

Full Length Research Paper

A unique water soluble formulation of β -asarone from sweet flag (*Acorus calamus* L.) and its *in vitro* activity against some fungal plant pathogens

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Accepted 7 July, 2011

An antifungal substance β -asarone was isolated from ethyl acetate extract of *Acorus calamus* L. As β -asarone is lipophilic, it was made into water soluble formulation by encapsulating into beta methyl cyclodextrin (BMCD). At β -asarone concentration of 30 ppm, this formulation completely inhibited mycelial growth of major plant pathogenic fungi, *Phytophthora capsici*, which was isolated from infected black pepper (*Piper nigrum* L.) leaf and identified by internal transcribed spacer polymerase chain reaction (ITS PCR) and sequencing. Further, this formulation completely inhibited the germination of zoospore and complete cessation of zoospore motility was observed within a minute.

Key words: *Acorus calamus* L., β -asarone, beta methyl cyclodextrin (BMCD), *Phytophthora*, internal transcribed spacer polymerase chain reaction (ITS PCR), zoospore.

INTRODUCTION

Sweet flag (*Acorus calamus* L.) is a widespread, semi-aquatic plant of temperate to sub-temperate regions. Herbaceous perennial sweet flag has long, erect, narrow, aromatic leaves ascending from a branched, underground rhizome. β -Asarone (cis-2,4,5-trimethoxy-1-propenylbenzene) is a major active principle found in *A. calamus* (sweet flag), along with fatty acids, myristic (1.3%), palmitic (18.2%), palmitoleic (16.4%), stearic (7.3%), oleic (29.1%), linoleic (24.5%) and arachidic (3.2%). Apart from glucose, fructose and maltose (Balakumbaran et al., 2010) occurrence of terpenoids and flavonoids were reported from this plant (George et al., 1986). *Acorus* plants have been found use in herbal medicine to cure fevers, for asthma, bronchitis and as an all-round sedative (Leung, 1980; Wren, 1988). The volatile oil from the tetraploid "Indian" form of *A. calamus* L. contains β -asarone as a major component (70 to 80%). Whereas, the oil from the "European" or triploid form contains less than 10%. Antimicrobial activity of β -asarone was reported earlier (Asha et al., 2009; Masuda et al., 1991). Differential antifungal activity of β -asarone *in vitro* against various plant pathogenic fungi was observed

(Lee et al., 2004). β -Asarone though known for its antifungal activity but its lipophilic nature prevented its widespread use. Hence, the need to develop a water soluble formulation arose. Current study deals with encapsulation of β -asarone into cyclodextrin derivative to convert it into water soluble form (Petrovic et al., 2010).

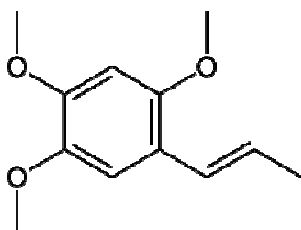
Cyclodextrins (α, β, γ) have been widely used as encapsulating agents which are cyclic oligosaccharides consisting of glucose units fused together as a ring (Loftsson and Brewster, 1996; Sztetli, 1994; Loftsson and Duchene, 2007; Reineccius et al., 2002). Encapsulation of bioactive materials into cyclodextrins not only enhances their stability, but also their bioavailability apart from improving their solubility factor (Hedges et al., 1995; Pagington, 1986; Fernandes et al., 2004; Reineccius et al., 1986).

In the present study we have used β -methyl cyclodextrin (BMCD) as its water solubility is better than other cyclodextrins. We have used different ratios of β -asarone vs BMCD to reach the optimal ratio and to study its activity against the plant pathogens with main emphasis against *P. capsici*. *Phytophthora* species are devastating plant pathogens in both agricultural and natural environment (Blair et al., 2008) causing annual damages of billions of dollars in temperate and tropical regions. *Phytophthora* is a genus that is mainly parasitic

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Table 1. Optimization of encapsulation of β -Asarone into BMCD (β -methyl cyclodextrin).

