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Rice pathogens intercepted on seeds originating from 11 African countries and from the USA

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Abstract

1,916 rice seed samples from 11 African countries and the USA were tested for the presence of pathogenic microorganisms or those affecting seed quality. *Bacillus* spp., *Pantoea* spp., *Sphingomonas* sp. and the fungi *Acremoniella* sp., *Alternaria* sp., *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Chaetomium* sp., *Curvularia* sp., *Fusarium oxysporum*, *F. solani*, *Fusarium* sp., *Helminthosporium* sp., *Microdochium oryzae*, *Nigrospora oryzae*, *Penicillium* sp., *Pestalotia* sp., *Phoma* sp., *Magnaporthe oryzae*, *Rhizopus* sp., *Sarocladium* sp. and *Tilletia barclayana* were isolated. The highest incidence values were obtained with *Curvularia* sp., *Microdochium oryzae*, *A. flavus, F. solani* and *Nigropora* sp. In contrast, these fungi were not isolated from seeds of many countries with Togo having the least affected seeds (nine out of the 24 potential organisms diagnosed). The highest frequencies of these organisms were found on seed samples from Benin (20/22), Burundi and Tanzania (19/24), and Senegal (18/24). Across countries, *A. flavus*, *A. fumigatus*, *Curvularia* sp., *F. solani*, *Nigropora* sp., *Rhizopus* sp. and *Microdochium oryzae* were the most frequently isolated organisms. Concerning the major diseases, blast was diagnosed only once despite the high number of samples tested.

Keywords: biosafety, pathogen detection, plant quarantine, rice pathogens, seed movement

Introduction

Rice is a monocot plant with two main cultivated species *Oryza sativa* L. and *O. glaberrima* Steud, also respectively known as Asian and African rice. It is the most widely consumed cereal and the staple food in a large part of the world including Asia and Sub-Saharan Africa (SSA). Despite the 51% increase in production over the period of 2000 to 2010, rice consumption has also increased faster than that of any other staple food in Africa, at about 5.5% per year (Seck *et al*., 2013). However, yields in SSA are still relatively low [2.7 t ha^{-1} compared with > 4.6 t ha^{-1} in Asia and developing countries (FAOSTAT, 2014)] and production is not able to meet the growing demand. Consequently, most African countries import 50-99% of their rice (Seck *et al.*, 2013). Analysis of the seed systems in the Southern African Development Community (SADC) and the Economic Community of West African States (ECOWAS) revealed several challenges, including the lack of

operational infrastructural facilities and equipment for implementing seed health testing according to international standards to prevent the entry and dissemination of quarantine pests (Diallo, 2016).

Rice seed movement across countries is a matter of concern as the crop is attacked by several fungal, bacterial, nematode and viral pathogens, with some being seed-borne. Those microorganisms associated with rice seeds can be classified into two categories, those causing characteristic disease symptoms and those that do not normally cause any disease but affect grain quality during storage or seed germination, or cause seed rot. Among the pathogens found on or in seeds, bacterial leaf blight [BLB; *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings], bacterial leaf streak [BLS; *X. oryzae* pv. *oryzicola* (Fang) Swings], rice blast [*Magnaporthe oryzae* (Couch)], brown spot [*Cochliobolus miyabeanus* (Ito &. Kurib.) Drechsler ex Dastur) *anamorph Bipolaris oryzae*), leaf scald [*Monographella albescens* (Hashiola & Yakogi) Samuels & Hallett) anamorph *Rhynchosporium oryzae*] and narrow brown spot [*Sphaerulina oryzina* (Hara) anamorph *Cercospora oryzae*]. Because the common seed regulations are not yet operational in SADC as well as in ECOWAS countries (Kuhlman, 2012; Diallo, 2016; Kawonga, 2016), seed health testing must be done in each importing/exporting country to minimise the dissemination of pathogens via seeds.

