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Antifungal activity of fungicides and plant extracts against yellow sigatoka disease causing *Mycosphaerella musicola*

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Abstract

Mycosphaerella musicola causes yellow sigatoka disease in banana plantations, and affects overall yield and quality of the fruit. Synthetic fungicides are used to control disease. An integrated approach by using plant based extracts and synthetic fungicides can control the disease efficiently by reducing the usage of fungicides. *In vitro* studies were carried out to test methanolic extracts of ten plants belonging to seven different families having previous reports on antimicrobial activity against *Mycosphaerella musicola, methanolic extracts of two plants* showed significant antifungal activity as well as significant inhibition of spore germination in spore germination inhibition assay. All eight fungicides exhibited inhibitory action on *M. musicola* in poison food technique, where as MIC range of each fungicide varied significantly. The results revealed that integrated disease management by using efficient plant extracts and effective fungicides can control the disease and the pathogen in fields efficiently.

Keywords – Antifungal activity – fungicides – *Mycosphaerella musicola* – plant extracts – spore germination inhibition assay.

Introduction

Sigatoka disease in banana plantation is one of the serious disease after panama wilt, considered as a potential threat to the banana production globally (Arzanlou et al. 2008) *Mycosphaerella* sp, causes sigatoka disease (Irish et al. 2013), as disease progress to advanced stages large photosynthetic area of leaf will be destructed, resulting in premature falling of leaves and premature mature ripening of fruits (Ramsey et al. 1990). *Mycosphaerella* genus contains several species, known to cause foliar spots on the banana plantain (Henderson et al. 2006). *Mycosphaerella fijiensis* Morelet causes black sigatoka disease, *Mycosphaerella musicola* Leach, causes yellow sigatoka and *Mycosphaerella emusae* causes Eumusae leaf spots of banana (Ramsey et al. 1990, Jones 1999). Sigatoka disease is wide spread in all major banana growing areas, specifically in South East Asia, Pacific, Latin America and Africa (Jones 1999). Asian countries including West Malaysia, Thailand, Vietnam, Southern India and Sri Lanka the disease is found serendipitously (Carlier et al. 2000, Crous & Mourichon 2002). In India Sigatoka disease have been reported from several states such as Tamil Nadu, Kerala, Karnataka, Andhra Pradesh, Maharashtra, Gujarat, West Bengal and North eastern hilly states of India (Selvarajan et al. 2001).

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Management of Sigatoka disease is usually done by the application of fungicides in commercial plantations (Herrmanto et al. 2010). Due to the increased usage of chemical fungicides, fungicides consume 25% of overall banana production cost (Ploetz 2000). Annually 20–25 cycles of fungicide application is done for the disease management (Peterson et al. 2002). Due to the excess usage of the fungicides, there are reports indicating the fungicide resistance for contact fungicides such as mancozeb, chlorothalonil from the major banana growing areas such as Belize, Cameroon, Central America and Costa Rica (Bellaire et al. 2009, Smith 1988, Churchill 2011). Due to the emergence of fungicide resistance has led to the application of fungicides up to 40–45cycles annually (Bellaire et al. 2009). The long term usage of the fungicides also has adverse impact on environment, contaminating the food sources and water bodies (Ndoumbe–Nkeng & Sache 2003). Several disease management strategies such as phyto–sanitation, application of bio control agents, disease forecasting in the commercial banana growing areas have planned and executed for the reduction of fungicide usage (Ngongo 2002). Conversely sigatoka disease of banana remains as a major constrain in the global banana production (Raut & Ranade 2004). Controlling the spread of Sigatoka disease can also achieved by screening of Mycosphaerella isolates for the fungicide resistance and recommending the banana growing farmers, about the fungicides to which the pathogen is highly susceptible (Fullerton & Olsen 1991, Mouliom–Pefoura et al. 1996). Current work focuses on the *in–vitro* screening of *Mycosphaerella musicola* against the different systemic and contact fungicides and plant extracts

Materials & Methods

Collection and preparation of plant material and fungicides for *in vitro* evaluation

Fresh disease free foliar and bark samples were collected from surrounding hilly areas of Sakleshpur and Subramanya, Karnataka state, India, in the month of March 2014 **(Table 1)**. Leaves and bark samples were washed thoroughly in running tap water to remove dust and shade dried. Leaf samples were blended in Waring blender (Waring International, New Hartford, CT, USA) for 5 minutes to form coarse powder. 20 g of powder was extracted with100 ml of Methanol for 8 hours, centrifuged at 10,000 x g for 20 min and the supernatant was flash evaporated using rotary flash evaporator (Buchi R–3 rotavapor, USA). The extracts were preserved at 4 ºC for further usage. Commonly used commercial grade contact and systemic fungicides were procured from the local agro–chemical vendors were used in this study **(Table 2).**

Table 1 List of medicinal plants and their parts used in the study.

