diseases affecting cassava production worldwide, including regions of Brazil in the eastern Amazon. The use of beneficial microorganisms, such as endophytic plant growth-promoting bacteria, has emerged as an effective tool for controlling diseases in many crops. Here, two *Klebsiella* endophytic isolates (26Y and 29Y) isolated from cassava were evaluated for the control of CBB through antagonistic assays and biological control of the disease in plants inoculated by irrigating the substrate and by foliar spray under greenhouse conditions. The two isolates were able to inhibit the *in vitro* growth of the pathogen, as well as to control the disease severity by at least 90% in plants inoculated by both inoculation methods. We report the first *Klebsiella* strains to control CBB in the eastern Amazon, though their risk assessment for drug-resistance in humans is still pending.

KEYWORDS: antagonistic activity, biological control, *Manihot esculenta*, plant growth-promoting bacteria, *Xanthomonas phaseoli*

Bactérias endofíticas *Klebsiella* controlam a bacteriose da mandioca na Amazônia Oriental

RESUMO

A bacteriose da mandioca, causada por *Xanthomonas phaseoli* pv. *manihotis*, é uma das doenças mais importantes que afetam a produção de mandioca no mundo, incluindo a Amazônia Oriental brasileira. O uso de microrganismos benéficos, como bactérias endofíticas promotoras de crescimento de plantas, é uma ferramenta eficaz no controle de doenças de muitas culturas. Nesse estudo, dois isolados endofíticos de *Klebsiella* (26Y e 29Y) obtidos de mandioca foram avaliados para o controle da bacteriose da mandioca, por meio de ensaios antagônicos e controle biológico da doença em plantas inoculadas por irrigação do substrato e pulverização foliar em condições de casa de vegetação. Os dois isolados inibiram o crescimento *in vitro* do patógeno, e controlaram pelo menos 90% da severidade da doença em plantas inoculadas por ambos métodos de inoculação. Reportamos as primeiras cepas de *Klebsiella* a controlar a bacteriose da mandioca na Amazônia Oriental, embora sua avaliação de risco para resistência a drogas em humanos ainda esteja pendente.

PALAVRAS-CHAVE: atividade antagonista, controle biológico, *Manihot esculenta*, bactérias promotoras de crescimento vegetal, *Xanthomonas phaseoli*

Cassava (*Manihot esculenta* Crantz) is one of the most important crops in Africa, Asia and Latin America and can be infected by *Xanthomonas phaseoli* pv. *manihotis* (previously *Xanthomonas axonopodis* pv. *manihotis*) (Constantin et al. 2016), which causes cassava bacterial blight (CBB), a major disease affecting cassava production worldwide (López and Bernal 2012). In Brazil, CBB occurs in all regions where cassava is cultivated, including the state of Pará, in the eastern Amazon (Ishida et al. 2016). The symptoms of CBB include angular leaf spots, creamy white and later yellow to orange

exudates, blight and wilting. Due to the systemic nature of CBB and the lack of curative methods for its control, the use of resistant cultivars has been the most effective strategy to cope with the disease. However, this resistance is strain-specific, and it can be broken down by sub-groups of pathogens that evade the plant's recognition system (Restrepo et al. 2004; López and Bernal 2012).

The use of beneficial microorganisms for plants has emerged as an effective tool for controlling diseases (Ozaktan et al. 2012; Rahma et al. 2022). Endophytic plant growth-

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promoting bacteria (PGPB) may benefit plant growth by controlling phytopathogens and/or producing bio-stimulating substances, helping plants to cope with stress conditions. Defense against phytopathogens can include the production of antibiotic substances by beneficial bacteria or induced systemic resistance (ISR), when plants primed by beneficial bacteria respond more intensely to pathogen attack (Eid et al. 2021; Zou et al. 2023). Recently, some PGPB were identified from cassava roots, that were able to control soft root rot caused by *Phytophthium* sp., as well as promoting the growth of cassava and cowpea (Ferreira et al. 2021). Our aim here was to further evaluate two of the isolates by Ferreira et al. (2021) for the control of CBB using antagonistic assays and biological control of disease in cassava plants under greenhouse conditions.

We used isolate 29Y (*Klebsiella pneumoniae*, Accession MT845802 in GenBank) and isolate 26Y (with no molecular identification yet), which were promising to control CBB in previous tests. Both isolates were stored at Universidade Federal do Pará (Belém, Pará state, Brazil). We used *Xanthomonas phaseoli* pv. *manihotis* (strain Xam 17), collected in Acará county (Pará, Brazil) and previously selected by pathogenicity test, from the Microbiological Collection at Embrapa Amazônia Oriental (Belém, Pará, Brazil) and a cassava variety susceptible to CBB (accession CPATU 312) from the Cassava Germplasm Bank at Embrapa Amazônia Oriental.

