Australian Journal of Crop Science

AJCS

AJCS 13(02):309-314 (2019) ISSN:1835-2707
doi: 10.21475/ajcs.19.13.02.p1474

Reduction of initial occurrence of rice blast (*Pyricularia oryzae*) inocula on seeds by microbial and hot water seed treatments

Ibrahim Hashim[1, 2,] *, Delphine Mamiro[2], Robert B. Mabagala[2], Tadele Tefera [3]

[1]International Center of Insect Physiology and Ecology (ICIPE), P.O. Box 30772-00100 Nairobi, Kenya
[2]Sokoine University of Agriculture (SUA), Department of Crop Science and Horticulture, P. O. Box 3005, Morogoro, Tanzania
[3]International Center of Insect Physiology & Ecology, ILRI Campus, Gurd Shola, P.O Box 5689, Addis Ababa, Ethiopia

***Corresponding author: ibrahimmhashim@gmail.com; ihashim@icipe.org**

Abstract

Rice blast disease resulting from infected rice seed can be avoided by using treated seeds. Seed treatment using chemical fungicide has many limitations such as development of resistance to pathogens, damage to the natural environment and the health of farmers and consumers. Such limitations have raised the need for alternative non-chemical seed treatment methods such as antagonist microbial agents and hot water. Laboratory and the screen house experiments were carried out with the aim of evaluating the efficacy of *Trichoderma asperellum, Bacillus subtilis* and hot water (50°C/15 min) seed treatments against rice blast disease inocula on rice seeds. The results showed that, seeds treated with microbial agents (*T. asperellum, B. subtilis)* and hot water reduced the percentage of infected rice seeds by 4.3 to 52.7% relative to non-treated seeds. The germination percent and seedling vigour index increased from 12.3 % to 17.1 % . Rice seeds treated with *B. subtilis* reduced the incidence and severity of rice blast disease from 10% to 72.4 %. Seed treatment using *B. subtilis* followed by *T. asperellum* were the best in reducing the number of infected seeds and rice blast disease incidence and severity on rice seedlings. Therefore, the use of these microbial agents has a potential for effective management of rice blast disease.

Key words: *Bacillus subtilis*, hot water, *Pyricularia oryzae,* Seed treatment, *Trichoderma asperellum.*
Abbreviations: RBD_ rice blast disease, PDA_ potato dextrose agar, CRD_ completely randomized design, Vi _ vigor index, RL_ root length, SL_ shoot length, GP_ germination percentage.

Introduction

Rice is the second crop after maize in world production (FAOSTAT, 2014). It is a staple food security crop of economic importance in Asian and many African countries (FAO, 2017). Some of the diseases affecting rice production include rice blast disease (RBD) caused by *Pyricularia oryzae* Cav. The disease is the main constraint to rice production worldwide (Kihoro et al., 2013; Aravindan et al., 2016; Mgonja et al., 2016). In the absence of control measures and where susceptible cultivars are grown, *P. oryzae* can cause yield losses ranging from 60% to 100% (Kihoro et al., 2013; Aravindan et al., 2016). When *P. oryzae* infects rice plants in the field at the reproductive stage (flowering and mature panicles) infested rice seeds are produce (Faivre-Rampant, 2013). When used for planting infected seeds act as the primary source of inocula resulting into poor germination and abnormal seedlings (Imolehin, 1983; Webster, 2000; Long et al., 2001; Faivre-Rampant, 2013). Spores of *P. oryzae* produced on contaminated rice seeds are transmitted to newly formed leaves and roots during seed germination (Faivre-Rampant, 2013). Rice blast disease resulting from infested and infected rice seeds can be avoided by using treated seeds. Currently, seed treatment using chemical fungicide has resulted into many limitations such as development of resistance to pathogens, damage to the natural environment as well as the health of farmers and consumers (Burgess, 1997; Birgit Jensen, 2000). Such effects have raised the need for alternative non-chemical seed treatment methods such as antagonist microbial agent and hot water. Hot water seed treatment has been used successfully on different vegetable crops against different pathogens (Nega *et al.*, 2003; du Toit and Hernandez-Perez, 2005; Mtui et al., 2010; Koch and Roberts, 2014). Microbial seed treatment has been reported to control seed-borne pathogens and provide protection against soil-borne pathogens (Chung et al., 2008; Raaijmakers et al., 2009; Goudjal et al., 2014). However, there are few reports showing application of hot water and microbial seed treatment for management of RBD (Faruq et al., 2015). Several studies on alternative management methods of RBD have focused mainly on folia application of microbial agents (Jayaraj et al., 2004; Kumar et al., 2012; Singh et al., 2012). The present study therefore was undertaken in order to test the efficacy of *Trichoderma asperellum, Bacillus subtilis* and hot water seed treatment for reduction of rice blast inocula on rice seeds.

