Journal of Applied Biosciences 89:8272– 8280

ISSN 1997–5902

In vitro evaluation of the effect of aqueous extracts of *Agave sisalana* and *Cymbopogon citratus* on mycelial growth and conidia production of *Pyricularia oryzae*, causal agent of rice blast

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Original submitted in on 24th March 2015. Published online at www.m.elewa.org on 30th May 2015 http://dx.doi.org/10.4314/jab.v89i1.7

ABSTRACT

Objectives: To evaluate the effects of aqueous extracts of *Agave sisalana (sisal)* and *Cymbopogon citratus* (lemon grass) on mycelial growth and conidia production of *Pyricularia oryzae*, causal agent of Rice Blast.

Methodology and Results: The plants aqueous extracts were used at concentrations 0.1; 0.2; 0.3; 0.4; 0.5;1; 2; 3; 4; 5; 10; 20; 30% concentrations for *Agave sisalana* extracts and 0.5; 3; 5; 10; 15; 20% for *C. citratus* extracts. Fisher randomized block design with five (5) replicates was used to test the two extracts. All the two extracts tested had an inhibitory effect on the growth and spore production of the fungus. The Minimum inhibitory concentration (MIC) of *Agave sisalana* extracts was 3% (PI ≤ 97%).For the *C. citratus* extract, the MIC was 20% and the concentration less than 20% had a mild effect on mycelial growth.

Conclusion and application potential of the results: The use of pesticides of plant origin has been suggested by some researchers as alternatives to synthetic chemicals, in order to counter the potential hazards and pollution problems associated with the use of synthetic chemicals. The plants extracts tested showed antifungal activity. This result should enable use of aqueous extract of *Agave sisalana* to control blast diseases.

Key words: In vitro, plant extracts, mycelial growth, spore production, rice blast, *Pyricularia oryzae.*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereals in the world and is consumed by 50% of the world population (FAO, 2012). In Burkina Faso, the total rice production covers about 30% of the needs of the country. This situation is mainly due to the very low productivity of rice fields. Among the diverse constraints that rice producers face, diseases are causing serious yield losses. Rice blast caused by *Pyricularia oryzae* Cavara [synonym *Pyricularia grisea* Sacc. the anamorph of *Magnaporthe grisea* (Herbert) Yaegashi and Udagawa], is one of the most destructive and widespread diseases (Jia *et al*., 2000). This disease has caused significant yield losses in many rice-growing countries: heavy yield losses (up to 100%) were reported by farmers in Ghana (Nutsugah *et al*.,2004) and in some locations in The Gambia (Jobe *et al*., 2002). In Japan the yield loss due to blast constituted 45.9% of the total loss

induced by all diseases (MAFF 2012); 36–63% in Burkina Faso , 64% in Togo and up to 80% in Côte d'Ivoire (Séré *et al* ,2013). In Burkina Faso, rice blast is considered as one of the major yield constraints (*Sere et al, 2007*). The disease can be managed by the use of fungicides, resistant cultivars, agronomic practices and biotechnological methods (Ribot *et al.,* 2008). Chemicals are commonly applied for controlling rice blast disease (Anwar *et al*., 2002; Oh, 2007; Gohel *et al*. , 2008). However, the frequent use of fungicides on crops may cause hazards to human beings, plant health, useful microorganisms, and develop fungicide resistance in the pathogens populations. Moreover, some botanical pesticides have proved to be most secure and to have reduced adverse impact on the environment (Iftikhar *et al.,* 2010; Babar *et al.,* 2011). The use of pesticides of plant origin has been suggested by some researchers as alternatives to synthetic chemicals, in order to counter the potential hazards and pollution problems associated with the use of synthetic chemicals (Amadioha, 2000). *Sisal* is a perennial succulent plant that belongs to the family of Asparagaceae (Figure 1B).It is cultivated as a source of fibres traditionally used in the production of twine, ropes, carpets, mattresses and handicrafts. Leaf waste also has been used as a material to produce bio-fuel (methane). This species is also used as "live fences" or as an ornamental plant in gardens. Other products developed from sisal fibres include spa and cosmetic products, cat scratching posts, lumbar support belts, rugs, slippers, and cloths (FAO, 2012; PROTA, 2012). Some potential innovations include the use of the material as an organic fertilizer, a supplement in ruminant feed (Bandeira and Silva, 2006) and a raw material in the production of medicine (Debnath *et al*., 2010). In Africa, extracts of *A. sisalana* leaves and leaf waste are used in traditional medicine as a fungicide. A study evaluating the antimicrobial activity of extracts of the leaves and leaf waste discarded in the process of obtaining the hard fibres of *A. sisalana* showed significant inhibition of *Candida albicans* when treated with sisal extracts (Santos *et al*., 2009).