Investigations on rice seed pathogens are numerous. Ou (1985) reported 56 pathogens on rice seeds of which 41 were seed-borne. Sevilla and Mamicpic (1988) reported 70 rice seed-borne fungi in the Philippines and Archana and Prakash (2013) isolated 27 fungal species on samples collected in different Indian states, the predominating fungi isolated being *B. oryzae* (82.1%) and *Alternaria padwickii* (63.4%). In Pakistan, infection by the same fungus ranged from 17 to 83.3% (Habib *et al.*, 2012; Ashfaq *et al*., 2015). In Nigeria, Imolehin (1983), Aluko (1988) and Ibiam *et al.* (2008) found a number of fungi on rice seeds, including *Helminthosporium oryzae*, *Fusarium moniliforme*, *F. oxysporum*, *Penicillium* sp., *Curvularia lunata*, *Aspergillus* sp., *Alternaria terreus*, *Alternaria* sp., *Chaetomium globosum*, *Rhizopus arrhizus*, *Geotrichum* sp., with *H. oryzae, Aspergillus sp., F. moniliforme* and *R. arrhizus* being the predominant fungi that negatively affected seed germination. In Ghana, the testing of seven rice samples revealed low infection by *Bipolaris oryzae*, *Michrodochium oryzae* and *Sarocladium oryzae* with, respectively, 0.9, 0.7 and 0.5% incidence (Nutsugah *et al.*, 2004). Danquah *et al.* (1976) examined normal-looking and discoloured seeds from Ghana and diagnosed several fungal pathogens including *Curvularia* spp., *Cladosporium cladosporioides*, *Drechslera oryzae*, *Fusarium semitectum*, *Myrothecium* sp., *Nigrospora oryzae*, *Pyricularia oryzae* and *Verticillium* sp. on both types of seeds. The authors also reported that germination of discoloured seeds was affected.

For bacteria, Cottyn *et al.* (2001) detected 27 bacterial species from seeds collected in 1995 in the Philippines. In Tanzania, *Acidovorax avenae* subsp. *avenae* was diagnosed in 60% of the samples indicating that the bacterium was wide-spread in the country (Kihupi *et al.*, 1999). Ashura *et al.* (1999) confirmed the presence of *A. avenae* subsp. *avenae* in 63% of the samples but also diagnosed *Pantoea agglomerans* (causing palea browning), *Xanthomonas oryzae* pv. *oryzae* (causing bacterial leaf blight) and *Burkholderia glumae* (causing grain rot).

The Breeding Task Force led by AfricaRice is exchanging seeds with more than 28 African countries in and outside SADC and ECOWAS where advanced lines are tested for yield and adaptation. In addition, the AfricaRice genebank is shipping 8-10,000 seed samples each year to countries around the world and is receiving 4-5,000 samples each year from collaborators. AfricaRice has therefore set up a Plant Quarantine Unit at Cotonou, Benin to check the phytosanitary status of all incoming and outgoing seeds. This paper reports on the microorganisms intercepted on 1,916 accessions which is a subset of the rice accessions analysed between 2013 and 2016. The importance of the results obtained for the seed health and Plant Quarantine Services is discussed.

Materials and methods

Characteristics of the samples
Seeds of 1,916 lowland and upland rice accessions meant for distribution to partners or storage in the genebank and originating from 11 SSA countries namely Benin (393), Burundi (148), Cameroon (53), Central African Republic (CAR; 20), Democratic Republic of Congo (DRC; 138), Cote d'Ivoire (1), Mali (20), Nigeria (103), Senegal (608), Tanzania (302) and Togo (69), and from the USA (61) were tested for their health. The characteristics (e.g. year of production, season, ecology) of all those samples were not always available and thus not compiled.

Handling of the samples and microorganism identification
Twenty-five of both asymptomatic and symptomatic seeds for each seed sample were randomly selected and surface-sterilised in a 10% sodium hypochlorite solution for two minutes, rinsed in sterile distilled water then dried on sterilised Wattaman paper. The seeds were then plated on nutrient broth yeast extract agar (NBY: 8 g nutrient broth, 2 g yeast extract, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 2 g glucose, 15 g agar powder and 1000 ml distilled sterile water) in 90 mm-diameter Petri dishes. The plated Petri dishes were incubated at 25°C with 12 hours fluorescent light and 12 hours darkness per day. After 5-7 days, conidia and mycelium of all cultures were mounted on glass slides in water and examined under a microscope. Fungal identification was done using the identification keys of Barnett and Hunter (1972). Bacterial strains were enumerated after sub-culturing of single colonies on peptone sucrose agar (PSA) medium (10 g peptone, 10 g sucrose, 1 g glutamic acid, 15 g agar powder and 1000 ml sterile distilled water). This sub-culturing was done by streaking single bacterial colonies on new plates for isolating pure strains. Plates were then incubated at 28°C for 24 to 48 hours. Cultures that produced colonies were purified and maintained at -20°C in liquid PSA with 15% glycerol until further identification to genus and species. For this purpose, the following methodologies were applied:

DNA Extraction
For DNA extraction, bacteria strains were grown overnight in nutrient broth with shaking at 150 rpm and incubated at 28°C. Then, the method described by Wonni *et al.* (2014) was used to extract the DNA.