The molecular identification of isolate 26Y, based on the 16S rDNA gene, was performed according to Ferreira et al. (2021), using PCR assays with primers Y1F (5'-tggctcagaac gaacgtctggcggc-3') and Y3R (5'-taccttgttacgacttcacccagtc-3') (Cruz et al. 2001). Nucleotide sequences were compared to sequences available in GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST Program (Altschul et al. 1990).

The antagonistic assay followed Mariano and Souza (2016). The pathogen was cultured in 523 medium (Kado and Heskett 1970) at 28°C for 48 hours, followed by preparation of bacterial suspension in saline solution (NaCl 0,8%) with optical density at 570 nm ($OD_{570} = 0.3$). Then, 100 µL of suspension were spread in fresh Petri dishes, where filter paper discs (8 mm-diameter) were placed in equidistant positions and 10 µL of endophytic suspension $OD_{570nm} = 0.52$ were added (prepared in the same way as the pathogen suspension). Petri dishes were incubated at 28°C for 72 hours. Each filter paper disc containing endophytic suspension was considered one repetition, with four repetitions for each endophytic isolate. As a negative control, we used filter paper discs containing saline solution only.

Biocontrol assays of CBB by the endophytic bacteria were performed in a 3x2 randomized factorial block design with four repetitions per treatment: two endophytic isolates + one disease control containing the pathogen only vs. two

inoculation methods (substrate irrigation and foliar spray). Disease severity was evaluated at 2-day intervals during 14 days after pathogen inoculation, using the injury rating scale by Azevedo (1997). The values of disease severity served as a basis for calculating the area under the disease progress curve (AUDPC) (Shaner and Finney 1977). The data were submitted to analysis of variance, and means were compared by the Scott-Knott test ($p \leq 0.05$) using SISVAR Software (Ferreira 2010). Cassava plants were planted individually in 6-L pots filled with sterile coconut. At 20 days after planting (DAP) each plant was inoculated with 100 mL of suspension of endophytic isolate in saline solution $OD_{570nm} = 0.52$ prepared as described above. Seven days after inoculation with endophytic isolates, plants were inoculated with *X. phaseoli* pv. *manihotis* suspension $OD_{570} = 0.3$ by spraying the leaves until completely soaked. Plants were kept under greenhouse conditions during 14 days after inoculation with the pathogen.

Isolate 26Y was genetically close to *Klebsiella pneumoniae*, accession MG946801.1 from GenBank (99.28% identity with the partial 823-bp 16S rRNA gene sequence of isolate 26Y). The sequence was registered in GenBank under accession OP709764. In the antagonistic assay, 26Y and 29Y inhibited the growth of *X. phaseoli* pv. *manihotis* (Figure 1) with zones of inhibition values of 19.31 ± 0.23 and 8.85 ± 0.13 mm, respectively. By foliar spray, 29Y and 26Y controlled CBB severity by 91.54% and 90%, respectively (Table 1).

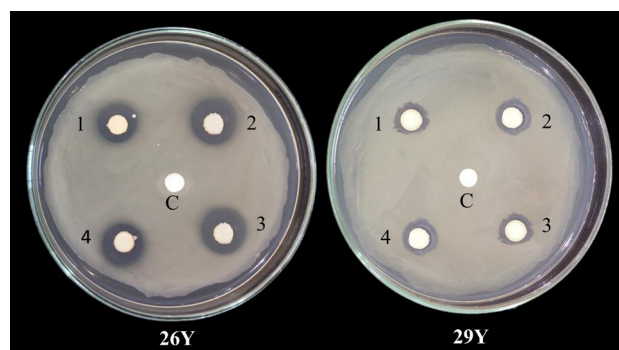


Figure 1. Antagonistic assays of *Klebsiella pneumoniae* endophytic bacteria (strains 26Y and 29Y) against *Xanthomonas phaseoli* pv. *manihotis*. Numbers 1-4 = repetitions for each isolate; C = negative control.

Table 1. Values for AUDPC (area under the disease progress curve) and CBB disease severity in cassava plants inoculated with *Klebsiella pneumoniae* endophytic bacteria (strains 26Y and 29Y) by irrigation of substrate and foliar spray. CV = coefficient of variation.

Treatment	AUDPC		Disease severity (%)	
	Irrigation	Foliar spray	Irrigation	Foliar spray
26Y	1.75 ± 0.13 b	15.18 ± 0.29 b	99.52	90.00
29Y	1.75 ± 0.13 b	12.83 ± 0.21 b	99.52	91.54
Control (pathogen only)	245.00 ± 0.55 a	151.80 ± 0.18 a	0.00	0.00
CV	41.18	11.52		

Values are the mean \pm standard deviation of four replicates. Different letters after the means in the same column indicate significant differences according to a Scott-Knott test ($p \leq 0.05$).