Sl. No.	Name of the plant	Code	Family	Parts used
1	*Aristolochiatagala* Cham	AT	Aristolochiaceae	Leaves
2	*Casearia tomentosa* Roxb.	CT	Salicaceae	Leaves
3	*Garcinia cambogia* Gaertn	GC	Clusiaceae	Leaves
4	*Garcinia xanthochymus* Hook.f.	GX	Clusiaceae	Leaves
5	*Salacia fruticosa* Wall.	SF	Hippocrateaceae	Leaves
6	*Terminalia belerica* Roxb.	TB	Combretaceae	Leaves
7	*Terminalia chebula* Retz	TC	Combretaceae	Leaves
8	*Terminalia paniculata* Roth	TP	Combretaceae	Leaves
9	*Orthosiphon diffusus* Benth.	OD	Lamiaceae	Leaves
10	*Redermachera xylocarpa* Roxb	RX	Bignoniaceae	Bark

Table 2 List of commercial available fungicides used in the study.

Sl. No.	Name	Active ingredients	Mode of action	Code
1	Baylethon	Triadimefon 125g/1000 ml	Systemic	F1
2	Taqat	Captan 70% + Hexaconazole 5%	Contact + Systemic	F2
3	Ridomil MZ 72	Metalaxyl 8% + Mancozeb 64%	Systemic	F3
4	Contaff plus	Hexaconazole 5%	Systemic	F4
5	Lurit	Dimethomorph 50%	Systemic	F5
6	Mirador	Azoxystrobin 23%	Systemic	F6
7	Sectin	Fenamidone 10% + Mancozeb 50%	Systemic	F7
8	Bavstin	Carbendazim 50%	Systemic	F8

Isolation and maintenance of fungal culture

Fungal isolate *Mycosphaerella musicola* MM1 (GenBank accession No.KM369832) was selected for the current study (Aman & Rai 2015). The fungal culture was maintained by repeated sub culturing on to potato dextrose agar (PDA) in monthly basis.

Screening of plant extracts against *Mycosphaerella musicola*

Methanolic extracts (25, 20, 15, 10, 5%) of all the plants were amended with MEPDA (Malt extract potato dextrose agar media) prior to solidification of medium. 5 mm mycelial plugs of *M. musicola* was inoculated on treated media and three replicates were maintained for each treatment (Kumar & Prasad 1992). The percent inhibition of mycelial growth was calculated using the formula: Percent inhibition = C – T / C X 100 where C = Fungal growth in control and T = Fungal growth in treatment.

Spore germination inhibition assay

Induction of vegetative spores in *M. musicola* was done by inoculating fungus on V–8 agar media (Himedia, Mumbai, India), and incubated for 5 days at 28 $^{\circ}$C. 50 µL of plant extracts (1mg/ml) exhibiting antifungal activity were treated with spore suspension (10^8 spores/ ml) and incubated for 24 hours at 28 $^{\circ}$C. Enumeration of germinated and non germinated spores was done by hemocytometer under 40 X magnification (Begum et al. 2010)

Screening of fungicides against *M. musicola*

Screening of fungicides against *M. musicola* was done at their recommended dosage (2 g /L) for antifungal activity by poisoned food technique as described above.

Minimum inhibitory concentration (MIC)

All 8 fungicides were used to determine MIC. Different concentrations of each fungicide (1, 3, 7, 10, 30, 60, 120, 250, 500, 1000 µg/ml) were prepared separately by dissolving their requisite amount in 950µl of distilled water. 50µl of spore suspension was inoculated and incubated for 7 days. Mycelial biomass was separated from all treated aliquots dried and weighed to determine MIC. A triplicate of each concentration was done.

