Fungitoxic spectrum of Amalab-a against fungal pathogens in rice under *in-vitro*

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ABSTRACT

Through the present study a botanically derived product Amalab-a was developed by combining *Aegle marmelos* leaf extract with a formulating agent (coded A+) and tested against three fungal pathogens of rice viz. *Helminthosporium oryzae* , *Curvularia lunata* and *Fusarium moniliforme*. Product registered complete inhibition of conidial germination at 0.01% in *H.oryzae*, *C. lunata* and *F.moniliforme* and mycelial growth inhibition at 1% in *H.oryzae*, *F.moniliforme* and at 0.1% in *C. lunata* whereas the extract alone could not at the same concentration. The shelf-life test displayed greater fungitoxic effect against all the three tested pathogens and retained its fungitoxicity upto 0.01% concentration till 12 months storage period. This formulated product has therefore improved the efficacy and shelf-life of this botanical product compared to the unformulated botanical extract tested under *in-vitro*, hence possesses greater potential for its integration against rice brown spot and grain discolouration control strategy but needs to be tested under *in-vivo* .

Key words: *Aegle marmelos, Curvularia lunata, Fusarium moniliforme*, fungitoxic spectrum, *Helminthosporium oryzae*

INTRODUCTION

To protect the crop and deterioration in produce quality, synthetic fungicides have commonly been recommended in the past but in recent years, a continuous use of these chemicals is known to cause undesirable effects such as residual toxicity, development of resistance, environmental pollution, health hazards to humans and animals. The products prepared from green plants constitute a suitable alternative to synthetic agrochemicals (Kamalkannan *et al*., 2001; Dev *et al*., 2002; Singh and Kumar, 2005; Sharma and Kumar, 2009). These products are environmentally non-pollutive and non- hazardous in preparation and use, and therefore merits attention of all concerned to look into the potential of integrating in the management of economically important diseases. However, the products prepared from these being organic in origin often tend to decompose easily and lose their efficacy faster than any petro-based agrochemicals. Therefore the present work being reported as hereunder is aimed at enhancing the efficacy and shelf- life of botanical extract with a specific formulating agent and tested against three fungal pathogens viz., *Helminthosporium oryzae* Breda de Haan, *Curvularia lunata* Boedijin and *Fusarium moniliforme* Sheld inciting brown spot and grain discolouration disease of rice causing serious loss in yield and deterioration in grain value.

MATERIALS AND METHODS

Preparation of aqueous extract

Fresh leaves of *A. marmelos* Corr. were collected, washed in sterilized distilled water and excess water removed before grinding these to a paste. The extract was prepared in distilled water (1:4w/v), and filtered through muslin cloth. The homogenized extracts thus obtained was treated as 100% crude aqueous mother extract (AME) which was diluted to 10%, 1%, 0.1%, 0.01%, 0.001%, 0.0001%, and 0.00001% and utilized for further studies. Care should be taken to pick up the leaves neither too old or neither too young and to be used fresh.

Preparation of formulated product

The formulating agent (FA), a surfactant (coded A^+) was also diluted from 100% to, 10%, 1%, 0.1%, 0.01%, 0.001%, 0.0001%, and 0.00001% and each of these dilutions was combined separately with each dilutions of AME(1:1v/v) to

Table 1. Fungitoxic effect of a formulated product Amalab-a against *Helminthosporium oryzae*

AME/FA Concentration(%)	Conidial germination (%)								
	100	**10**	**1**	**0. 1**	**0.01**	**0.001**	**0.0001**	**0.00001**	**Control(FA)**
0.01	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	2^{a}	50^{c}
0.001	2^{a}	54^{bg}	67^{bg}	98	98	98	98	98	80^{c}
0.0001	2^{a}	75^{c}	95^{c}	98	98	98	98	98	98
0.00001	2^{a}	86^{c}	94^{c}	98	98	98	98	98	98
Control(AME)	2^{a}	50^{b}	55^{c}	73^{c}	95^{c}	98	98	98	-
Control(S.D.W.)	98	98	98	98	98	98	98	98	-

C.D. at P= 0.05 = 0.76; AME- Aqueous mother extract; FA- Formulating agent; S.D.W.-Sterilised distilled water; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; ^{a}= completely inhibited conidia, ^{b}= reduced germ tube, ^{g}=granulated germ tube, ^{c}= excessively longer and highly branched germ tube

be treated as Amalab-a and utilized for further studies.