<i>Acorus calamus</i> L. oil: BMCD ratio	Observed β -asarone encapsulated (%)	Percentage of β -asarone encapsulation into BMCD
1:2	4.0	6.66
1:4	3.42	11.4
1:8	2.97	19.8
1:16	2.84	37.8
1:20	2.71	72.2
1:24	2.52	84.0
1:32	2.38	95.2
1:40	1.61	89.4
1:50	1.19	79.3
1:60	0.76	63.3
1:70	0.58	58.0

**Scheme 1.** Structure of β -asarone.

on various plant hosts. *P. capsici*, a soil borne pathogen is known to infect many species of pepper, tomato, and other members of the Solanaceae family. Several diseases caused by it are fruit rot of peppers (*Capsicum* spp.), leaf spot and blight of black peppers (*Piper nigrum* L.) etc. *Phytophthora capsici* infection commonly occurs in temperate, subtropical, and tropical environments. The crop loss due to this disease varies from country to country and also from region to region within a country. It has been reported to cause diseases on tropical hosts such as cocoa, rubber, papaya, betevine (Roy et al., 2009), and black pepper (Erwin and Rebeiro, 1996). The major diseases identified in black pepper are 'quick wilt and slow wilt' and are referred to as *Phytophthora* foot rot and slow decline (Anandaraj and Sarma, 1995).

MATERIALS AND METHODS

Extraction and identification of β -asarone from *Acorus calamus* L.

Fresh rhizome of *A. calamus* was collected during spring season from marshy areas of Kunigal, Karnataka. The rhizome was dried and powdered and 500 g of powdered rhizome was extracted with ethyl acetate using a soxhlet extractor and after evaporation of the ethyl acetate was obtained brown colored calamus oil with a sweet

smell. The concentrated oil ethyl acetate extract was subjected to column chromatography over silica gel (60 to 120 mesh) and eluted with Hexane/EtOAc (96:4), isolated the β -asarone having HPLC purity of 98.5% (column; Nova-Pak; C18, 4 μ m; 3.9 \times 150 mm; Waters. Mobile phase: 30 mM ammonium acetate in 0.1% acetic acid: acetonitrile 60:40, flow rate: 1 ml/min, detection at: 254 nm) and its structure (1) was confirmed by ¹H NMR (in CDCl₃ using tetramethylsilane as internal standard, δ : 1.88(3H, dd, J = 7.0, 2.0); 3.81(3H, s), 3.84(3H, s), 3.90(3H, s); 5.78(1H, m); 6.52(1H, m); 6.53(1H, s); 6.84(1H, s). These values matched well with the literature data (Lee et al., 2004), and mass spectrometry (GC-MS-QP20105, direct probe and on a Q-TOF MicroTM AMPS MAX 10/6A system).

Encapsulation of β -asarone into BMCD

A. calamus L. containing about 60% (using HPLC method) of β -Asarone was added to a 20% aqueous solution of β -methyl cyclodextrin in different ratios. The contents were continuously stirred using magnetic stirrer at room temperature for 8 h then filtered through celite bed. The clear filtrate was lyophilized to get dried off-white colored powder having differing amounts of β -asarone content as shown in Table 1. This powder will be referred here as β -methyl cyclodextrin encapsulated powder (BMCDEP). Method adopted by Liu et al. (2005) has been used for encapsulation of β -asarone into BMCD. 60% β -asarone present in crude oil was taken for the previous encapsulation. Based on the previous encapsulation data, it is evident that as the ratio of BMCD went up, the inclusion of β -asarone increased. This inclusion efficiency was found to be maximum at 1:32 ratio of β -asarone vs BMCD where about 95% of β -asarone gets encapsulated. This inclusion complex is freely soluble in water (2 g in 1 ml). Thus, by this technique lipophilic β -asarone was made into hydrophilic material and which has been tested against the plant pathogen for its efficacy. It is for the first time β -asarone was made into water soluble powder to check its activity against *P. capsici*.

Isolation and maintenance of *Phytophthora* and other fungal isolates

The pure culture of *P. capsici* was obtained from the infected black pepper leaf tissue following standard microbiological methods (Wang et al., 2009; Erwin and Ribeiro, 1996). The identity of the pathogen was confirmed by performing ITS region PCR, sequencing the ITS region (automated DNA sequencing facility at

Table 2. Inhibition of major fungal plant pathogens *in-vitro* after 72h by BMCDEP.

Concentration	<i>Phytophthora capsici</i>	<i>Sclerotium rolfsii</i>	<i>Alternaria alternata</i>	<i>Nigrospora spherica</i>	<i>Fusarium oxysporum</i>
BMCDEP containing β -asarone @10 ppm	58.33	0	-54.52	05.00	02.55
BMCDEP containing β -asarone @30 ppm	95.83	16.84	-24.24	11.10	03.88
BMCDEP containing β -asarone @50 ppm	98.95	14.61	-15.50	15.50	10.80
BMCDEP containing β -asarone @100 ppm	98.60	51.82	10.00	32.40	12.33
BMCDEP containing β -asarone @200 ppm	99.20	60.12	54.00	63.41	32.00
BMCDEP containing β -asarone @300 ppm	99.80	70.74	63.78	61.11	52.90
BMCDEP containing β -asarone @400 ppm	100.0	82.66	70.00	83.33	73.50
Ridomil MZ @1 g/L	100	89.24	83.70	92.38	86.70

