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ANTIFUNGAL ACTIVITY OF 2-HYDROXY-4-METHOXYBENZALDEHYDE ISOLATED FROM *DECALEPIS HAMILTONII* (WIGHT & ARN.) ON SEED-BORNE FUNGI CAUSING BIODETERIORATION OF PADDY

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Abstract: *In vitro* antifungal activity assay of different concentrations of 2-hydroxy-4-methoxybenzaldehyde isolated from *Decalepis hamiltonii* against six important seed-borne fungal pathogens viz., *Alternaria alternata*, *Drechslera tetramera*, *Fusarium oxysporum*, *F. proliferatum*, *Pyricularia oryzae* and *Trichoconis padwickii* isolated from paddy seeds revealed that, the compound 2-hydroxy-4-methoxybenzaldehyde showed significant antifungal activity. Among the fungi tested, *F. proliferatum* showed highest inhibitory activity, whereas *P. oryzae* showed least inhibitory activity. The minimal inhibitory concentration (MIC) varied between 350 µg/ml and 650 µg/ml depending on the fungal species. Comparative evaluation of the active compound with the synthetic fungicide thiram at recommended dosage revealed that, the antifungal activity of the active compound obtained from the plant was almost equivalent. Evaluation for nutritional parameters and dry matter losses (DML) revealed that, total carbohydrates, water soluble proteins, lipids and dry matter losses were significantly confined in 2-hydroxy-4-methoxybenzaldehyde treated paddy seeds compared with control seeds. This plant being an edible one can be exploited in the management of seed-borne pathogenic fungi and in the prevention of biodeterioration of grains and mycotoxin production during storage in an eco-friendly way.

Key words: *Decalepis hamiltonii*, 2-hydroxy-4-methoxybenzaldehyde, antifungal activity, seed-borne fungi, seed treatment, biodeterioration

INTRODUCTION

Rice (*Oryza sativa*) is one of the important cereal crops of the world. More than 50 fungal pathogens have been reported to be seed-borne in paddy (Agrawal 1999). Seed serves as important microcosm for saprophytic and pathogenic microorganisms and paddy seeds are no exception to this (Agrios 1997; Domijan *et al*. 2005). Thus many fungi are known to colonize and invade paddy seeds both at pre-and post-harvest stages causing considerable loss in yield and their nutritive value (Agrios 1997; Rocha *et al*. 2005). Seed treatment is the safest and the cheapest means to control seed-borne fungal plant diseases and to prevent biodeterioration of grains (Chandler 2005; Bagga and Sharma 2006). A large number of chemical fungicides are being used in the form of dusting, slurry and soaking treatment. Even though the use synthetic chemical fungicides can achieve effective and efficient control of seed-borne fungi, it is known that chemical fungicides cause serious environmental problems and are toxic to non-target organisms (Anon 2005). The toxic effect of synthetic chemicals can be overcome only by persistent search for new and safer pesticides accompanied by wide use of pest control methods, which are eco-friendly and effective.

Plant metabolites and plant-based pesticides appear to be ones of the best alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic pesticides (Varma and Dubey 1999). Extracts of many higher plants have been reported to exhibit antifungal properties under laboratory trails (Aliero and Afolayan 2006; Buwa and Staden 2006; Ergene *et al*. 2006; Parekh *et al*. 2006).

In view of these, a large number of plants are routinely screened in our laboratory for antifungal properties. *Decalepis hamiltonii* Wight & Arn. (*Asclepiadaceae*), an edible plant (Anon 1952), showed highly significant antifungal activity *in vitro*, against many phytopathogenic fungi. The active compound responsible for antifungal activity was 2-hydroxy-4-methoxybenzaldehyde (Mohana *et al*. 2008). The present study evaluates a dose dependent antifungal activity of the active compound against important fungal pathogens isolated from paddy and efficacy of the compound on prevention of fungi inducing biodeterioration of paddy grains during storage.

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MATERIALS AND METHODS

Plant material

Fresh rhizomes of *D. hamiltonii* free from diseases were collected and washed thoroughly 2–3 times with running tap water and once with sterile water, shade dried, powdered and used for soxhlet extraction.