Lemon grass is originating from Indonesia and belongs to the family of Poaceae (Figure 1A). This plant is a widely used herb in tropical countries. The compounds identified in Cymbopogon citratus are mainly terpenes, alcohols, ketones, aldehyde and esters. Some of the reported phytoconstituents are essential oils that contain Citral α, Citral β, Nerol Geraniol, Citronellal, Terpinolene, Geranyl acetate, Myrecene and Terpinol Methylheptenone. The plant also contains reported phytoconstituents such as flavonoids and phenolic compounds, which consist of luteolin, isoorientin 2'-O-rhamnoside, quercetin, kaempferol and apiginin. Studies indicate that Cymbopogon citratus possesses various pharmacological activities such as anti-amoebic, antibacterial, antidiarrheal, antifilarial, antifungal and anti-inflammatory properties. (PubMed,2011). Others studies indicate the positive effect of plant extracts in inhibiting mycelia growth. The results of P. grisea growth on PDA amended with plant extracts showed that A. indica, A. vera, A. sativum, C. arabica, C. coccineum, D. stramonium, C. sinensis, Z. officinalis and N. tabacum had antifungal properties against P. grisea at high (25%) but not at low (1%) concentrations in in-vitro. Therefore, the efficacy of plant extracts needs to be investigated. In the present study, the aqueous extract of *A. sisalana* (sisal) and *C. citratus* (lemon grass) have been used *in vitro* to test the inhibition of mycelial growth and the spore production of P*. oryzae, the* causal agent of rice blast.

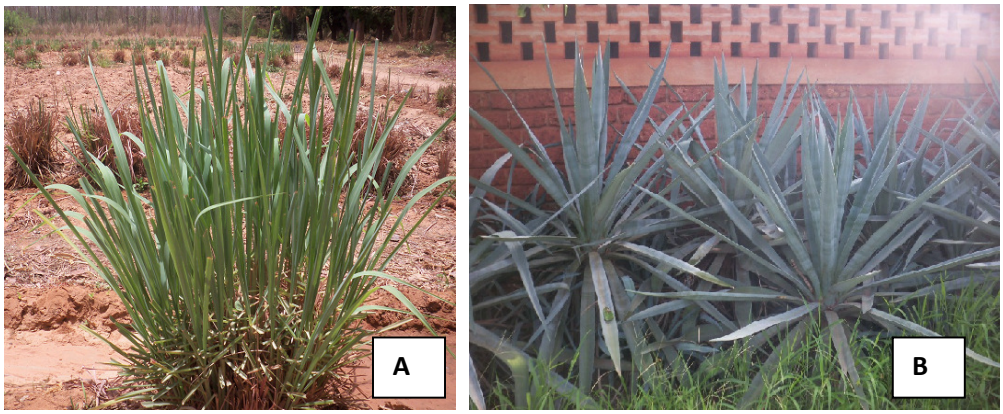

Figure 1 : (A) *C. citratus* (lemon grass) plant **et (B)** *A. sisalana* (sisal) plant

