Journal of General Plant Pathology (2019) 85:424–427
https://doi.org/10.1007/s10327-019-00862-w

BACTERIAL AND PHYTOPLASMA DISEASES

A semi-selective medium to isolate and identify bacteria of the genus *Pantoea*

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Received: 16 November 2018 / Accepted: 22 January 2019 / Published online: 30 May 2019

Abstract

The isolation, purification and accurate diagnosis of *Pantoea* and many other bacterial species that infect rice are essential for upstream studies. However, some isolates of *Pantoea* and other bacteria such as *Sphingomonas* have similar biochemical and morphological features on common culture media and are thus difficult to isolate selectively and accurately diagnose. We thus developed a semi-selective medium containing 65 g/l (65%) NaCl that allows growth of all *Pantoea* strains, but inhibits other microorganisms. It can be used to isolate and purify *Pantoea* spp. for preliminary diagnosis.

Keywords *Pantoea* genus · Rice pathogens · Africa · Semi-selective medium · Disease diagnosis

Numerous pathogens (e.g., nematodes, fungi, viruses and bacteria) cause diseases on rice in Africa and around the world (Séré et al. 2013). Bacteria causing diseases include *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, *Burkholderia* spp., *Pseudomonas* spp., *Sphingomonas* spp., and *Pantoea* spp. (Cui et al. 2016; Duveiller et al. 1988; Kini et al. 2017a, b, c; Poulin et al. 2015; Wonni et al. 2011, 2014; Zhu et al. 2008). The three *Pantoea* species that were reported earlier as rice pathogens in other continents have only recently been described as such in Africa (Kini et al. 2017b, c) and were isolated from rice seeds and leaves in several countries (Dossou and Silue 2018). On certain culture media, *X. oryzae* pathovars and species of *Burkholderia*, *Pseudomonas*, *Sphingomonas* and *Pantoea* have similar phenotypic characteristics (e.g., pigmentation, shape, colony elevation), so biochemical and molecular methods are needed to differentiate them. In addition, seeds and leaves can be co-infected by *Pantoea* spp. and *Sphingomonas* spp. (Afolabi et al. 2014; Dossou and Silue 2018). Consequently, it is difficult to obtain pure colonies of the bacteria from each genus, so that eliminating contamination and making accurate, reliable diagnosis are a challenge. Methods to obtain pure cultures and identify them are needed. Present semi-selective media for fungi and bacteria including *Pantoea* (Mamede et al. 2018) do not discriminate between the bacterial species mentioned above.

The genus *Pantoea* has 27 species, five of which cause damage to several crops (Arnold et al. 2003; Block et al. 1998; Coutinho and Venter 2009; Cruz et al. 2007; De Maayer et al. 2014). *P. ananatis, P. agglomerans* and *P. stewartii* are responsible for more than 80% of the reported cases of plant disease. Because *Pantoea* isolates are more or less halophilic and can establish in environments with varying concentrations of salt (Silini-Cherif et al. 2012; Silvi et al. 2013), this property can be used to develop culture media, such as PA 20 developed by Goszczynska et al. (2006) specific for *P. ananatis*. PA 20 contains 20% NaCl (w/v), crystal violet, and thallium nitrate, with a pH of 8.0 and suppresses the growth of bacteria and fungi (Goszczynska et al. 2006; Norris et al. 1976). The aim of the present study was to develop an easily prepared, inexpensive medium that is specific for the growth and isolation of strains of the genus *Pantoea* using these halophilic properties. Such a medium would facilitate the diagnosis and isolation of *Pantoea* strains collected from different rice plant organs (leaves, stems and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10327-019-00862-w) contains supplementary material, which is available to authorized users.

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seeds) especially when these organs are apparently healthy (e.g., seed).

The new medium we developed contains 1 l of sterile distilled water (pH 7.1 ± 0.2); 65 g NaCl; 0.001 g crystal violet; 8.5 g sodium thiosulfate; 13.5 g agar; 10 g peptone; and 10 g sucrose and prepared as follows:

1. Heat the mixture while stirring until all the constituents have dissolved.
2. Autoclave at 121 °C for 20 min.
3. Allow medium to cool but not solidify. Then pour it into Petri dishes and allow medium to solidify in a laminar flow cabinet.
4. Place the Petri dishes of medium in a plastic bag, seal and store either in a laminar flow cabinet or refrigerator (± 5 °C) until used.

Peptone sucrose agar (PSA) comprises 1 l distilled sterile water, 10 g peptone, 10 g sucrose, 16 g agar, and 1 g glutamic acid, pH adjusted to 7.1 ± 0.2 using 1 M KOH and NaOH buffers. Nutrient agar (NA) contains 1 l distilled sterile water, 0.5% peptone, 0.3% beef extract yeast extract, 1.5% agar, 0.5% NaCl, and 28 g nutrient agar powder; pH adjusted to neutral (6.8) using 1 M KOH and NaOH buffers at 25 °C. Each medium is heated while stirring to fully dissolve all ingredients, autoclaved at 121 °C for 20 min, cooled but not solidified, then poured into Petri dishes.