Results

Effects of seed treatments on infected rice seeds

Results showed that rice seeds treated with *B. subtilis*, *T. asperellum*, Apron star and hot water gave similar percentage of infected seeds detected in both blotter and PDA plating techniques (Figures 1, 2 and 3). The percentage of infected rice seeds on microbial agents and hot water treated seeds were significantly lower (P ≤ 0.05) than the negative control.

Effects of seed treatments on seed germination and seedling performance

Results indicated that there were significant differences (P ≤ 0.05) between treatments in rice seed germination and performance of seedlings (Table 1). The highest germination was recorded when the seeds were treated with *Bacillus subtilis* (98%) followed by Apron star® (94.9%), hot water (87%) treatment and the negative control (80.9%). There were significant differences (P ≤ 0.05) between treatments in the number of normal and abnormal seedlings, percent of dead seeds, root length and seedling vigour index. Treatment of rice seeds with *B. subtilis*, *T. asperellum*, Apron star and hot water gave similar results for root length and seedling vigour. Higher levels of dead seeds were observed in hot water treated rice seeds and the negative control than in rice seeds treated with *B. subtilis*, *T. asperellum* and Apron star (Table 1).

Seedling emergence and rice blast disease incidence and severity

Significant differences (P ≤ 0.05) were observed among seeds treated with microbial agents, hot water treatment, Apron Star and the negative control on disease incidence and severity (Table 2). Rice seeds treated with *B. subtilis*, *T. asperellum*, Apron star and hot water significantly reduced disease incidence and severity compared to the negative control (Table 2).

Discussion

Seed treatment has been reported to reduce the population of the target pathogen on the seeds. The percentage of rice seeds infected with *P. oryzae* detected using the blotter and PDA methods were similar where *B. subtilis*, *T. asperellum*, hot water and Apron star seed treatments were used (Figure 1 and 2). However, the application of *B. subtilis* and Apron star on infected rice seed were more effective in reducing the percentage of infected seeds detected than *T. asperellum* and hot water treatments (Figure 1 and 2). Similar effect of *Bacillus* spp and chemical fungicide on controlling rice blast disease has previously been documented (Shan et al., 2013; Meng et al., 2015). In this study, seeds treated with microbial agents and hot water reduced the percentage of *P. oryzae* infected rice seeds by 4.3 to 52.7% compared to the negative control. This implies that the two microbial pesticides and hot treatments were effective in reducing *P. oryzae* inocula on rice seeds.

The germination of rice seeds treated with *B. subtilis* significantly increased by 17.1 % followed by seeds treated with *T. asperellum* (14.0 %) and hot water treatment (7.1 %) (Table 1). Some of the rice seeds died due to blast disease establishment on emerging coleoptiles and primary roots. A significantly low (P < 0.01) percentage of dead seeds were observed on rice seeds treated with *B. subtilis* and Apron star. Previous study, Faivre-Rampant (2013) indicated that dead seeds and seedlings in the soil were the source of *P. oryzae* infecting healthy seedlings. This indicates that rice seed treatment can reduce the percentage of dead seeds which may act as primary source of inocula for blast disease.