CBB symptoms, such as angular leaf spot and rust, were predominantly observed on the control plants (Figure 2). The lowest AUDPC average was observed in irrigated plants with both 26Y and 29Y (Table 1), producing 99.5% of disease severity control.

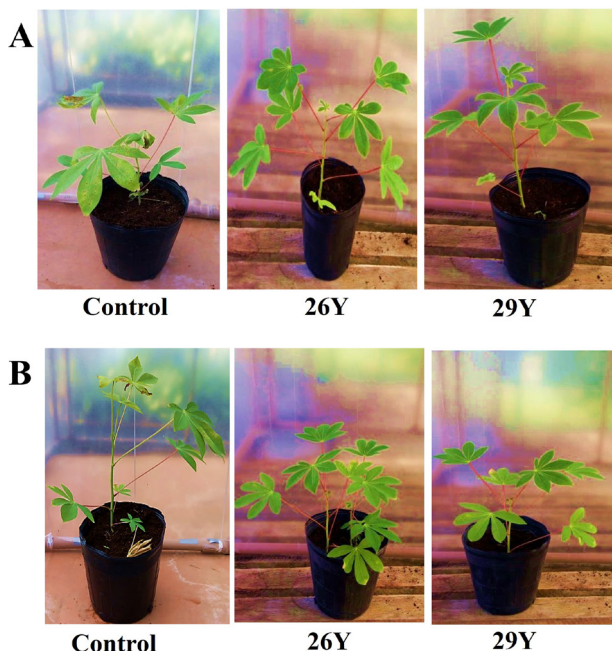


Figure 2. Biocontrol of CBB in cassava plants inoculated with *Klebsiella pneumoniae* endophytic bacteria (strains 26Y and 29Y) by irrigation of substrate (A) and foliar spray (B) under greenhouse conditions. Disease symptoms were evaluated in plants inoculated with endophytes in comparison to the control plant (inoculated with pathogen only) during 14 days after inoculation with the pathogen.

This is the first report on *Klebsiella* endophytic bacteria controlling CBB in the eastern Amazon. Our results for antibiosis effects against *X. phaseoli* pv. *manihotis* agree with Ferreira et al. (2021), who observed that 29Y was able to inhibit the *in vitro* growth of *Phytophthium* sp. Secondary metabolites produced by *K. pneumoniae* strain ST2501 showed inhibitory activity against *Pythium insidiosum* (Wittayapipath et al. 2019). Inhibition of other phytopathogens by *K. pneumoniae* strains has been reported by Dey et al. (2019). Bacteria able to control *Xanthomonas arboricola* pv. *juglandis*, which causes bacterial blight in walnut, produced inhibition zones from 3 to 13 mm (Ozaktan et al. 2012). The difference between the zones of inhibition produced by our *Klebsiella* isolates may be due to the chemical nature of their antagonistic molecules.

Isolates 26Y and 29Y controlled CBB by at least 90% regardless of the inoculation method. Likewise, *K. pneumoniae* HR1 controlled rot root disease in *Vigna mungo* (Dey et al. 2019), and bacterial leaf streak in rice was controlled by *Streptomyces* strains at 81% (Hata et al. 2021). When inoculated via irrigation, colonization by 26Y and 29Y most likely occurred in the cassava roots, with the bacterial effects

spreading later to the other parts of the plant, such as the leaves, where the pathogen was inoculated. This suggests that the endophytes activated the plant's defense system against CBB through ISR. Furthermore, 26Y showed an inhibition zone two times larger than that by 29Y, while in the control of disease severity this difference was not observed, indicating other disease control mechanisms in addition to antagonistic activity. Future studies on genome sequencing will enable the identification of bacterial genes related to the beneficial agronomical properties exhibited by our *Klebsiella* isolates, contributing to the elucidation of the mechanisms by which they control CBB. It is also important to determine the pathogenic potential of strains 26Y and 29Y for animals, as *K. pneumoniae* can be an opportunistic pathogen, also to humans, with significant multi-drug resistance (Aguiar et al. 2020) and its pathogenicity mechanisms may be related to its antagonistic effect against *X. phaseoli*. For example, *K. pneumoniae* 342 has genes related to its endophytic habit as well as to virulence and antibiotic resistance, but with attenuated pathogenicity (Fouts et al. 2008).

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DATA AVAILABILITY

The DNA sequence corresponding to the partial 16S rRNA gene sequence of isolate 26Y was registered in the NCBI GenBank under accession OP709764. The other data that support the findings of this study are not publicly available.