Isolation and maintenance of test pathogens

Fresh infected leaves having small dark brown circular/oval spots, were collected from a susceptible variety Tapaswini for *H.oryzae*, cut into small pieces and infected grains of susceptible variety Utkal prava for *C. lunata* and *F.moniliforme*, surface sterilized with 0.1% sodium hypo chloride solution for 30 seconds, washed thoroughly with sterile distilled water thrice and dried on sterilized blotting paper before transferring it to Potato Dextrose Agar medium (Peeled potato-250g; Dextrose- 15 g; Agar Agar-20g; Distilled water-1L; P^{H}-7) aseptically in petriplate. The isolate thus obtained from each pathogen was purified by successive subculturing and maintained on PDA slants. These slants were incubated for seven days at 24^{0} C, and stored at 4^{0} C for further studies.

Bioassay test

Conidial germination test

Aliquots, 0.1 ml from each concentrations of Amalab-a, the formulated product was pipetted out on to cavity slides separately and evaporated to dryness. Conidial suspension of 7-day old culture of *H.oryzae, C. lunata* and *F.moniliforme* with 30-35 conidia each per microscopic field(Nene and Thapliyal, 1979) were placed separately on each glass slide with equal quantity and incubated in moist chamber at 24^{0}C for 24hours. Observations on conidial germination (%) and the pattern of fungitoxicity were recorded using Olympus microscope BX51 at magnification 10X after 24 hours of incubation. Appropriate controls (AME, FA& sterilized distilled water) were maintained by keeping three replications in each case and the experiment was repeated thrice. Data on germination was statistically analyzed using Cropstat 7.2 developed by IRRI.

Poisoned food Technique

The formulated product was combined with sterilized melted PDA medium separately to get the final concentration of 10%, 1%, 0.1%, 0.01%, 0.001%. The extract mixed medium was poured into the petriplates aseptically and left for 24hr to check contaminations if any. Actively growing mycelia of *H.oryzae, C. lunata* and *F.moniliforme* were cut with a sterile cork-borer (0.5mm) and inoculated separately in the center of petriplates aseptically. All plates were incubated at 28 ± 2^{o}C for seven days. Appropriate control (sterilized distilled water) was maintained by keeping three replications in each case and the experiment was repeated thrice. The mycelial growth (cm) was observed and recorded when it grew to periphery in control petriplates and was computed through $3.14\times r^{2}$ methods (Tewari and Shukla, 1990). No mycelial growth was recorded with numerical value 0.1cm, for the purpose of statistical analysis.

Shelf-life effect

To assess the fungitoxic effect of the formulated product under the storage condition Amalab-a, FA and AME (100%) were kept at room temperature for fresh, 1, 3, 6, 9 and 12 months in a clean, sterilized glass vial with an air tight stopper. The product was then bioassayed separately against *H.oryzae, C. lunata* and *F.moniliforme* through conidial germination at 10%, 1%, 0.1%, 0.01%, 0.001% and 0.0001% concentrations as stated above. Appropriate control (sterilized distilled water) was maintained by keeping three replications in each case and the experiment was repeated thrice. Observations on conidial

Table 2. Fungitoxic effect of a formulated product Amalab-a against *Curvularia lunata*

AME/FA Concentration(%)	Conidial germination (%)								
	100	10	1	0. 1	0.01	0.001	0.0001	0.00001	Control(FA)
0.01	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	70[c]
0.001	2[a]	77[b]	87[b]	98	98	98	98	98	90[c]
0.0001	2[a]	79[c]	98	98	98	98	98	98	98
0.00001	2[a]	87[c]	98	98	98	98	98	98	98
Control(AME)	2 a	68[c]	76[c]	85[c]	98	98	98	98	
Control(S.D.W.)	98	98	98	98	98	98	98	98	

C.D. at P= 0.05 = 0.46; AME- Aqueous mother extract; FA- Formulating agent; S.D.W.-Sterilised distilled water; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; [a]= completely inhibited conidia, [b]= reduced germ tube, [c]= excessively longer germ tube

germination (%) and the pattern of fungitoxicity were recorded after 24 hours of incubation. Data on germination was statistically analyzed.