Bangalore Gene, India) which matched with already defined sequences (BLAST search against the Genbank nucleotide sequence databases) (Cooke et al., 2000). The ITS rDNA region of the *Phytophthora* isolates were amplified using the universal primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT G 3') (White et al., 1990) and the sequence was compared with NCBI database (National Center for Biotechnology Information, USA). Other standard fungal cultures namely: *Sclerotium rolfsii*, *Nigrospora spherica*, *Alternaria alternata*, *Fusarium oxysporum* were obtained from MTCC (Microbial Type Culture Collection, IMTECH, Chandigarh, India).

***In vitro* analysis of β -asarone on fungal plant pathogens**

A stock solution containing 500 ppm β -asarone was prepared by dissolving suitable amount of BMCDEP in 100 ml of water and sterilized. The calculated quantities of the stock solution were added then to the autoclaved potato dextrose agar (PDA) media under aseptic conditions to make all the test concentrations (10, 30, 50, 100, 200, 300 and 400 ppm) and poured in sterile Petri plates and solidified. All treatments were replicated thrice. While PDA plates without β -asarone were kept as untreated control, three plates with 0.1% Ridomil MZ58 were used as chemical control. Using sterilized 0.8 mm diameter cork borer, disks were punched onto the three days' old fungal cultures. Each disk was picked using a sterile needle and placed in the similar well made at the center of the test plates. The plates were wrapped with cling film and incubated in dark at 27°C. Growth of the fungi was observed after 72 h and the percentage inhibition was worked taking the fungal growth in untreated control vis-à-vis with all other treatments.

Zoospore motility and spore germination studies for *Phytophthora*

Zoospore release by *P. capsici* was triggered by replacing with prechilled sterile distilled water (at 4°C) and then warming for 20 min at room temperature. Zoospore concentration was adjusted to 100 per ml. To assess zoospore motility, 5 ml of an adjusted zoospore suspension was added to each of two replicate Petri dishes containing equal volumes of β -asarone solution at final concentrations of 0, 10, 20, 30, 50 ppm. Zoospore suspensions were maintained at 25°C and observed microscopically to record the time for complete cessation of motility. For zoospore germination test, 1 ml of an adjusted zoospore suspension was spread onto each of the two replicate Petri dishes of carrot juice agar containing β -asarone at a concentration of 0, 10, 20, 30, 50 ppm. After incubating for 2 days at 25°C in dark, the number of *Phytophthora* colonies appearing in each plate was recorded.

RESULTS AND DISCUSSION

Antifungal activity of BMCDEP on fungal plant pathogens

Different plant pathogenic fungi reacted differently to the varied concentrations of β -asarone in the form of BMCDEP incorporated in PDA (Table 2). Since β -asarone is insoluble in water it did not give consistent results when incorporated into PDA and hence, BMCDEP has been employed in determining the antifungal activity. The effect was very prominent against *P. capsici* with more than 50% inhibition even at 10 ppm β -asarone and more than 95% growth inhibition at 30 ppm β -asarone as shown in Figure 1. The BMCDEP formulation could reduce the growth of *S. rolfsii*, *A. alternata* and *N. spherica* by about 50 to 60% at 200 ppm levels of β -asarone (Figure 2). *F. oxysporum* needed more concentration (300 ppm) of β -asarone to cause a growth inhibition of more than 50% *in vitro* (Table 2 and Figure 3). The BMCDEP formulation completely inhibited the germination of zoospore at a concentration of 10 ppm and zoospore motility was ceased within a minute.

Conclusion

The lipophilic β -asarone has been successfully encapsulated into BMCD which was easily soluble in water. It was found that at a ratio of 1:32 of β -asarone and BMCD the encapsulation was found to be maximum (about 95%). The antifungal activity varied against different fungi. Among various plant pathogens tested, it was able to inhibit *P. capsici* more effectively at a concentration of 30 ppm while it needed a minimum 200 ppm to cause about 50% inhibition against *S. rolfsii*, *A. alternata* and *N. spherica*. *F. oxysporum* still need higher 300 ppm to get 60% inhibition *in vitro*.