A significant increase in seedling vigour index (> 12.3%, P ≤ 0.016) was found on rice seeds treated with *B. subtilis* and *T. asperellum* (Table 1). The increased rice seedling vigour index may be attributed by increase in germination percentage in rice seeds treated with *B. subtilis* and *T. asperellum* (Table 1). The fungicide Apron star was also found to increase rice seedling vigour index (12.9 %, P ≤ 0.016. The effects of growth promoting and antifungal activity of microbial agents has been reported to increase seedling vigour index (Andresen et al., 2015). The increased seedling vigour index on *B. subtilis* and *T. asperellum* treated seeds can be partly caused by antifungal activity and growth promoting effect of these microbial agents. *Trichoderma* spp. and *B. subtilis* have been reported to induce growth of young rice seedlings (Ali and Nadarajah, 2014).

A significantly lower (P < 0.001) incidence and severity of rice blast disease was observed on microbial and hot water seed treatments (Table 2). Together, *B. subtilis*, *T. asperellum,* hot water and Apron star were significantly (P < 0.001) more effective in reducing the incidence and severity of rice blast disease on rice seedlings than the negative. This implies that treating rice seeds with microbial and hot water reduced rice blast disease on young rice seedlings. Roumen *et al.* (1992) and Faivre-Rampant (2013) indicated that young rice seedlings were more susceptible to blast disease than older rice plants. All rice seeds treatments used in this study reduced blast disease on rice seedlings.

Materials and methods

Plant materials

Rice genotype (Supa) which is susceptible to rice blast disease was used (Chuwa et al., 2015). The genotype was obtained from the International Rice Research Institute, Dakawa, Morogoro, Tanzania.

Location and treatments

The experiment was conducted in the laboratory and screen house at the African Seed Health Centre, Sokoine University

Table 1. Analysis of seed germination and seedling morphology after 10 days following seed treatment with *Trichoderma asperellum*, *Bacillus subtilis* and hot water.

Treatments	Germination (%)	Seedling performance		Dead seeds (%)	Root length (cm)	Shoot length (cm)	Seedling vigour index
		Normal seedlings (%)	Abnormal seedlings (%)				
Trichoderma asperellum	94.9 bc	76.2 b	23.8 a	3.5 ab	15.2 b	12.3 a	126.5 b
Bacillus subtilis	98.0 c	82.8 b	17.2 a	1.0 a	14.6 b	12.5 a	126.1 b
Hot water	87.0 ab	58.8 ab	41.2 ab	12.8 bc	14.2 ab	11.8 a	122.1 ab
Apron Star®	98.0 c	79.6 b	18.6 a	0.5 a	15.0 b	12.7 a	126.7 b
Negatives control	80.9 a	33.5 a	64.1 b	18.7 c	12.4 a	9.3 a	113.8 a
P value	<.001	<.001	<.001	<.001	0.005	0.091	0.016
Mean	91.8	66.2	32.9	7.3	14.3	12.3	127.4
C.V	2.3	15.4	23.5	47.9	6.5	15.1	4.3
SE +/-	0.22	0.15	0.14	1.09	0.93	1.77	5.32

C.V = Coefficient of variation, S.E = Standard error of mean
Means on the same column followed by different letters are significantly different according to Tukey's test at P < 0.05.