Statistical analysis

Anti fungal activity of plant extracts and fungicides against *M. musicola* was analyzed using mean of 3 replicates ± standard deviation (SD) and One way Anova followed by Tukey's test. All statistical analysis was performed using Graph Pad Prism software.

Results

Antifungal activity of plant extracts against *M. musicola*

Result revealed that among 10 plants screened for antifungal activity, methanolic extracts from leaves of RX and OD showed high antifungal activity. Antifungal activity of all plants reduced with decrease in extract concentration. Inhibitory percentage of RX and OD was maximum (35.2±0.4 and 34±0.5) at 5% of plant extracts. AT, GC and GX showed moderate antifungal activity with percentage of inhibition ranging from 10.3±0, 8.5±1.6 and 9.0±0 respectively whereas methanolic extracts from the leaves of SF,CT, TB, TC and TP did not showed any inhibitory action against *M. musicola* at 5% concentration of plant extracts **(Table 3).**

Spore germination inhibition assay

Extracts (1mg/ml) from RX and OD exhibited highest inhibitory effect on spore germination of *M. musicola*. However leaf extract of GC had sporicidal activity, inhibiting spore germination partially. Extracts from the leaves of CT, TB, TC and TP did not show any spore germination inhibitory activity **(Fig. 1)**.

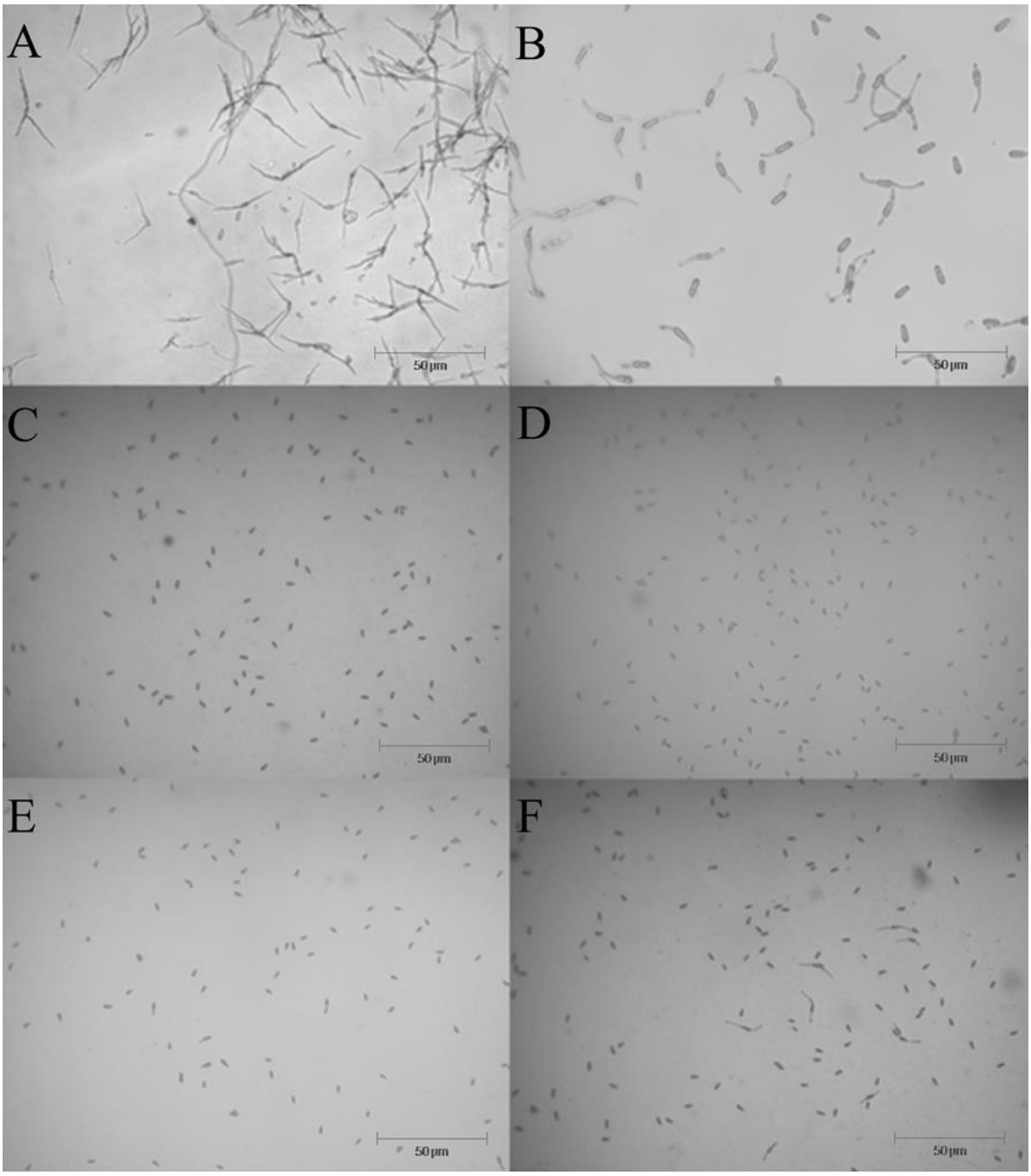

Fig. 1 – Inhibitory effect of methanolic plant extracts on germination and viability of *M. musicola* ascospores. Untreated ascospores (A, B) showing extensive spore germination with least spore mortality. Carbendazim treated ascospores (1mg/ml) (C) showing ungerminated conidia. Methanolic bark extracts of *R. xylocarpa* Roxb treated ascospores(1mg/ml) (D) showing ungerminated conidia with no viable