Isolation and identification of 2-hydroxy-4-methoxybenzaldehyde, from petroleum ether extract of *D. hamiltonii* by TLC

The bioactive compound was isolated by activity guided assay of phenolic fraction of petroleum ether extract of rhizomes of *D. hamiltonii* by TLC (Mohana *et al*. 2008). The pure active principle (band-5 with R_f value 0.77) was dissolved in $CDCl_3$ and subjected to ^1H NMR (Hydrogen Nuclear Magnetic Resonance) at 300.1315MHz, ^{13}C NMR (Carbon Nuclear Magnetic Resonance) at 75.4734MDz and Mass spectral analysis (MASPEC system [msw/9629]) to confirm the identity of the compound. The pure active compound was used for treatment.

Test fungi

Fourteen seed samples of different cultivars of paddy (IR-20, IR-60, Jaya, Rasi, Sona masuri, Jyothi, Mandya vijaya and Intan) were collected from farmers field, ware houses and market from different agro-climatic region of Karnataka during 2004–2005, and were plated on blotter for testing by Standard Blotter Method (SBM) and Czapek-Dox-Agar (CDA) to isolate frequently occurring important seed-borne pathogenic field and storage fungi associated with these seeds. *Alternaria alternata*, *Drechslera tetramera*, *Fusarium oxysporum*, *F. proliferatum*, *Pyricularia oryzae* and *Trichoconis padwickii* were identified and isolated in pure culture, seven-days-old pure cultures served as the test fungi for antifungal activity assay.

Antifungal activity assay

The pure active compound, 2-hydroxy-4-methoxybenzaldehyde was subjected to antifungal activity assay by poisoned food technique (Singh and Tripathi 1999). The pure active compound was added to the medium to achieve the desired different concentrations in the medium, autoclaved, poured into petri dishes (20 ml each) and allowed to cool. Five mm discs of 7-day-old cultures of the test fungi were inoculated. Four replicates were maintained for each concentration. For comparison, a synthetic fungicide thiram, commonly used for seed treatment, obtained from Mysore agrochemical market was tested at recommended dosage (2000 µg/ml). The petri dishes containing media devoid of the compound and thiram served as control. The plates were incubated at 22±1°C for seven days. The fungitoxicity of the bioactive compound in terms of percentage inhibition of mycelial growth was calculated by using the formula:

$$\% \text{ inhibition} = dc–dt \times 100/dc$$

where: dc – average increase in mycelial growth in control, dt – average increase in mycelial growth in treatment (Singh and Tripathi 1999).

***In vivo* effect of 2-hydroxy-4-methoxybenzaldehyde on fungi inducing biodeterioration of paddy during storage**

Freshly harvested and locally available paddy seeds (IR-20), which recorded high incidence of natural fungal infestation with diverse species of seed-borne fungi were selected for the study. Seed moisture content of the sample was maintained at 16%, above safe storage limit of 13% by the following formula:

$$W = A\ (b–a)/(100–b)$$

where W is the volume of water required (ml), A is the initial weight of the sample (g), a is the initial moisture content (%) and b is the required moisture content (%).

The seeds were treated with two concentrations (0.5 g/kg and 1 g/kg) of the compound 2-hydroxy-4-methoxybenzaldehyde and thiram at recommended concentration (2 g/kg) following the procedures of slurry treatment (Ghasolia and Jain 2004). Seeds without the active compound and thiram served as control. The treated and control seeds were stored in polythene bags at 20°C for 90 days in separate sets of 500 g per each treatment in quadruplets. Samples (100 g) were drawn at regular intervals of 30 days and subjected to SBM (ISTA 1996). Total carbohydrates content was determined by Phenol Sulphuric acid method (Dubois *et al*. 1956). The total proteins content was determined by Folin-phenol reagent method (Lowry *et al*. 1951). The total crude lipid content was determined following the procedure of Fabbri *et al*. (1980) and dry matter losses by hot air oven method (Reed 1987).

RESULTS

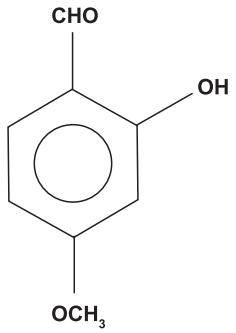
Isolation and identification of 2-hydroxy-4-methoxybenzaldehyde, from petroleum ether extract of *D. hamiltonii* by TLC