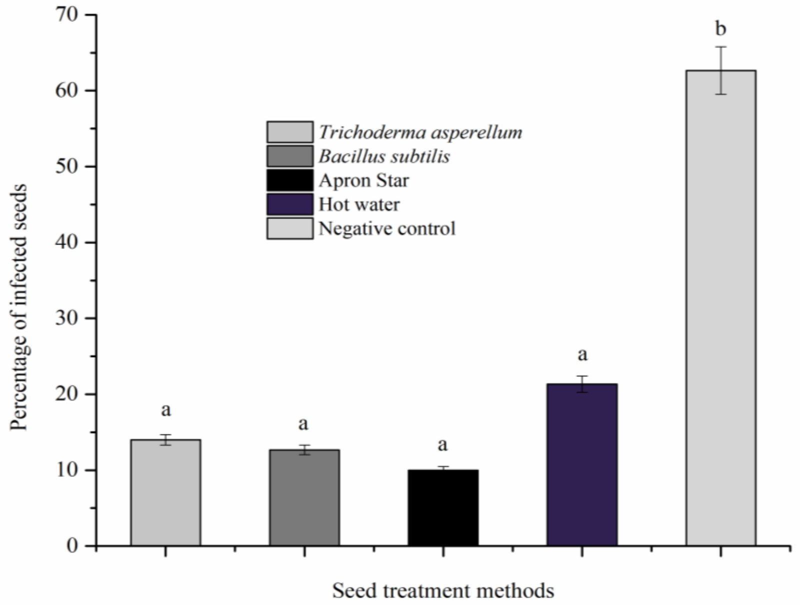

Fig 1. Effect of seed treatments on percentage of *Pyricularia oryzae* infected rice seeds detected using the Blotter test. Bar values are means ± standard error of means. Means with the same letters are not significantly different according to Tukey's HSD test at P < 0.05.

Table 2. Effects of seed treatment on seedling emergence, rice blast disease incidence and severity under screen house conditions.

Treatments	Emergence (%)	RBD incidence (%)	RBD severity (%)
Trichoderma asperellum	99.0 a	15.1 a	5.6 a
Bacillus subtilis	99.0 a	12.9 a	4.5 a
Hot water	95.9 a	17.7 a	5.6 a
Apron Star	99.0 a	14.4 a	5.0 a
Negative control	91.9 a	98.1 b	15.7 b
P value	0.196	<0.001	<0.001
Mean	97	31.6	7.3
C.V	2.5	17.0	23.3
SE +/-	0.24	0.10	1.69

RBD = Rice blast disease, C.V = Coefficient of variation, S.E = Standard error of meanMeans on the same column followed by the same letters are not significantly different according to Tukey's test at P < 0.05.

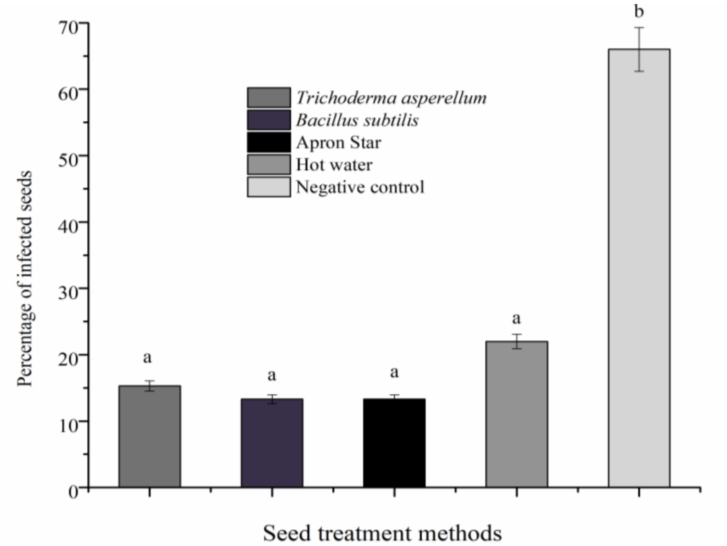

Fig 2: Effect of seed treatments on percentage of *Pyricularia oryzae* infected rice seeds detected using the Potato Dextrose Agar test. Bar values are means ± standard error of means. Means with the same letters are not significantly different according to Tukey's HSD test at P < 0.05.

of Agriculture, Morogoro, Tanzania. Commercial microbial pesticides (*Trichoderma asperellum* and *Bacillus subtilis*) were obtained from Real IPM Pvt., Nairobi, Kenya. Apron Star® (20% Thiamethoxam+2% Metalaxyl +2% Difenoconazole) was obtained from the agrochemical shop in Morogoro, Tanzania. Apron star is a commonly used seed coating fungicide in Tanzania.

Inoculum preparation and inoculation

Rice blast fungus was isolated in May, 2017 from infected rice leaves collected in rice fields in Morogoro and Tanga regions. Isolation and sporulation of *P. oryzae* was done as described by Mathur and Kongsdal (2003). Rice seeds were surface sterilized with 0.1% sodium hypochlorite solution for 2 minutes thereafter, rinsed with distilled water and dried on blotter paper as described by Singh et al. (2012). Inoculation was done by soaking rice seeds in *P. oryzae* suspension adjusted to 1×10^5 spores/ml using Haemocytometer. Inoculated seeds were then allowed to dry on three layers of sterile blotting paper placed under the laminar flow chamber for one hour.