spores. Methanolic leaf extract of *O. diffusus* Benth inhibited spore germination effectively (E), small proportions of viable ascospores were visible. Leaf extracts from *G.cambogia* Gaertn reduced conidial germination and incidence of viable spores was increased marginally (F).

Effect of fungicides on the growth of *M. musicola*

Poisoned food technique was conducted to observe the effect of eight systemic and contact on the growth of *M. musicola*. All fungicides inhibited the fungal growth completely at the concentration recommended by the manufacturers; however MIC range of all fungicides varied significantly **(Table 4)**, F2 was more efficient with an MIC range of 7.8µg/ml, followed by F4, F7 and F8 had MIC range of 31.2µg/ml, were as F5 and F6 exhibited least MIC range of 500µg/ml.

Table 3 Inhibitory activity of Methanolic plant extracts against *M. musicola* at different concentrations by poison food technique.

Sl. No	Concentration of extract	Percentage of Inhibition*									
		AT	CT	GC	GX	SF	TB	TC	TP	OD	RX
1	25%	50.3±2.1[a]	31.6±1.2	68±1.6[b]	40±1.7	32.1±1	35.8±0.8	34.5±1.2	25.4±1.2	67.2±0.6[b]	73.4±0.7[b]
2	20%	35.2±2.7[a]	14.5±1.1	59.4±0.7[b]	33.4±0.6	20.7±0.8	24.3±0.6	24.3±0.2	21.2±0.9	64.3±0.7[b]	60.7±0.3[b]
3	15%	28±0.3 [a]	12.7±1.7	42.5±1.7[b]	24.9±1	14.5±1.1	11.6±0.5	15.2±0.6	8.5±0.2	52.7±0.3[b]	52.7±0.8[b]
4	10%	21.2±0.9[a]	12.1±0.7	26.7±1.1[b]	19.4±0.9	4.9±0.3	4.3±0.4	9.0±0	8.5±0.6	40.7±0.2[b]	42.5±0.3[b]
5	5%	10.3±0 [a]	7.2±0.2	8.5±1.6[b]	9.0±0	4.9±0.2	6.7±0.2	5.4±0.1	4.9±0.5	34±0.5[b]	35.2±0.4[b]

*Values are mean ±SD of three parallel measurements. The results were analyzed by One Way Anova followed by Tukey's test [a]P<0.05, [b]P<0.01

Table 4 MIC values of different fungicides at varied concentrations against *M. musicola.*