Statistical analysis

Data on conidial germination and mycelial growth of FA and botanical have been taken as individual treatments and were statistically analyzed using Cropstat 7.2 developed by IRRI after transforming the data to angular values. Hence, there is only one critical difference (C.D.) provided to compare between the treatment means for all FA and botanical. The treatments mean values have been provided in a tabular form in order to economize space in publication of the paper.

RESULTS

Conidial germination

Amalab-a at 0.01% concentration, exhibited complete inhibition of *H. oryzae*, *C. lunata* and *F. moniliforme* conidial germination (Table 1-3). There was also significant reduction in germination at 0.001% combination of FA with 10-1% concentrations of AME against *H. oryzae C. lunata* and *F. moniliforme* but the reduction was significantly more in AME alone at these concentrations. Excessively longer and/or highly branched germ tube was registered at 0.0001% and 0.00001% combination of FA with 10-1% concentration of AME against *H. oryzae* and only at 10% against either *C. lunata* or *F. moniliforme*. Control with FA at 0.1% and AME only at 100 % registered no germination, whereas, another control maintained with sterilized double distilled water recorded normal germination 98%. This experiment on fungitoxic effect of a formulated product Amalab-a with FA/AME to optimize and determine the optimum concentration for combination of the two i.e FA and AME as seen in the table so as to determine the minimum volume of products (FA/AME) needed for developing formulation without compromising its efficacy as shown in the table through vertical and horizontal concentration of FA and AME. The treatment 10% to 0.1% concentration produced identical effect on conidial germination. Hence the data on conidial germination (Table 1-3) are displayed from 0.01% to 0.00001% concentration.

Poisoned food Technique

Amalab-a (AME+FA) at 0.1% concentration produced complete mycelial growth inhibition

Table 3. Fungitoxic effect of a formulated product Amalab-a against *Fusarium moniliforme*

AME/FA Concentraion(%)	Conidial germination (%)								
	100	10	1	0. 1	0.01	0.001	0.0001	0.00001	Control (FA)
0.01	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	2[a]	80[c]
0.001	2[a]	83[b]	91[b]	98	98	98	98	98	98
0.0001	2[a]	86[c]	98	98	98	98	98	98	98
0.00001	2[a]	97[c]	98	98	98	98	98	98	98
Control(AME)	2[a]	70[b]	85[b]	94[c]	98	98	98	98	
Control(S.D.W.)	98	98	98	98	98	98	98	98	

C.D. at P= 0.05 = 0.61; AME- Aqueous mother extract; FA- Formulating agent; S.D.W.-Sterilised distilled water; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; [a]= completely inhibited conidia, [b]= reduced germ tube, [c]= excessively longer germ tube

Table 4. Shelf-life and bio-efficacy effect of a formulated product Amalab-a against *H. oryzae*

Treatments concentration (%)	Storage period(months)																	
	Fresh			1			3			6			9			12		
	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA
10	2[a]	50[b]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
1	2[a]	55[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.1	2[a]	73[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.01	2[a]	95[c]	50[c]	2[a]	98	50[c]	2[a]	98	5[c]	2[a]	98	57[c]	2[a]	98	60[c]	2[a]	98	70[c]
0.001	60[b]	98	80[c]	60[b]	98	80[c]	62[c]	98	83[c]	65[c]	98	85[c]	65[c]	98	85[c]	70[c]	98	90[c]
0.0001	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98
Control(S.D.W.)	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98

C.D. at P= 0.05 = 0.54; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; [a]= completely inhibited conidia, [b]= reduced germ tube, [c]= excessively longer and highly branched germ tube

Table 5. Shelf-life and bio-efficacy effect of a formulated product Amalab-a against *C. lunata*

Treatments concentration (%)	Storage period(months)																	
	Fresh			1			3			6			9			12		
	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA
10	2[a]	68[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
1	2[a]	76[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.1	2[a]	85[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.01	2[a]	98	70[c]	2[a]	98	70[c]	2[a]	98	75[c]	2[a]	98	77[c]	2[a]	98	80[c]	2[a]	98	80[c]
0.001	70[b]	98	90[c]	70[b]	98	90[c]	70[c]	98	90[c]	70[c]	98	93[c]	70[c]	98	95[c]	70[c]	98	95[c]
0.0001	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98
Control(S.D.W.)	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98