ACKNOWLEDGEMENT

The authors are thankful to DBT, government of India for the financial support (Grant No:

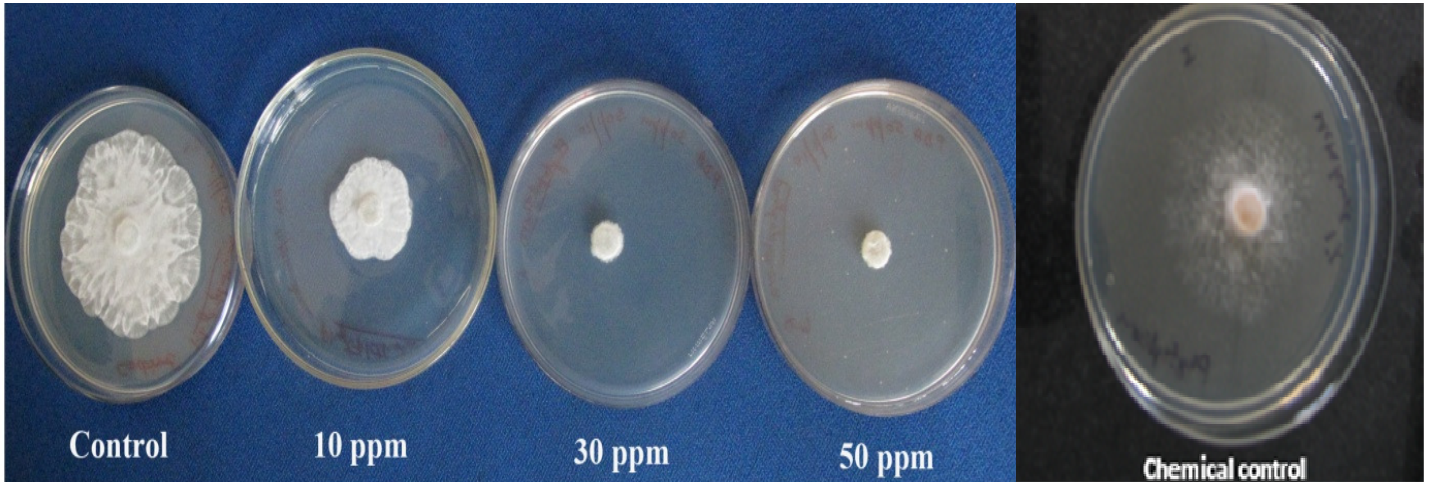


Figure 1. *In-vitro* Inhibition of mycelia growth of *Phytophthora capsici* by β -asarone incorporated in PDA.