Sl. No	Concentration In µg/ ml	Dried mycelial biomass in mg							
		F1[a]	F2[c]	F3[a]	F4[c]	F5[b]	F6[b]	F7[c]	F8[c]
1	1000	0	0	0	0	0	0	0	0
2	500	0	0	0	0	3.2±0.2	1.5±0.2	0	0
3	250	0	0	0.9±0.1	0	4.4±0.1	2.1±0.2	0	0
4	125	6.1±0.4	0	5.4±0.2	0	5.3±0.1	3.1±0.4	0	0
5	62.5	6.2±0.4	0	7.1±0.5	0	5.8±0.1	4.9±0.1	0	0
6	31.2	7.5±0.3	0	8.7±0.2	0.9±0.1	6±0.2	6.7±0.1	0.6±0.2	1.3±0.1
7	15.6	8.2±0.4	0	9.6±0.6	1.1±0.2	6.3±0.3	7.2±0.1	4.8±0.1	1.6±0.3
8	7.8	10.2±0.6	0.8±0.1	9.8±0.1	2.1±0.5	6.6±0.3	7.4±0.1	6±0.3	1.6±0.1
9	3.9	10.3±0.2	1.7±0.2	10.2±0.3	4.8±0.2	9.2±0.3	8.3±0.3	7.6±0.2	2.3±0.1
10	1.9	10.3±0.3	2.2±0.5	10.9±0.1	8±0.1	9.7±0.1	10.0±0.1	8.8±0.5	5.1±0.3

*Values are mean ±SD of three parallel measurements. The results were analyzed by One Way Anova followed by Tukey's test [a]P<0.05, [b]P<0.01, [c]P<0.001

Discussion

Methanolic extracts from *O. diffusus* Benth (Leaves) and *R. xylocarpa* Roxb (Bark) completely inhibited the growth of *M. musicola* (Poison food technique) and maximum inhibition of germinating spores was observed (spore germination inhibition assay). Current work is first to report on antifungal activity of methanolic leaf extract from *O. diffuses* and *R. xylocarpa* Roxb (Bark) against *M. musicola.* Large number of evidence indicates the folk medicinal importance of *G. cambogia* Gaertn in complimentary and alternate systems used to treat various human diseases (Shivakumar et al. 2013). Methanolic crude extracts from *G. atroviridis* leaves, fruits, roots, stem and trunk bark exhibited antimicrobial, cytotoxic, antitumour promoting and anti oxidant activities (Mackeen et al. 2000). Fruit extracts from *G. atroviridis* exhibited significant antifungal activity against *Cladosporium herbarum* (Mackeen et al. 2000). However in current study methanolic extracts from *G. cambogia* Gaertn inhibited ascospore germination efficiently but failed to exhibit any antifungal activity on actively growing fungal colonies. *O. diffusus* Benth plant paste is used to cure skin infections in folk medicine (Murugesan et al. 2011). Studies on the extracts and oil from *O. diffusus* Benth showed significant cytotoxicity and hepato–protective nature (Ghaffari et al. 2013, Sadashiva et al. 2013). Limited work has been done on ecofriendly management of fungal diseases in agricultural crops using extracts of *O. diffusus* Benth. However anti microbial studies using ethanolic extracts of *O.stamineus* Benth showed significant anti bacterial and anti fungal activity (Neharkar & Laware 2013). Secondary metabolite profiling from different parts of *R. xylocarpa* Roxb conducted by Ekade & Manik (2014) indicated that all parts of plant are rich in steroidal compounds. Anti microbial study conducted using flower extracts of *R. xylocarpa* Roxb against *B. subtilis* and *E. coli* showed strong antibacterial property (Sukumar & Anandi 2014). Even though *R. xylocarpa* Roxb having high folk medicinal value, based on literature, limited work has been done on antifungal aspects against fungal plant pathogens. Further investigation on isolation and characterization of active molecules responsible for antifungal activity from *R. xylocarpa* Roxb and *O. diffusus* Benth is in progress.

In vitro screening of fungicides for their efficacy against *M. musicola* showed that even though all fungicides inhibited the growth of the pathogen, MIC range varied contrastingly among the fungicides. F2 (Taqat) a mixture of contact and systemic fungicide was more effective against *M. musicola,* with an MIC range of 7.8µg/ml. Present work suggested that extracts from *O. diffusus* Benth, *G. cambogia* Gaertn and *R. xylocarpa* Roxb can be used in ecofriendly management of sigatoka disease in banana plantations. In commercial banana plantations application of F2 can reduce the number of fungicides spraying cycles. Reduction in fungicide spraying cycles can reduce overall consumption of fungicides for disease management. Combination of plant extracts (*O. diffusus* Benth, *G. cambogia Gaertn* and *R. xylocarpa* Roxb) with F2 will be more effective in integrated disease management strategy.

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