The selective efficacy of the media was tested using 89 isolates of *Pantoea* species, 23 of *P. agglomerans*, 32 of *P. ananatis*, and 148 of *P. stewartii* (Table 2 in supplemental data file) from the culture collection of the Africa Rice Center (and thus labeled as ARC). They were isolated from rice seeds and leaves in paddy fields or from seed stored in Benin and Togo as described by Kini et al. (2017a, b). In addition, seven reference strains from the French Collection of Phytopathogenic Bacteria (or CFBP, Angers, France), and the following referenced isolates from the Africa Rice strain bank were also used: *X. oryzae* pv. *oryzae* (6), *X. oryzae* pv. *oryzicola* (6), *Sphingomonas* spp. (5), *Baccilus* spp. (9), *Burkholderia* spp. (2) and *Pseudomonas* spp. isolates (2). The characteristics of all the isolates used are given in Table 1 of the supplemental data file. Strains of *Sphingomonas* and *Pantoea* from the AfricaRice strain bank were diagnosed using, respectively, a monoplex and a Multiplex PCR scheme developed by Kini et al. (2017a, 2018).

Selectivity and specificity of the *Pantoea* genus-specific agar (PGSA), was tested by plating the pure isolates and reference isolates mentioned above on the PGSA and the non-selective PSA and NA. The experiments were repeated tree times.

Each rice grain was soaked for 5 min in a solution of sodium hypochlorite (1% w/v) and then ethanol (70% v/v) and later washed 3 times with distilled sterile water for surface sterilization. A metallic forceps, sterilized with alcohol then with a dry bead sterilizer at 250 °C, was then used to place 25 sterilized seed in three circles around the surface of each plate of PGSA, PSA and NA. All seed plating was done in a laminar flow cabinet. Pieces of diseased rice leaves (1 mm^2) were surface-sterilized as described for the seeds, crushed or macerated in distilled sterile water, and the resulting sap was placed on plates of the three media.

Growth of the different bacteria, fungi and other microorganisms on the different media was monitored daily. The phenotypic characteristics of the different colonies of bacteria were then recorded.

Tests with the 301 *Pantoea* strains, including the 13 reference strains (Tables 1 and 2 in the supplemental data file), and 27 isolates of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, *Sphingomonas* spp., *Bacillus* spp., *Burkholderia* spp. and *Pseudomonas* spp., showed that (i) only *Pantoea* strains grew on PGSA (Fig. 1); (ii) bacteria of other genera did not grow on PGSA (Fig. 1); and (iii) no fungi grew on PGSA. Conversely, numerous bacterial and fungal colonies grew on PSA and NA (data not shown). After 48 h, an average of 20 *Pantoea* colonies grew on PGSA and had the characteristics described below but had purple color borders (Fig. 1). After 72 h for PSA and NA and 96 h for PGSA, colonies of *P. ananatis* and *P. agglomerans* were pale yellow, floury and very viscous, and had coalesced. Those of *P. agglomerans* were not floury. For *P. stewartii*, the colonies had coalesced after 48 h on PSA and NA and 72 h on PGSA, but the colonies were not very viscous but sticky. They were light yellow, and the presence of water reduced the color intensity in certain places. On the other hand, an average of 40 beige to yellow colonies grew on PSA and NA, 24 h after plating. These colonies were round, convex and smooth with well-differentiated borders. Most of the other 92 unknown (*Pantoea* sp.) formed colonies on NA, PSA and PGSA in less than 24 h (data not shown).

Pantoea genus-specific agar (PGSA) enabled growth of all isolates belonging to the *Pantoea* genus but not other bacteria and fungi. In fact, the 301 diverse *Pantoea* isolates tested were able to grow on PGSA and on NA and PSA, but their growth and diagnosis on NA and PSA were compromised by the proliferation of fungi and other bacteria (see Tables 1 and 2 in supplemental data file). By contrast, isolates of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, *Sphingomonas*, *Baccilus*, *Burkholderia* and *Pseudomonas* did not form colonies on PGSA but did so on NA and PSA. In addition, fungal colonies grew on PSA and NA but not on PGSA. PGSA is thus specific for the growth of isolates of the genus *Pantoea*. It contains sodium chloride (65% w/v), sodium thiosulfate, and crystal violet and has a pH of 7.1 ± 0.2. The PA 20 medium of Goszczynska et al. (2006) also contains crystal violet and thallium nitrate but inhibits the growth of fungi and other bacteria only at pH 8.0.