Application of seed treatments

Inoculated seeds were treated with liquid suspension of *T. asperellum* (1 ml/L)*, B. subtilis* (1 ml/L)*,* hot water (50°C/15 min) and Apron Star®. Rice seeds (200 seeds per treatment) pre-inoculated with *P. oryzae* as described above, were soaked separately in suspensions of each *Trichoderma asperellum* (1 ml/L), *Bacillus subtilis* (1 ml/L) and Apron Star® (2.5g/Kg) for one hour, as per the suppliers' recommendation. Hot water treatment was carried out by dipping seeds on water bath at 50°C for 15 min as described by Faruq et al. (2015). Rice seeds soaked in sterile distilled water were used as a negative control. Treated seeds were dried on sterile blotter paper laced under the laminar flow chamber and allowed to dry for one hour.

Detection of Pyricularia oryzae on infected seed

The blotter method and direct plating on potato dextrose agar (PDA) techniques were used to detect *P. oryzae* on pre-inoculated and treated rice seeds. Each treatment was made up of two hundred seeds (Mathur and Kongsdal, 2003). The blotter method consisted of 25 seeds per dish placed on three layers of moist sterile filter paper in 90 mm diameter Petri dishes as described by Mathur and Kongsdal (2003). Direct plating on PDA was done using 25 seeds per dish in 90 mm diameter Petri dishes. The inoculated plates were arranged into Completely Randomized Design (CRD) with four replications and incubated at 25°C - 26°C. After 7 days, rice seeds were examined under the stereo and compound microscopes for the presence of *P. oryzae* as described by Mathur and Kongsdal (2003).

Rice seeds germination test

The pre-inoculated and treated seeds as described above, were tested for germination using the between paper method as described by Mathur and Kongsdal (2003). Fifty seeds per treatment were placed on water soaked filter papers. Thereafter, seeds were loosely covered with another

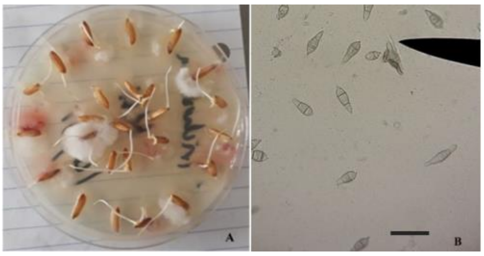

Fig 3. Colonies (A) and spores (B) of *Pyricularia oryzae* isolated from inoculated rice seeds on Potato Dextrose Agar media. The mycelia and spores were visible at 5 – 10 days after incubation. Scale bar = 10µ.

moisten filter paper rolled together and tied with a rubber band at each end. The experiment was arranged into a CRD with four replications and incubated at 25°C-26°C. After 10 days, the number of germinated and dead seeds, number of normal and abnormal (seedlings with deformed roots or shoots) were counted. Seedling vigor index (Vi) was determined after measuring the seedling shoot length and root length and calculated using the equation as described by Joe (2012) as shown below:

$Vi = (RL + SL) \times GP$

Where; RL = root length (cm), SL = shoot length (cm) and GP = germination percentage

Seedling emergence, rice blast disease incidence and severity

Pre-inoculated and treated rice seeds as described above were planted in trays (24 cm x 14 cm x 48cm), 20 seeds/tray arranged in CRD with four replications. The trays were kept under screen house conditions (26˚C - 30˚C and 75% - 90% RH). Seedlings emergence was assessed 15 days after sowing based on the presence of aboveground hypocotyls. Rice blast disease incidence and severity on rice seedlings was assessed 35 days after emergence. The percentage disease incidence was calculated as follows:

$$\text{Percentage disease incidence} = \frac{\text{Number of diseased seedlings}}{\text{Total number of inspected seedlings}} \times 100\%$$