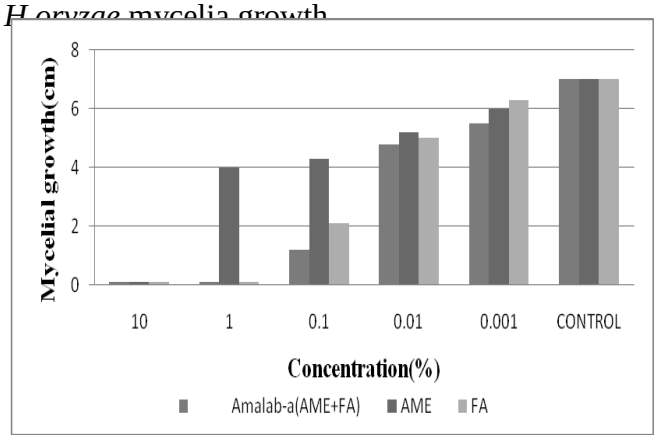
C.D. at P= 0.05 = 0.89; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; [a]= completely inhibited conidia, [b]= reduced germ tube, [c]= excessively longer and highly branched germ tube

Table 6. Shelf-life and bio-efficacy effect of a formulated product Amalab-a against *F. moniliforme*

Treatments concentration (%)	Storage period(months)																	
	Fresh			1			3			6			9			12		
	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA	Amalab-a	AME	FA
10	2[a]	70[b]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
1	2[a]	85[b]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.1	2[a]	94[c]	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]	2[a]	98	2[a]
0.01	2[a]	98	80[c]	2[a]	98	80[c]	2[a]	98	85[c]	2[a]	98	87[c]	2[a]	98	90[c]	2[a]	98	90[c]
0.001	70[b]	98	98	70[b]	98	98	70[c]	98	98	70[c]	98	98	70[c]	98	98	70[c]	98	98
0.0001	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98
Control(S.D.W.)	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98

C.D. at P= 0.05 = 0.69; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented as 2% and normal conidial germination is represented as 98%; [a]= completely inhibited conidia, [b]= reduced germ tube, [c]= excessively longer and highly branched germ tube

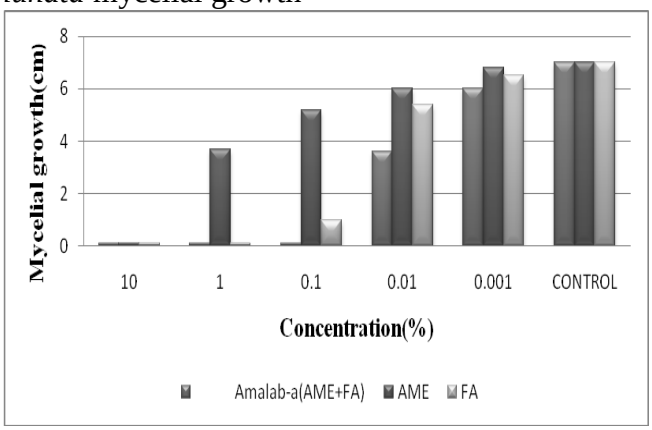
Figure 1. Fungitoxicity of Amalab-a against *H.oryzae* mycelia growth



C.D. at P= 0.05 = 0.12; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented by 0.1 cm

(0.1cm) in *C. lunata* (Figure 2), at 1% in *H. oryzae* and *F. moniliforme* (Figure 1 & 3). FA alone registered complete inhibition at 1% concentration in *H. oryzae, C. lunata* and at 10% in *F. moniliforme.* Amalab-a also displayed significantly reduced mycelial growth in all pathogens at each tested concentration (Figure 1-3). All the other treatments also did significantly reduce the mycelial growth compared to control.