MATERIALS AND METHODS

Collection and culture of fungal material: Blast lesions on diseased or infected leaves were used for the collection of *P. oryzae* spores (Figure 2A). These diseased organs were incubated on moist filter papers in Petri plates at room temperature and incubated under alternating cycles of 12 hours near ultra-violet (NUV) light and 12 hours darkness for 48 hours to induce spores production (Figure 2B). The conidia from the lesion surface were spread onto 3% water agar with a sterile loop and incubated overnight (Figure 2C). Single germinating conidia were isolated and grown on potato-dextrose agar (PDA) medium for 5-10 days.

Figure 2: (A) infected sample **(B)** incubation **(C)** isolation of conidia.

Preparation of plant extract and culture media: The aqueous extracts of A. sisalana were obtained from dried roots and C. citratus from dried leaves. The dried roots and the leaves were mechanically reduced. The aqueous extracts were prepared by soaking 1; 2; 3; 4; 5; 10; 15; 20; 30g of plant powder in 100 ml of distilled water for 24 hours at room temperature (25-28° C) (Figure 3 a). These different quantities were equivalent to 1; 2; 3; 4; 5; 10; 15; 20 and 30%. For concentrations lower than 1%, stock solution (10) was diluted. In addition, 100 ml of the filtrate was added to 4.2 g PDA. The studied concentrations were 0.1, 0.2, 0.3, 0.4, 0.5,1, 2, 3, 4, 5, 10, 20 and,30% for A. sisalana (sisal) and 0.5,3, 5, 10, 15, and 20% for C. citratus .The culture medium was autoclaved at 121°C for 15 minutes and then poured into 9 cm diameter Petri plates (Figure 3b). After the solidification of the medium, 5 mm disc of pure culture of the test fungus (P. oryzae) was placed in the center of Petri plates and incubated at 30 oC (Figure 3 c). Plates of PDA without aqueous extract served as controls. There were five replications of each treatment.

Figure3: Extract of *A. sisalana* **(a);** culture media preparation **(b);** inoculation **(c)**

Evaluation of mycelial growth: Radial mycelial growth of the test fungus was measured at right angles on the different concentrations the 3rd, 5th, 7th, 10th 12th and 14th days after incubation (DAI) (Figure 4).Percentage of inhibition of mycelial growth by plant extracts was calculated using the formula: % inhibition of mycelia growth = (DC - DT) X 100 / DC Where DC= mean diameter of control DT= mean diameter of test

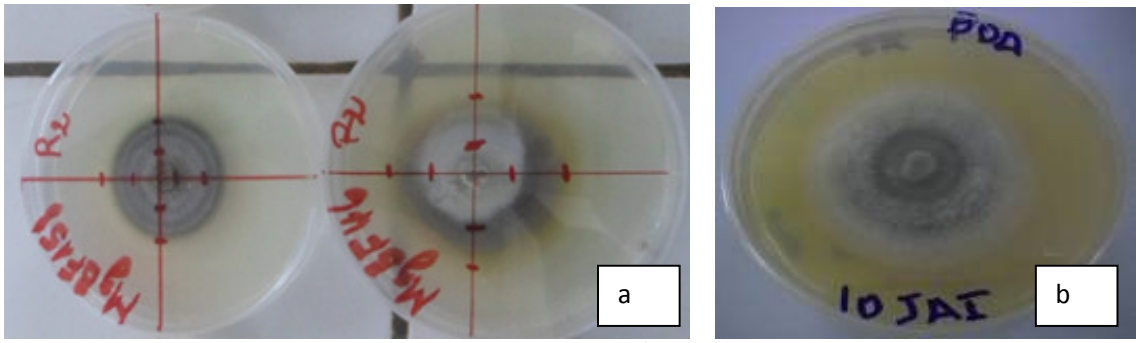

Figure 4: Evaluation of mycelial growth of *Pyricularia oryzae (a and b)*

Evaluation of fungus spore production on different concentrations of plant extract: After day 14, the colony mycelium of *P. oryzae* containing the spores was scraped into a test tube and the content was filtered using a filter. Spore production counting was conducted using Thomas cell. The volume used to extract the spores was 10ml and the count was conducted at a magnification of 40.