Rice blast disease severity was scored using the 0 - 9 scale (IRRI 1996). The scores were converted into percent disease severity using the formula below:

$$\text{Percentage disease severity} = \frac{\text{Sum of scores X 100\%}}{\text{Number of observations x highest number on the rating}}$$

Statistical data analysis

Analysis of variance was performed on percentage seed germination, abnormal seedlings, dead seeds, seedling vigor index, percentage disease incidence and severity. Tukey's honestly significant difference (HSD) test was used to compare means at $P \leq 0.05$. To obtain homogeneity of variance, data on percentage seed germination, abnormal seedlings, dead seeds and disease incidence were ArcSine transformed before analysis, while data on percentage seedling emergence were square root transformed before analysis (Gomez and Gomez, 1984). All analyses were conducted using Genstat software 15th Edition

Conclusion

This study showed the potential of using microbial agents and hot water for reducing inoculum of rice blast disease on rice seeds. The study indicated that infested rice seeds treated with microbial agents increased the germination percent and seedling vigour index, while it reduced the incidence and severity of rice blast disease on rice seedlings. Further studies to determine the incidence and severity of rice blast disease on combinations of seed treatment and folia application of microbial agents are needed. Although hot water (50 °C for 15 minutes) seed treatment is simple and practical for use by farmers, there is a need to standardize the temperature and time for hot water seed treatment on different rice genotypes, to reduce any side effect on the seeds.

Acknowledgement

We gratefully acknowledge the financial support for this research by USAID Feed the Future IPM Innovation Lab, Virginia Tech, Cooperative Agreement No. AID-OAA-L-15-0000.

References

Ali H, Nadarajah K (2014) Evaluating the efficacy of *Trichoderma* spp and *Bacillus substilis* as biocontrol agents against *Magnaporthe grisea* in rice. Aust J Crop Sci. 8(9): 1324.

Andresen M, Wulff EG, Mbega ER, Stokholm MS, Glazowska SE, Zida PE, Mabagala RB, Lund OS (2015) Seed treatment with an aqueous extract of *Agave sisalana* improves seed health and seedling growth of sorghum. Eur J Plant Pathol. 141(1): 119-132.

Aravindan S, Yadav MK, Sharma P (2016) Biological control of rice blast disease with *Trichoderma spp*. under upland rice system. Int J Ric. 53(2): 167-173.

Birgit Jensen I, Knudsen, MB, Dan Funck J (2000) Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: Biocontrol efficacy against *Fusarium culmorum*. Eur J Plant Pathol. 106(233–242.

Burgess DR, Keane PJ (1997) Biological control of *Botrytis cinerea* on chickpea seed with *Trichoderma* spp. and *Gliocladium roseum*: indigenous versus non-indigenous isolates. Plant Pathol. 46(910–918).

Chung S, Kong H, Buyer JS, Lakshman DK, Lydon J, Kim SD, Roberts DP (2008) Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Appl Microbiol Biot*. 80(1): 115-123.

Chuwa C, Mabagala RB, Reuben M (2015) Assessment of grain yield losses caused by rice blast disease in major rice growing areas in Tanzania. Int J Sci and Res. 4(10): 2211-2218.

du Toit LJ, Hernandez-Perez P (2005) Efficacy of hot water and chlorine for eradication of *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* from spinach seed. Plant Dis. 89(12): 1305-1312.

Faivre-Rampant O, Genies L, Piffaneli P, Thareau D (2013) Transmission of rice blast from seeds to adult plants in a non-systemic way. Plant Pathol. 62: 879 – 887.

FAO (2017). Rice market monitor. Food and Agriculture Orgaization of the United Nation, p. 39.

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations. Accessed on 7 July, 2017.

Faruq A, Amin M, Islam M, Islam M, Alam M (2015) Evaluation of some selected seed treatments against leaf blast, brown spot and narrow brown leaf spot diseases of hybrid rice. Adv Agric Biol. 4(1): 8-15.

Goudjal Y, Toumatia O, Yekkour A, Sabaou N, Mathieu F, Zitouni A (2014) Biocontrol of R*hizoctonia solani* damping-off and promotion of tomato plant growth by endophytic *Actinomycetes* isolated from native plants of Algerian Sahara. Microbiol Res. 169(1): 59-65.