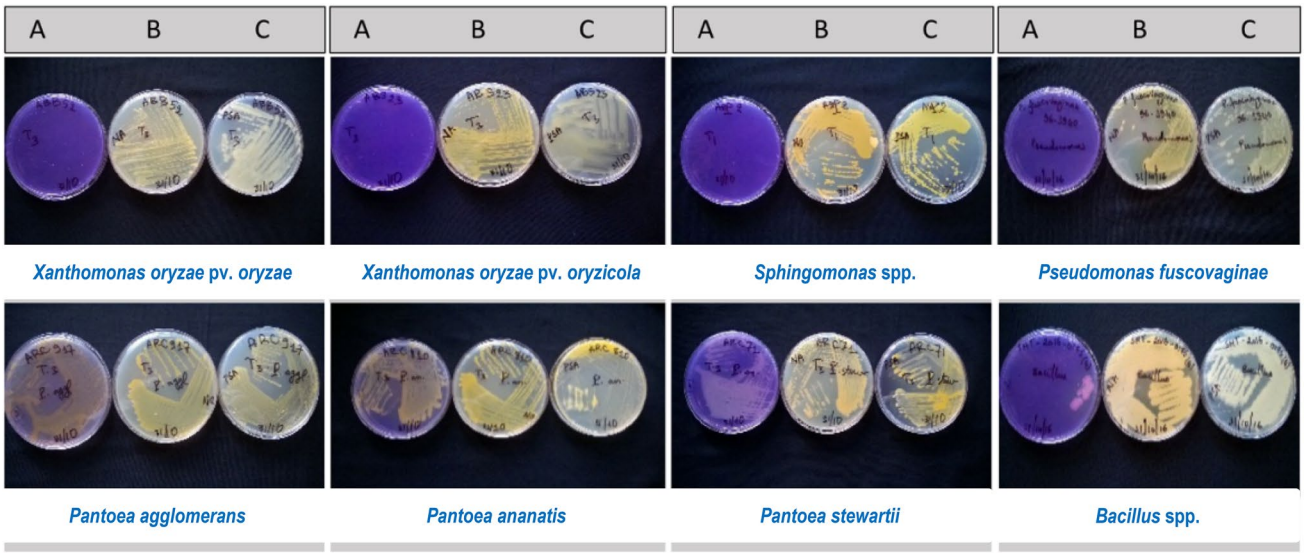

Fig. 1 Bacterial colonies after 10–12 days growth on *Pantoea*-genus-specific agar (A), peptone sucrose agar (PSA) (B) and nutrient agar (NA) (C). *Pantoea ananatis, P. stewartii* and *P. agglomerans* grew well on all media, whereas *Xanthomonas, Sphingomonas, Bacillus,* and *Pseudomonas* and fungi were inhibited by the selective medium but grew well on PSA and NA

Therefore, the role of pH in the selective efficacy of the PGSA medium needs to be further investigated.

Crystal violet and NaCl (65% w/v) in PGSA probably contributed to the lack of growth of other bacteria and fungi as suggested by Goszczynska et al. (2006) and Norris et al. (1976). In fact, Goszczynska et al. (2006) mentioned that the combination of D-arabitol, pH, NaCl concentration and thallium nitrate contributed to the selectivity of PA 20. In addition, the unwanted fungi and bacteria likely did not have the halophilic properties of *Pantoea* spp., which would explain their inhibition on PGSA. It is unclear, however, whether the halophilic properties of the *Pantoea* species are responsible for the successful isolation of *Pantoea* strains using PA 20 or PGSA. Further investigations are therefore needed.

Pantoea genus-specific agar will complement PA 20 for diagnosing *Pantoea* species and is not meant to be used alone. It can be used as a preliminary diagnostic tool that complements existing cultural, biochemical and molecular diagnostic methods (Kini et al. 2017a, b, 2018). It will facilitate the isolation of strains collected on seeds or from sap from crushed leaves.

In conclusion, the newly developed PGSA medium can be used to purify isolates contaminated by other bacteria such as *Sphingomonas* spp. It requires only a few affordable ingredients and can be easily prepared in less-equipped laboratories, including those of Plant Quarantine Services in Africa. It is will be particularly useful for preliminary diagnostic tests when *Pantoea* spp. are suspected, especially on seeds. However, it cannot replace further testing of other characteristics, including pathogenicity of any isolates obtained. In fact, some of these *Pantoea* species might not be pathogenic to crops. Thus, the usual procedures for handling microbial cultures should be strictly respected.

Acknowledgements Dr. Kossi Kini benefited from a PhD scholarship from the French Research Institute for Sustainable Development (IRD) within the programme "Allocation de Recherche pour une Thèse au Sud" (ARTS), a grant from the International Foundation for Science (IFS), and financial support from the Ministry of Foreign Affairs (Japan) and the Global Rice Science Partnership (GRiSP) through AfricaRice. All these institutions are gratefully acknowledged. We thank Toyin Afolabi (AfricaRice, Cotonou, Benin), Sandrine Fabre and Florence Auguy (IRD, CIRAD, University of Montpellier, IPME, Montpellier, France) for excellent technical support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human/animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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