Inhibition after 72 h

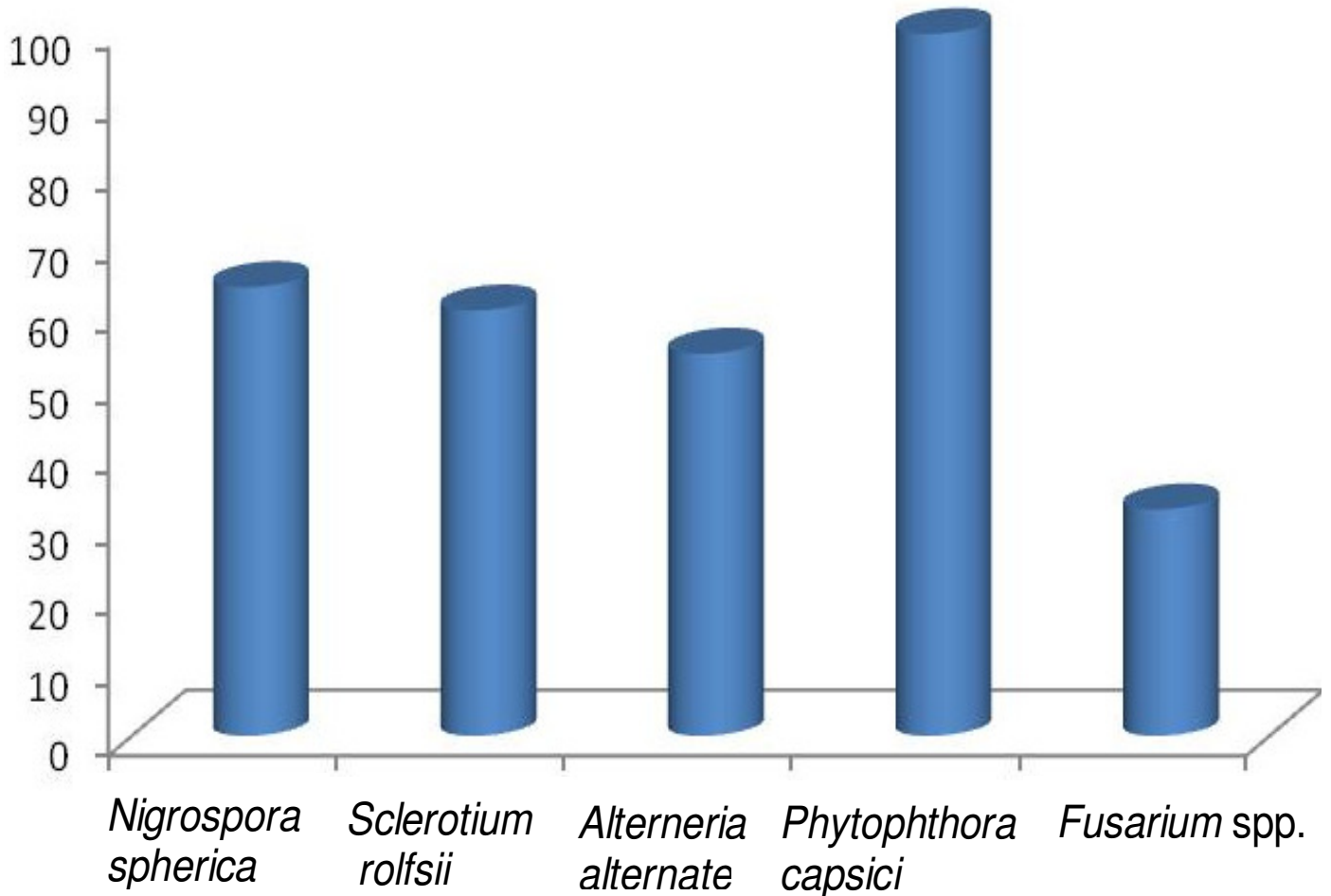


Figure 2. Inhibition of fungal pathogens with BMCDEP at 200 ppm β -asarone.

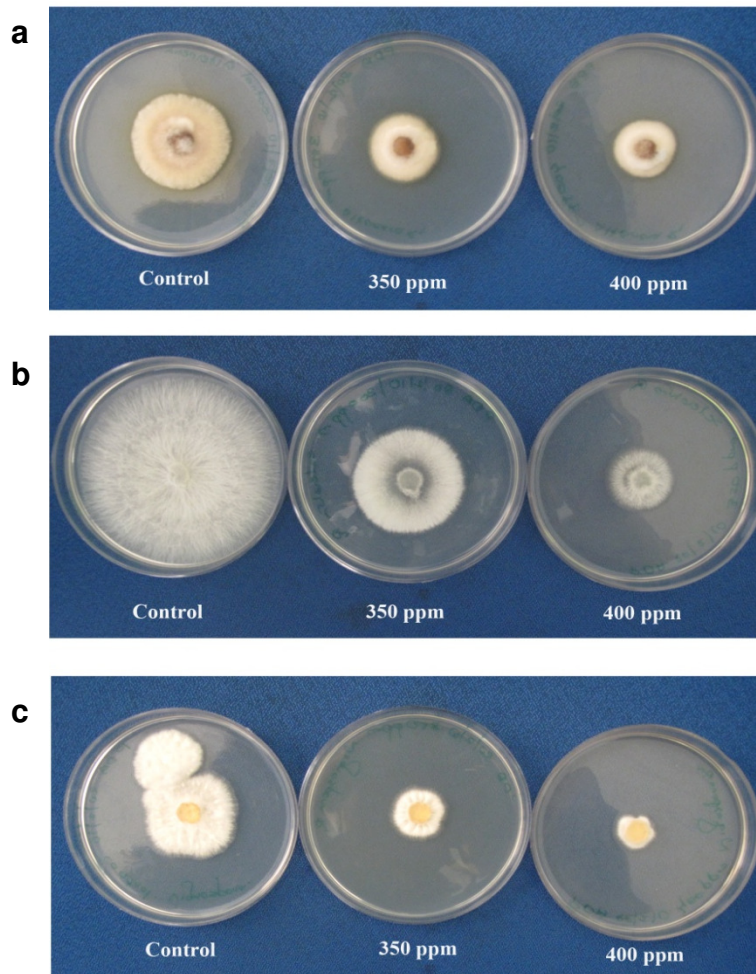


Figure 3. Inhibition of mycelia growth of fungi on β -asarone incorporated in PDA. (a) *Alternaria alternata* (b) *Sclerotium rolfsii* (c) *Nigrospora spherica*.

BT/PR11756/AGR/05/456/2009t) to take up the research work on botanical pesticides.

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