Imolehin E (1983) Rice seedborne fungi and their effect on seed germination. *Plant Dis*. 67(12): 1334-1336.

IRRI (1996). Standard evaluation system for rice, 4th ed, Manila, Philippines, p. 7.

Jayaraj J, Yi H, Liang G, Muthukrishnan S, Velazhahan R (2004) Foliar application of *Bacillus subtilis* AUBS1 reduces sheath blight and triggers defense mechanisms in rice. J Plant Dis Prot. 111(2): 115-125.

Joe MM, Rashedul I, Karthikeyan B, Bradeepa K, Sivakumaar PK, Sa T (2012) Resistance responses of rice to rice blast fungus after seed treatment with the endophytic A*chromobacter xylosoxidans* AUM54 strains. Crop Prot. 42(141-148.

Kihoro J, Bosco NJ, Murage H, Ateka E, Makihara D (2013) Investigating the impact of rice blast disease on the livelihood of the local farmers in greater Mwea region of Kenya. Sprin Plus. 2(1): 308.

Koch E, Roberts SJ (2014). Non-chemical seed treatment in the control of seed-borne pathogens, Global perspectives on the health of seeds and plant propagation material. *Sprin, Dordrecht*, pp. 105-123.

Kumar KVK, Yellareddygari SK, Reddy M, Kloepper J, Lawrence K, Zhou X, Sudini H, Groth D, Raju SK, Miller M (2012) Efficacy of *Bacillus subtili*s MBI 600 against sheath blight caused by *Rhizoctonia solani* and on growth and yield of rice. *Rice Sci*. 19(1): 55-63.

Long D, Correll J, Lee F, TeBeest D (2001) Rice blast epidemics initiated by infested rice grain on the soil surface. Plant Dis. 85(6): 612-616.

Mathur S, Kongsdal O (2003). Common laboratory seed health testing methods for detecting fungi. Bassersdorf, Switzerland. 89pp.

Meng X, Yu J, Yu M, Yin X, Liu Y (2015) Dry flowable formulations of antagonistic *Bacillus subtilis* strain T429 by spray drying to control rice blast disease. Biol Cont. 85(46-51.

Mgonja EM, Balimponya EG, Kang H, Bellizzi M, Park CH, Li Y, Mabagala RB, Sneller C, Correll J, Opiyo S (2016) Genome-wide association mapping of rice resistance genes against *Magnaporthe oryzae* isolates from four African countries. Phytopath. 106(11): 1359-1365.

Mtui HD, Bennett MA, Maerere AP, Miller SA, Kleinhenz MD, Sibuga KP (2010). Effect of seed treatments and mulch on seedbornebacterial pathogens and yield of tomato (*Solanum lycopersicum* Mill.) in Tanzania. *J Anim Plant Sci*. 8(3): 1006- 1015.

Nega E, Ulrich R, Werner S, Jahn M (2003) Hot water treatment of vegetable seed an alternative seed treatment method to control seed-borne pathogens in organic farming. J Plant Dis Prot. 110(3): 220-234.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil. 321(1-2): 341-361.

Roumen E, Bonman J, Parlevliet J (1992) Leaf age related partial resistance to *Pyricularia oryzae* in tropical lowland rice cultivars as measured by the number of sporulating lesions. Phytopathol. 82(12): 1414-1417.

Shan H, Zhao M, Chen D, Cheng J, Li J, Feng Z, Ma Z, An D (2013) Biocontrol of rice blast by the phenaminomethylacetic acid producer of *Bacillus methylotrophicus* strain BC79. Crop Prot. 44(29-37.

Singh P, Singh A, Singh H, Dhakad B (2012) Biological control of rice blast disease with *Trichoderma harzianum* in direct seeded rice under medium low land rainfed conditions. Env Ecol. 30(3B): 834-837.

Webster RK (2000) *Rice blast disease identification guide*. University of Calfornia, Davis. Accessed on 5 Aug, 2017.