Multiplex PCR

DNA or pure cultures were then subjected to a diagnostic multiplex polymerase chain reaction (PCR) amplification method described by Lang *et al.* (2010) and that is diagnostic for *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *X. oryzae* pv. *oryzicola* (Xoc). The reference isolates ABB 1 (Afolabi *et al*., 2015) and Ug 1 (Afolabi *et al*., 2014) were respectively used for Xoo and Xoc PCR diagnosis. For *Pantoea* species, the methods of Brady *et al.* (2008) and Kini *et al.* (2016a, b) were used along with the described primers and control isolates ARC 22, ARC 570 and ARC 570 described by Kini *et al.* (2016a, b). For the identification of *Sphingomonas* species which are pathogenic to rice (Kini *et al*., 2017), the primers and the protocols described Buonaurio *et al.* (2001) and control isolate ASP 3 (Kini *et al*., 2017) were used. In addition, some unknown cream-coloured bacteria cultures were amplified using the bacteria 16S rRNA universal primers (27F AGAGTTTGATCCTGGCTCAG and 1525R AAGGAGGTGWTCCARCC) and the PCR products were purified using (QIAGEN QIAquick PCR purification kit) as described by the manufacturer. They were further analysed by sequencing and analysing the 16S rRNA gene using the BLASTn and the standard approximation likelihood ratio (Dereeper *et al*., 2008).

Incidence calculation and total occurrence of the target microorganisms in the countries

The incidence of a given microorganism on the tested accessions originating from a given country was calculated by dividing the number of affected accessions by the total number of the tested accessions originating from this country and multiplying the obtained ratio by 100. For the determining the occurrence of these microorganisms in the target countries termed as ‘’total occurrence’’ in the text, all identified microorganisms were summed and captured against the total diagnosed (table 1).

Results

Bacteria

Results obtained on all 1,916 seed samples are presented in table 1. The first important group of prevailing bacteria is *Bacillus* spp. that were diagnosed on seeds from all 11 countries, with incidences varying from 5 to 95%. Seeds from Mali had the highest incidence (95%). *Bacillus* spp. was not found in samples originating from the USA. All samples except those from DRC, where *Pantoea* spp. was not diagnosed, had varying incidences (for the two other groups of bacteria namely *Pantoea spp.* and *Sphingomonas* spp. Incidence varied from 1.89 (Cameroon) to 26.97% (Benin) for *Pantoea* spp. and 0.72 (DRC) to 40.98% (USA) for *Sphingomonas* spp. *Bacillus* sp., *Pantoea* spp. and *Sphingomonas* spp. were common and widespread at respectively over 50% in seven countries, over 10% in three countries and over 10% in five countries.

Several other bacteria were present on the seeds of seven countries (Benin, Burundi, Cameroon, DRC, Senegal, Tanzania and USA), but were not identified. Their incidences are shown in Table 1 and varied from 8.8 (Burundi) to 94.3 (Cameroon) and 99.3% (DRC). Samples from the USA showed 65.6% incidence while those from Senegal and Tanzania

Table 1. Incidences of the different microorganisms isolated from seeds of 1916 accessions originating from Benin (393), Burundi (148), Cameroon (53), Central African Republic (CAR; 20), Democratic Republic of Congo (DRC; 138), Cote d'Ivoire (1), Mali (20), Nigeria (103), Senegal (608), Tanzania (302), Togo (69) and USA (61).