Experimental design and data analysis: The test was conducted according to Fisher randomized block design with five (5) replicates for the two tested extracts. For *A. sisalana* extracts, fourteen (14) treatments were used while for *C. citratus* extracts seven (07) treatments were used. Data were analyzed by the software Xlstat-Pro using analysis of variance (ANOVA) and mean differences were separated at p≤0.05 level of significabce using Newman Keuls test.

RESULTS AND DISCUSSION.

Effectiveness of *Agave sisalana* and *Cymbopogon citratus* extracts on mycelium growth of *Pyricularia oryzae*:The data indicated that the different treatments had a repressive effect on fungal growth rate. The statistical analysis revealed statistically significant differences among several groups (P < 0. 0001). The general observation showed that 18 DAI, the control (T0) had recorded the highest radial growth (7 cm). The 3%concentration of the extract of *A. Sisalana* (T8) appeared to be the minimum inhibitory concentration of radial growth of *P. oryzae*. Above this concentration, no fungal growth was observed.

Table 1: Effect of *Agave sisalana* extracts on mycelial growth of *Pyricularia oryzae*

Treatment	3DAI	5DAI	7DAI	10DAI	12DAI	15DAI	18DAI
T0 (PDA)	0.85b	1.51a	2.18a	3.53a	4.56a	5.78a	7.50a
T1 (0.1%AS)	0.90a	1.45a	2.08b	3.31b	4.16b	5.35b	7.11b
T2 (0.2%AS)	0.76c	1.15b	1.65c	2.41c	3.45c	4.46c	5.86c
T3 (0.3%AS)	0.70d	0.96c	1.35d	2.16d	2.96d	3.58d	4.70d
T4 (0.4%AS)	0.60e	0.86cd	1.21e	1.86e	2.55e	3.18e	4.08e
T5 (0.5%AS)	0.60e	0.80d	1.06f	1.50f	2.28f	2.68f	3.80f
T6 (1%AS)	0.00 f	0.66e	0.80g	1.16g	1.45g	1.55g	2.11g
T7 (2%AS)	0.00f	0.66e	0.78g	0.85 h	0.93h	1.08h	1.18h
T8 (3%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
T9 (4%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
T10 (5%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
T11 (10%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
T12 (20%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
T13 (30%AS)	0.00f	0.00f	0.00h	0.00i	0.00i	0.00i	0.00i
Value of F	487.61	241.46	587.65	345.21	791.16	886.17	1579.58
Probability	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Level of signification	VHS	VHS	VHS	VHS	VHS	VHS	VHS

Values with the same letter on the same column have no significant differences at 5% level (Newman-Keuls test). VHS: Very Highly Significant. T0: control PDA; T1-T13: different concentrations of *Agave sisalana* extract; AS *Agave sisalana*; DAI: Day After Incubation.

Table 2: Effect of *Cymbopogon citratus* extracts on mycelium growth of *Pyricularia oryzae*

Treatment	3DAI	5DAI	7DAI	10DAI	12DAI
T0 (PDA) control	1.283b	2.167c	3.200c	4.850c	6.267b
T1(0.5%Cc)	1.417b	2.667b	3.683bc	5.767ab	7.217a
T2(3%Cc)	1.633a	3.117a	4.433a	6.233a	7.567a
T3(5%Cc)	1.433b	2.850b	3.683bc	5.283bc	6.183b
T4(10%Cc)	1.050c	2.117c	3.250c	5.167c	5.050c
T5(15%Cc)	0.500d	1.433d	2.300d	4.050d	4.333c
T6(20%Cc)	0.000e	0.000e	0.000e	0.000e	0.000d
Value of F	184.002	172.405	105.170	135.555	96.049
Probability	0.0001	0.0001	0.0001	0.0001	0.0001
Level of signification	VHS	VHS	VHS	VHS	VHS

Values with the same letter on the same column have no significant differences at 5% level (Newman-Keuls test). VHS: Very Highly Significant. T0: control (PDA); T1-T6: different concentrations of Cymbopogon citratus extract; Cc: *Cymbopogon citratus*; DAI: Day After Incubation.