The ^1H NMR analysis of the compounds shows [d 3.85 (s,- OCH3), 6.52 (dd, J=2 HZ; 3-H), 6.55 (d, J=7 H_Z, 5-H), 7.40 (d, J=7 H_Z; 6-H), 9.70 (s, CHO),11.6 (s, -OH) functional groups. ^{13}C NMR analysis of the compounds shows eight carbon signals135.6(1-CH), 108.7(3-CH), 167.2(C of carbonyl), 101.05(5-CH), 164.8(2-C), 115.5(C), 194.7(6-CH), and 56.09(CH_3) and its identity conformed by the mass spectral analysis [m/z(% abundance): 57(48), 95(46), 108(24), 121(20), 151(100), 152(70). The strong molecular ion peak (m/z, 152) and stronger M-1 ion peak (m/z, 151) observed were characteristic of aromatic aldehyde. The melting point of this compound is 46°C. These results revealed that the compound is 2-hydroxy-4-methoxybenzaldehyde reported in literature (Nagarajan *et al*. 2001; Mohana *et al*. 2008).

Antifungal activity assay of 2-hydroxy-4-methoxybenzaldehyde isolated from *D. hamiltonii* and synthetic fungicide thiram

The per cent inhibitory activity on mycelium of, the active compound 2-hydroxy-4-methoxybenzaldehyde and thiram against 6 phytopathogenic fungi is presented in table 1. Tukey-HSD analysis of data revealed that, the highest inhibitory activity was observed in *F. proliferatum*,

followed by *D. tetramera* and the lowest inhibitory activity was observed in *P. oryzae*. The inhibitory activity increases with increasing dosage. The minimal inhibitory concentration (MIC) of the compoun 2-hydroxy-4-methoxybenzaldehyde against *D. tetramera* and *F. proliferatum* was 350 μg/ml, while for *F. oxysporum* and *A. alternata* the MIC was 400 μg/ml and 450 μg/ml respectively. *T. padwickii* and *P. oryzae* were totally inhibited (MIC) at 600 μg/ml and 650 μg/ml respectively. The synthetic fungicide thiram completely inhibited the fungal growth of all test fungi.



2-hydroxy-4-methoxybenzaldehyde

***In vivo* effect of 2-hydroxy-4-methoxybenzaldehyde on fungi inducing biodeterioration of paddy during storage**

The per cent incidence of different fungi in control, active compound 2-hydroxy-4-methoxybenzaldehyde (0.5 g/kg and 1 g/kg) and thiram (2 g/kg) treated paddy seeds is presented in table 2. In control paddy seeds, species of *Alternaria*, *Aspergillus*, *Curvularia*, *Drechslera*, *Fusarium*, *Penicillium*, *Pyricularia* and *Trichoconis* which were present in higher percentage, with increasing the storage period, the fungal incidence gradually increased both in diversity and percentage. The active compound and thiram treated paddy seeds, showed highly significant control of all these seed borne fungi up to 90 days of storage. Among the two concentrations of active compound tested, 1 g/kg treatment was highly significant in preventing fungal growth compared to 0.5 g/kg treatment.

Changes in nutritional parameters and dry matter losses of both treated and control seeds of paddy are presented in figure 1A–D. The results revealed that, total carbohydrate which was 0.68 g/g on '0' days was reduced to 0.58 g/g in control seeds, 0.62 g/g in 0.5 g/kg compound treated seeds, 0.65 g/g in 1 g/kg compound treated seeds and 0.67 g/g in thiram treated seeds after 90 days storage. Water soluble protein content on '0' day which was 4.2 mg/g was reduced to 3.9 mg/g in control seeds, 4.1 mg/g in 0.5g/kg compound treated seeds, 4.18 mg/g in 1 g/kg compound treated seeds and 4.2 mg/g in thiram treated seeds after 90 days storage. Lipid content which was 36 mg/g on '0' days was reduced to 22 mg/g in control seeds, 28 mg/g in 0.5 g/kg compound treated seeds and 33 mg/g in 1 g/kg compound treated seeds and 34 mg/g in thiram treated seeds after 90 days storage. The dry matter losses were significantly higher in control seeds (4.9%) when compared with 0.5g/kg compound treated seeds (1.8%), 1g/kg compound treated seeds (0.3%) and thiram treated seeds (0.1%) respectively after 90 days storage.