Figure 2. Fungitoxicity of Amalab-a against *C. lunata* mycelial growth

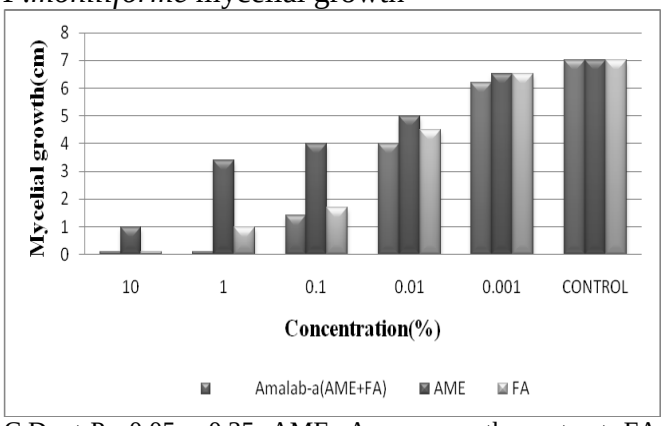


C.D. at P= 0.05 = 0.24; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented by 0.1 cm

Shelf-life effect

Complete conidial germination inhibition (2%) was recorded at 0.01% and 0.1% concentrations against *H. oryzae, C. lunata* and *F. moniliforme* respectively (Table 4-6). Excessively longer and/or highly branched germ tube was observed in formulated product at 0.001% concentration in a 3,

Figure 3. Fungitoxicity of Amalab-a against *F.moniliforme* mycelial growth



C.D. at P= 0.05 = 0.35; AME- Aqueous mother extract; FA- Formulating agent; Amalab-a (AME+FA)- Formulated product; complete inhibition is represented by 0.1 cm

6, 9, and 12 months storage test against all the three pathogens. Similar pattern of longer and/or highly branched germ tube was also observed in FA at two concentrations i.e. 0.01% and 0.001% against *H.oryzae, C. lunata* and only at 0.01% concentrations against *F.moniliforme* in a fresh, 1, 3, 6, 9, and 12 month storage period exposure test. All the other treatments produced normal germination and were found on par with control 98%. The fungitoxic effect of the product (AME) is very strong and effective and the values are consistently the same for all the months.

DISCUSSION

The botanically derived fungitoxicants which are easily decomposed largely due to their organic in nature are currently preferred, to be deployed in the strategy of disease management against the synthetic chemicals possessing residual toxic effect retained in edible products after the treatments, where the latter being considered hazardous to human health and the environment.

Nevertheless, readily decomposable botanical products loses the toxic strength against the targeted pathogens rapidly and require repeated treatment for an effective protection. The laboratory of natural plant product at CRRI is engaged in exploring and enhancing the efficacy of the botanical products against serious fungal pathogens of rice (Tewari and Nayak, 1991; Tewari, 1995). Botanically derived products have been reported to exhibit their antimicrobial potential (Parekh *et al*., 2006; Satish *et al*., 2010;

Mohana *et al*., 2011; Bakavathiappan *et al*., 2012; Uma *et al*., 2012)

In an *in-vitro* study, *A. marmelos* and *O. sanctum* ethanolic and essential oil extracts registered conidial deformities such as reduced or erratic germ tube growth, septal dissolution cytoplasmic content granulation, conidial shrinkage, collection of cytoplasmic content at either end in conidia and cell wall rupture in *P. grisea* in at various concentrations (Tewari, 1995; Tewari and Mishra, 1990). In the present investigation, the three major fungal pathogens viz. *H.oryzae* inciting brown spot and *C. lunata* and *F. moniliforme* associated with storage diseases of rice are responsible for causing substantial loss in yield and quality of rice. In this study, these pathogens were subjected to intensive bioassay test under *in-vitro* condition and it was found that the product Amalab-a not only completely inhibited the conidial germination and mycelial growth of pathogens but also produced a variety of conidial distortions (Table 1-3 and Figure 1-3). Prominent amongst the distorted and the abnormal growth of the infectious conidial propagules were reduction in germ tube length or excessively branched germ tube. The product producing such effect on fungal growth makes them redundant for causing successful infection and thus saves the product from loss in quantity and quality. The antimicrobial activity of *A. marmelos* (bael) has been found to have proven activity against other pathogens (Balakumar *et al*., 2011; Gheisari *et al*., 2011). This report is specific to the rice diseases of pathogens *H. oryzae, C. lunata* and *F. moniliforme* with the studies carried out in detail as indicated in various experimentation presented under result which is the first report particularly that includes shelf-life of the formulated product. The formulated product Amalab-a not only improved the fungitoxic strength but also strikingly enhanced the shelf-life for one year against the tested pathogens (Table 4-6). This product therefore holds a great potential if integrated in the management strategies against the above stated diseases but only after its field evaluation. Further work is in progress.

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