Different concentrations of *C. citratus* extracts yielded very highly significant different mycelia growth ($P < 0.0001$). The different aqueous extracts of *C. citratus* had a repressive effect on fungal growth. All studied treatments showed that the concentrations less than 5% enhanced the mycelium growth of *P. oryzae.* The 20% concentration (T6) proved to be the MIC along of experiment period.

Effectiveness of *Agave sisalana* and *Cymbopogon citratus* extracts on spore production of *Pyricularia oryzae:* The result showed that the number of spores counted 10 DAI was dependant on the concentrations of *A. sisalana* extract. The results of the analysis of variance showed very high significant (P<0.0001) differences between treatments for the number of conidia. Five statistically different groups were distinguished between treatments based on the number of spore counted. T0 (PDA) ; T1 (0.1%AS) and T2(0.2%AS) had inhibition percentages lower than 20%. As for T3 (0.3%AS); T4 (0.4%AS); T5(0.5%AS); T6(1%AS) and T7(2%AS), they had inhibition percentages between 60% and 97% while T8 (3% AS), T9 (4%AS), T10 (5%AS), T11 (10%AS), T12 (20% AS), T13 (30%AS) inhibited spore production and T8 was therefore considered as the MIC for spore production.

Table 3: Effect of *Agave sisalana* aqueous extracts on spore production of *Pyricularia oryzae*

Treatment	**Number of spores/ml (10 JAI)**	**Inhibition Percentage (PI) (10DAI)**
T0 (PDA) control	3.525 10^5 a	0.00 c
T1 (0.1%AS)	3.175 10^5 ab	7.46 c
T2 (0.2%AS)	2.800 10^5b	18.08 c
T3 (0.3%AS)	1.250 10^5 c	63.50 b
T4 (0.4%AS)	6.750 10^4d	79.19 a
T5 (0.5%AS)	4.470 10^4 de	86.45 a
T6 (1%AS)	3.500 10^4de	89.42 a
T7 (2%AS)	1.000 10^4 de	97.09 a
T8 (3%AS)	0.000 e	100 a
T9 (4%AS)	0.000 e	100 a
T10 (5%AS)	0.000 e	100 a
T11 (10%AS)	0.000 e	100 a
T12 (20%AS)	0.000 e	100 a
T13 (30%AS)	0.000 e	100 a
Value of F	73.65	49.29
Probability	0.0001	0.0001
Level of signification	VHS	VHS

Values with the same letter on the same column have no significant differences at 5% level (Newman-Keuls test). VHS: Very Highly Significant. T0: control PDA; T1-T13: different concentrations of *Agave sisalana* extract; AS: *Agave sisalana*; DAI: Day After Incubation; Pi: Percentage of inhibition;

The evaluation of the effect of lemon grass on spore production after 10 days of incubation revealed that only the 20% concentration (T6) was effective. The analysis of variance of the conidia count for the treatments revealed a very highly significant difference (P<0.0001) between treatments. The 10% ¨concentration (T4) and 15% concentration (T5) produced less spores compared to 0.5% concentration (T1) and 3% concentration (T2). The control T0 (PDA) produced the highest spore production. The results of the table show that the concentration between 0.5% and 15% had a partial inhibition of spore production compared to 20% concentration (T6) whose inhibition is complete (100%).