Microorganisms	Incidence by country (%)											
	Benin	Burundi	Cameroon	CAR	DRC	Cote d'Ivoire	Mali	Nigeria	Senegal	Tanzania	Togo	USA
Bacteria												
Bacillus spp.	13.99	93.92	26.42	90.00	5.07	100.00	95.00	85.44	65.79	64.24	86.96	0.00
Pantoea spp.	26.97	4.05	1.89	5.00	0.00	100.00	5.00	11.65	8.88	10.93	5.80	3.28
Sphingomonas spp.	18.58	6.76	1.89	5.00	0.72	100.00	5.00	9.71	11.02	15.56	11.59	40.98
Other	66.67	8.78	94.34	0.00	99.28	0.00	0.00	0.00	18.91	18.54	0.00	65.57
Fungi												
Acremoniella sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00
Alternaria sp.	0.25	2.03	0.00	0.00	0.00	0.00	0.00	0.00	4.61	0.66	0.00	0.00
Aspergillus flavus	20.10	8.11	39.62	25.00	24.64	0.00	5.00	10.68	30.26	12.91	0.00	1.64
Aspergillus fumigatus	1.53	1.35	5.66	0.00	9.42	0.00	0.00	0.00	0.16	3.64	0.00	0.00
Aspergillus niger	1.27	1.35	7.55	5.00	5.80	0.00	0.00	6.80	16.94	5.30	0.00	0.00
Chaetomium sp.	3.05	0.68	0.00	5.00	4.35	0.00	0.00	0.00	0.00	6.29	0.00	8.20
Curvularia sp.	19.59	6.08	22.64	0.00	1.45	100.00	75.00	2.91	28.95	41.72	2.90	62.30
Fusarium oxysporum	3.05	0.68	5.66	0.00	0.72	0.00	20.00	0.97	6.09	1.99	2.90	3.28
Fusarium solani	6.36	8.11	7.55	0.00	0.72	100.00	35.00	1.94	12.50	7.62	10.14	21.31
Fusarium spp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.35	0.99	0.00	0.00
Helminthosporium sp.	0.25	6.08	1.89	0.00	3.62	100.00	0.00	0.00	0.16	7.62	0.00	1.64
Microdochium oryzae	1.27	3.38	0.00	45.00	0.00	0.00	15.00	0.00	0.00	0.66	2.90	0.00
Nigropora sp.	7.89	27.70	3.77	0.00	1.45	100.00	0.00	0.00	0.99	2.32	0.00	0.00
Penicillium sp.	0.76	0.68	13.21	20.00	0.72	0.00	0.00	1.94	1.64	0.00	1.45	4.92
Pestalotia sp.	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phoma sp.	1.78	1.35	0.00	0.00	0.00	0.00	15.00	0.00	0.00	2.32	0.00	8.20
Magnaporthe oryzae	0.25	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhizopus sp.	0.25	2.03	3.77	5.00	3.62	0.00	0.00	27.18	4.77	0.33	0.00	0.00
Sarocladium sp.	2.80	0.00	1.89	5.00	5.80	0.00	15.00	0.00	0.16	3.64	14.49	0.00
Tilletia barclayana	0.00	0.00	0.00	0.00	0.72	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total occurrence	20/24	19/24	15/24	10/24	16/24	7/24	10/24	10/24	18/24	19/24	9/24	11/24

had respectively 18.9 and 18.5% incidence. The unique sample from Cote d'Ivoire was affected by only *Pantoea* spp., *Sphingomonas* spp. and *Bacillus* spp.

Fungi
Twenty fungal species belonging to 18 genera were identified: *Acremoniella* sp., *Alternaria* sp., *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Chaetomium* sp., *Curvularia* sp., *Fusarium oxysporum*, *F. solani*, *Fusarium* sp., *Helminthosporium* sp., *Microdochium oryzae*, *Nigrospora oryzae*, *Penicillium* sp., *Pestalotia* sp., *Phoma* sp., *Magnaporthe oryzae*, *Rhizopus* sp., *Sarocladium* sp. and *Tilletia barclayana.* The highest incidence values were obtained for *Curvularia* sp. (75% in the samples from Mali), *Microdochium oryzae* (45% in the samples from CAR), *A. flavus* (39.62% in the samples from Cameroon), *F. solani* (35% in the samples from Mali) and *Nigropora* sp. (27.7% in the samples from Burundi) and *Rhizopus* sp. with 27.18% for the Nigerian samples (table 1). Some of these fungi were not isolated from seeds from many other countries including CAR, Mali, Nigeria and Togo for *Helminthosporium* sp., CAR for *Curvularia* sp and Cameroon, CAR, DRC, Cote d'Ivoire, Mali, Nigeria, Togo and the USA for *Alternaria* sp. The unique sample from Cote d'Ivoire was affected with *Curvularia* sp., *F. solani*, *Helminthosporium* sp., *Nigropora* sp. and *A. flavus* (table 1).

Total occurrence
Togo had the least affected seeds with only nine out of the 24 microorganisms found on the seeds tested (table 1). The highest occurrence values of these potential organisms were found on seeds originating from Benin (20/22), Burundi (19/24), Senegal (18/24) and Tanzania (19/24). Among the big three major diseases, BLB and BLS were not diagnosed despite the high number (1,916) of sample tested. Blast was diagnosed in only one of the 393 samples from Benin. Pathogens of medium importance (e.g. *Cochliobulus* sp., *Alternaria oryzae*) and those affecting seed germination (e.g. *Penicillium* sp., *Aspergillus* sp.) were also diagnosed.