Table 1. Antifungal activity of 2-hydroxy-4-methoxybenzaldehyde isolated from *D. hamiltonii* and thiram against phytopathogenic fungi isolated from paddy

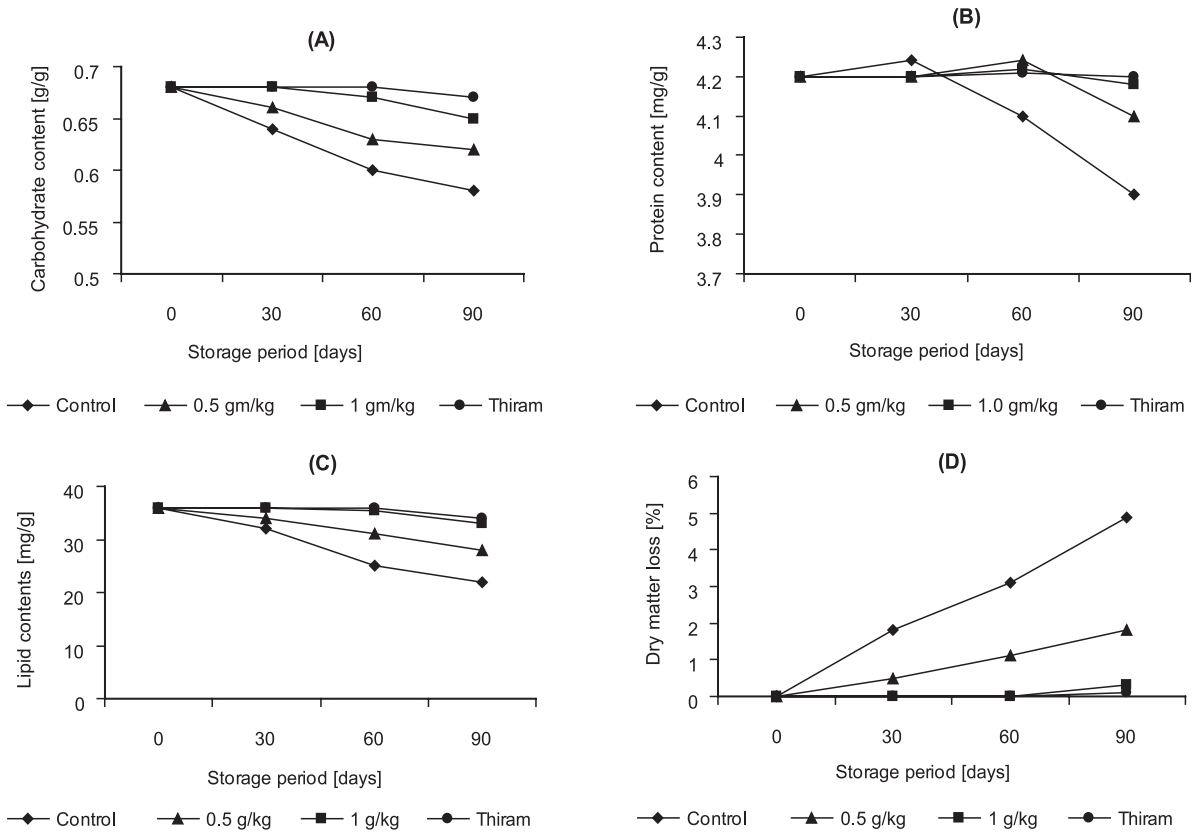
Compound concentration (μg/ml)	Per cent mycelium inhibition of pathogenic fungi of paddy					
	Alternaria alternata	*Drechslera tetramera*	*Fusarium oxysporum*	*Fusarium proliferatum*	*Pyricularia oryzae*	*Trichoconis padwickii*
40	3.25±0.3	3.34±0.2	11.47±0.3	12.72±0.4	0.00±0.0	2.10±0.5
60	8.09±0.5	6.6±0.6	24.33±0.3	23.35±0.1	5.66±0.4	3.61±0.5
80	11.33±0.4	19.72±0.4	39.31±0.4	42.30±0.4	10.34±0.2	8.84±0.6
10	16.71±0.5	24.20±0.6	53.05±0.3	51.63±0.2	14.36±0.4	11.88±0.4
100	21.82±0.7	34.60±0.5	56.90±0.5	57.58±0.4	17.77±0.4	12.64±0.5
150	34.34±0.7	45.89±0.6	68.53±0.3	68.06±0.5	22.64±0.2	24.67±0.6
200	43.07±0.6	67.10±0.3	71.26±1.2	79.06±0.3	26.60±0.3	26.55±0.5
250	59.94±0.7	86.69±0.4	83.14±0.4	86.21±0.3	42.76±0.7	31.41±0.7
300	64.78±0.7	91.35±0.5	90.07±0.4	92.78±0.6	56.57±0.6	37.18±0.5
350	76.14±0.5	100.0±0.0	98.33±0.7	100.0±0.0	63.54±2.3	41.75±1.0
400	91.49±1.1	100.0±0.0	100.0±0.0	100.0±0.0	67.94±0.4	49.56±0.5
450	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	78.19±0.5	65.00±0.6
500	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	85.88±0.7	76.19±0.6
550	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	89.79±0.6	90.08±0.8
600	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	97.57±0.3	100.0±0.0
650	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Thiram	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0