Table 4: Effect of *Cymbopogon Citratus* aqueous extract on the spore production of *Pyricularia oryzae*

Treatment	Number of spores /ml (10DAI)	Inhibition Percentage (PI) (10DAI)
T0 (PDA)	1.555 10^6 a	0.00e
T1 (0.5%Cc)	1.450 10^6 b	6.75d
T2 (3%Cc)	6.35010^5 c	59.15c
T3 (5%Cc)	1.600 10^5 d	89.70b
T4(10%Cc)	3.500 10^4 e	97.75a
T5 (15%Cc)	2.500 10^4 e	98.39a
T6 (20%Cc)	0.000 e	100.00a
Value of F	1829.519	1761.432
Probability	0.0001	0.0001
Level of signification	VHS	VHS

Values with the same letter on the same column have no significant differences at 5% level (Newman-Keuls test). VHS: Very Highly Significant. T0: control PDA; T1-T6: different concentration of *Cymbopogon citratus;* C c: *Cymbopogon citratus;* DAI: Day After Incubation; PI: Percentage of Inhibition;

DISCUSSION

The colony growth and spore production of P. oryzae was gradually reduced with increasing concentrations of the aqueous extracts. The reduction of the diameter of mycelial colonies of test fungus in the presence of the extract of A. sisalana and C. citratus showed that there is an active principle in antifungal properties that inhibit the growth of the test fungus. This inhibitory effect varies according to plant species, plant age and plant part and/or to applied concentration. The tests with A. sisalana aqueous extract showed that low concentrations (less than 3%) inhibited partially the mycelial growth of the fungus but higher concentration inhibited completely the growth of the parasite. The in vitro effect of aqueous extracts of A. sisalana on spore production of P. oryzae showed that low concentrations (less than 3%) partially inhibited the spore or conidia production of the fungus with a percent inhibition (PI) that was less than 100%. On the other hand, high concentrations (higher than 3%) have completely inhibited the spore production with a percent inhibition (PI) was 100%. These results confirm those reported by Jener and al (2009) and Ade-Ajayi and al (2011) whose reported that Agave sisalana juice revealed the presence of alkaloids, terpenoids, flavonoids, tannins, saponins and cardiac glycosides, which have been found in vitro to have antimicrobial properties in vitro. The effect of aqueous extract of C. citratus on the mycelial growth and conidia production showed that high concentrations (greater than 20%), inhibited completely the mycelial growth and conidia production of P. oryzae but the low concentrations (less than 20%), provided moderately reduction in the mycelial growth of the fungus. These results confirm those reported by Somda et al (2007) who observed that the concentration of C. citratus below 30% (≥ 30%) were inefficient to control P. sorghina and C. graminicola whereas concentrations above 30% were efficient.

CONCLUSION

Aqueous extracts of *A. sisalana* and *C. citratus* proved their antifungal activity by mycelial growth and spore production of *P. oryzae.* This extract also showed an inhibitory effect on spore production at the low concentration. The extract of *C. citratus* at high concentration (≥ 20%) inhibited completely the mycelial growth and spore production of *P. oryzae*. However, low concentrations (<20%), provided moderately reduction in the mycelial growth of the fungus . Therefore, an inappropriate use of this extract has dramatic consequences especially as it is the vegetative form of the fungus that is responsible for damage observed in the field. In spite of the *in vitro* efficiency of the extracts from plants, further experiments on their ability to control disease in greenhouse and in field to assess an efficient concentration of control of *P. oryzae*. Furthermore, the identification and characterization of the active compound will also be useful in the process of biopesticides

production. Research on the range of activity of plant extracts for control of other rice pathogens is recommended.

ACKNOWLEDGEMENTS:

The authors wish to express their sincere thanks to the Institute of Environment and Agricultural Research (INERA), for providing the financial support to carry out the research work. Special thanks go to Dr TARLA Divine Nfor, to Dr YODA Aristide and Dr KOITA Kadidia for critical reading of the manuscript.

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