Discussion

The first important group of bacteria found on the rice seeds tested in this study was *Bacillus* spp. Cottyn *et al.* (2001) reported upto 22% incidence of *Bacillus* spp. on seeds originating from the Philippines. Some of these bacteria might not be pathogenic to rice but their presence on the seeds is known to negatively affect seed germination (Cottyn *et al*., 2001). No satisfactory explanation regarding the high occurrence of these bacteria on the seeds can be drawn but this could be due to the polluted environment where these seeds were produced, processed and stored.

Pantoea spp. and *Sphingomonas* spp. were diagnosed in most samples except those from DRC in which only *Sphingomonas* spp. were not detected. This diagnosis was possible after partial DNA sequence analysis and comparison with reference sequences deposited in genebanks. These two bacteria were reported to be associated with seeds (Ashura *et al.*, 1999; Cottyn *et al.*, 2001), but using the stem inoculation method, Ashura

et al. (1999) were not able to elucidate the pathogenicity of the *Pantoea* strains they isolated. Both bacteria have several known species but so far only *P. ananatis* and *P. agglomerans* have been reported to cause blight diseases on rice. *P. ananatis* was reported to be pathogenic to rice in Italy (Cortesi and Pizzatti, 2007), China (Yan *et al.*, 2010), Cambodia (Cother *et al.*, 2010), India (Mondal *et al.*, 2011) and Russia (Egorova *et al.*, 2015). In contrast, *P. agglomerans* was only reported as a rice pathogen in Korea (Lee and Hong, 2010) and Venezuela (González *et al.*, 2015). Recent investigations in our laboratory also indicated for the first time in Africa that some species originating from eleven African countries namely *P. ananatis*, *P. stewartii* (Kini *et al.*, 2016a, b), *P. agglomerans* (Kini *et al.*, unpublished) and *Sphingomonas* spp. from several African countries are pathogenic to rice (Kini *et al.*, 2017). These three diseases that are new to Africa pose plant quarantine concerns for the continent because of their association with seeds (Oyaizu-Masuchi, 1988; Ming *et al.*, 1991; Ashura *et al.*, 1999) and because of the intense seed distribution activity implemented by the AfricaRice Breeding Task Force. These new diseases require special plant quarantine measures to prevent their dispersal through seeds.

Most of the mycota diagnosed in the present study were also isolated by several authors in Africa (Danquah *et al.*, 1976; Imolehin, 1983; Natsugah *et al.*, 2004) and elsewhere (Archana and Prakash, 2013; Islam and Borthakur, 2012). Information on the seasons during which those seeds were produced, their drying and conservation were not available but if available could provide clues on these high seed infections incidences.

Across countries, *A. flavus*, *A. fumigatus*, *Curvularia* sp., *F. solani*, *Nigropora* sp., *Rhizopus* sp. and *Microdochium oryzae* were the most frequently isolated organisms (table 1). Fungi that were absent or rarely isolated from seed samples were *T. barclayana* (present only in DRC on 0.72% of the samples), the unidentified *Fusarium* species present only on seeds of Senegal and Tanzania at respectively 11.35 and 0.99% incidence, *Chaetomium* sp. present only in six countries (Benin, Burundi, Central African Republic, DRC, Tanzania and the USA) with low incidences ranging from 0.68 and 8.20%.

In this work, the seeds tested had 24 potential organisms. Togo had the least affected seeds with only nine out of these 24 microorganisms found on the seeds tested. In contrast, Benin had 20, Burundi and Tanzania 19, Senegal 18 and DRC 16.

Except for blast diagnosed in only one of the 393 Beninese samples, BLB and BLS were not diagnosed despite the high number (1,916) of samples tested. In Africa, only Danquah *et al.* (1976) and Afouda *et al.* (2010) documented blast on Ghanaian and Beninese rice seeds at 14-28 and 4-15.75% incidence, respectively. Other scientists reported this fungus elsewhere on seeds and Archana and Prakash (2013) reported an incidence of up to 13% while Manandhar *et al.* (1998) reported 5.0-31.0%. Whether the environments where the tested seeds were produced was not conducive for the disease to occur on seeds is not known. As RYMV found in seeds is not infectious (Konate *et al.*, 2001), we did not therefore check for its presence in the tested seeds although its detection is possible with serological and molecular techniques.

The ultimate goal of the seed health testing conducted at AfricaRice is to diagnose the pathogenic microorganisms associated with seeds that may present important threats to rice production through seed movement. In fact, if virulent pathogens are moved from one

country to another, they can induce severe attacks and yield losses in the new locations where they were absent. Investigations are currently ongoing to identity products that can disinfect all microorganisms found on seeds.

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