Data given are mean of four replicates ± standard error
Analysis of variance (ANOVA) d.f. = 15 at $p < 0.0001$

Table 2. Efficacy of the active compound of *D. hamiltonii* and thiram against seed-borne fungi of paddy up to 90 days storage

Concentration	Untreated				0.5 g/kg active compound				1 g/kg active compound				Thiram [2 g/kg]			
	per cent incidence of seed-borne fungal species in paddy seeds															
Storage Periods (days)	0	30	60	90	0	30	60	90	0	30	60	90	0	30	60	90
Alternaria spp.	22±0.5	26±0.5	28±0.3	27±0.8	4±0.5	12±0.6	14±0.6	19±0.6	0.0	0.0	7±0.4	11±0.7	0.0	0.0	8±0.6	11±0.7
Aspergillus spp.	42±0.8	51±1.2	64±0.5	72±0.4	9±0.3	14±0.3	26±0.4	35±0.5	0.0	5±0.2	12±0.6	17±1.1	0.0	5±0.6	23±0.5	32±0.5
Curvularia spp.	28±0.6	27±0.5	31±0.5	39±0.8	0.0	8±0.8	13±0.3	18±0.8	0.0	0.0	4±0.3	6±0.4	0.0	0.0	0.0	3±0.3
Drechslera spp.	41±0.8	40±0.8	44±0.4	48±0.7	0.0	5±0.5	9±0.5	14±0.8	0.0	0.0	0.0	4±0.3	0.0	0.0	0.0	1±0.2
Fusarium spp.	36±0.3	42±0.7	49±0.9	54±0.8	0.0	6±0.3	10±0.5	15±0.8	0.0	0.0	6±0.3	13±0.6	0.0	0.0	3±0.4	8±0.5
Penicillium spp.	21±0.4	34±0.8	42±0.5	58±0.9	4±0.3	11±0.5	19±0.4	28±0.5	0.0	0.0	8±0.3	18±0.5	0.0	8±0.6	15±0.6	22±0.5
Pyricularia spp.	22±0.5	26±0.8	27±0.8	25±0.4	0.0	6±0.5	8±0.8	12±0.5	0.0	0.0	2±0.9	4±0.8	0.0	0.0	0.0	2±0.3
Trichoconis padwickii	23±0.7	25±0.5	21±0.8	19±0.5	0.0	0.0	4±0.5	8±0.7	0.0	0.0	0.0	2±0.3	0.0	0.0	0.0	1±0.6
Trichothecium spp.	13±0.4	16±0.8	21±0.5	26±0.5	0.0	0.0	5±0.3	10±0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Per cent incidence is based on 4 replicates with 100 seeds each, $F = 321.64$, $p < 0.001$



(A) Changes in total carbohydrates; (B) Changes in proteins;

(C) Changes in lipids; (D) Per cent dry matter loss

Fig. 1. (A, B, C and D) Comparative efficacy of the bioactive compound 2-hydroxy-4-methoxybenzaldehyde isolated from *D. hamiltonii* (0.5 g/kg and 1 g/kg)and Thiram (2 g/kg) on fungi inducing nutritional losses in paddy grains stored up to 90 days

DISCUSSION

Rhizome of *D. hamiltonii* is largely used in South India for pickling along with curds or limejuice (Anon 1952). Earlier reports of the phytochemical analysis of the roots revealed that 2-hydroxy-4-methoxybenzaldehyde is the important and major component (Nagarajan *et al*. 2001). *In vitro* production of this compound has also been attempted by George *et al*. (2000), considering the usefulness of the compound. Antimicrobial properties of *D. hamiltonii* have been reported (Elizabeth *et al*. 2005; Thangadurai *et al*. 2002; George *et al*. 1999a; Phadke *et al*. 1994). In all these reports, the test organisms are human pathogenic microorganisms. The insecticidal property of this compound against important storage insects (*Sitophilus oryzae* L., *Rhizopertha dominica* F. and *Tribolium castaneum* Hbst.) has also been demonstrated (George *et al*. 2000, 1999b). Mohana *et al*. (2008) have reported antifungal property of *D. hamiltonii* against important phytopathogenic fungi. The efficacy of this compound for prevention of biodeterioration of grains during storage has not been worked out. In the present investigation the antifungal active compound has been evaluated for the first time to improve grain quality and to prevent loss in nutritional quality of paddy. The present investigation demonstrates *in vitro* antifungal property of the active compound 2-hydroxy-4-methoxybenzaldehyde isolated from *D. hamiltonii* against six important disease causing phytopathogenic fungi isolated from paddy. Similarly the *in vivo* efficacy of the active compound to prevent biodeterioration of paddy grains during storage has also been demonstrated.

Synthetic fungicide thiram is generally used in the management of fungal pathogens in agriculture (Ghasolia and Jain 2004; Sagar and Sugha 2004; Chandler 2005; Bagga and Sharma 2006). *In vitro* comparative evaluation of the synthetic fungicides with that of active compound, 2-hydroxy-4-methoxybenzaldehyde isolated from *D. hamiltonii* has revealed that the antifungal activity is almost equal to the synthetic fungicide thiram.

The analysis of seed-borne fungi, food reservoir and dry matter losses (DML) of paddy seeds treated with the active compound (0.5 g/kg and 1 g/kg) were significantly effective in controlling pathogenic fungi, fungal induced nutritional changes and dry matter losses. None of the earlier investigators have evaluated the efficacy of the compound 2-hydroxy-4-methoxybenzaldehyde to prevent nutritional loss in paddy seed during storage. The observations of the present investigations suggest that 1 g/kg treatment with the active compound is ideal to prevent nutritional loss during storage of paddy grain. Even though 1 g/kg treatment is appropriate for preventing nutritional quality loss of paddy seeds further investigations are necessary on the toxicological aspects of this treatment before it is finally recommended for the commercial exploitation.

The present investigation it is an important step in developing plant-based pesticides, which are eco-friendly for the management of the seed-borne fungi and development of commercial formulation of botanicals. Further investigations are necessary for developing commercial formulation based on field trail and toxicological experiment.

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POLISH SUMMARY

AKTYWNOŚĆ PRZECIWGRZYBOWA 2-HYDROXY-4-METHOXYBENZALDEHYDU WYOSOBNIONEGO Z *DECALEPIS HAMILTONII* (WIGHT & ARN.) PRZECIWKO GRZYBOM PRZENOSZONYM PRZEZ NASIONA I POWODUJĄCYM BIODEGRADACJĘ NASION RYŻU

Badania przeciwgrzybowej aktywności 2-hydroxy--4-methoxybenzaldehydu wyosobnionego z *Decalepis hamiltonii* przeciwko sześciu ważnym, przenoszonym przez nasiona grzybom patogenicznym, w tym: *Alternaria alternata*, *Drechslera tetramera*, *Fusarium oxysporum*, *F. proliferatum*, *Pyricularia oryzae* i *Trichoconis padwickii*, które zostały wyizolowane z ryżu wykazały, że związek ten ma znaczącą aktywność przeciwgrzybową. Spośród badanych grzybów najwyższą aktywność przeciwgrzybową wykazał *F. proliferatum*, natomiast najniższą *P. oryzae*. Minimalne stężenie tego związku, inhibitujące grzyby, wahało się pomiędzy 350 ug/ml i 650 ug/ml, zależnie od rodzaju grzyba. Porównawcza ocena aktywnego związku z syntetycznym fungicydem thiaram użytym w zalecanej dawce wykazała, że przeciwgrzybowa aktywność związku uzyskanego z rośliny była prawie taka sama, jak aktywność thiaramu. Stwierdzono, że straty węglowodanów ogółem, białek rozpuszczalnych w wodzie, lipidów i suchej masy w nasionach ryżu potraktowanych 2-hydroxy-4-methoxybenzaldehydem były znacznie ograniczane przez ten związek, w porównaniu do nie traktowanych nasion kontrolnych. Ta jadalna roślina w sposób przyjazny dla środowiska może być wykorzystana do ograniczania patogenów przenoszonych przez nasiona, degradacji nasion oraz wytwarzania mykotoksyn podczas przechowywania.