Indian J. Fish., 61(2) : 90-96, 2014

Lagenidium sp. infection in the larval stages of the freshwater prawn *Macrobrachium rosenbergii* (DeMan)

A. KRISHNIKA AND P. RAMASAMY

Department of Biotechnology, University of Madras, Guindy Campus, Chennai – 600 025, Tamil Nadu, India

e-mail: krishnika@gmail.com, ramasamy_p@hotmail.com

ABSTRACT

This study investigated the occurrence, infectivity and pathogenecity of *Lagenidium* sp. in the hatchery of *Macrobrachium rosenbergii.* Microscopic examination revealed that the *Lagenidium* sp. infected zoea appeared white in colour. The presence of non-septate fungal hyphae was observed within the body of the exposed larvae replacing nearly all the muscles. Light and scanning electron microscopy demonstrated that 24 h post-exposure, the infection began to appear externally. After 32 h, zoospores appeared on external hyphae of infected larvae. The fungus *Lagenidium* sp. exhibited optimum growth at 30 ^{0}C, 0-2% NaCl and slow growth at 5% NaCl on potato dextrose agar (PDA). Antimicrobials, clotrimazole and griseofulvin were found to be more effective than miconazole, itraconazole and fluconazole in inhibiting the growth of *Lagenidium* sp. Under experimental conditions, *Lagenidium* sp. causes 100% mortality within two days of infection, producing 10^3 spores $ml^{-1,}$

Keywords: Fungal disease, Infection, *Lagenidium*, *Macrobrachium rosenbergii.* Zoospore

Introduction

Species of *Lagenidium* have been encountered in various hosts from both freshwater and marine habitats (Sparrow, 1973) and found to be particularly troublesome in the aquaculture of marine decapod crustaceans (Lightner, 1977). *Lagenidium callinectes* has been described from ova of the blue crab, *Callinectes sapidus* (Bland and Amerson, 1973), from the barnacle, *Chelonibia patula* (Johnson and Bonner, 1960) and even from several species of marine algae (Fuller *et al.,* 1964). *Lagenidium chthamalophilum* has been described from ova of the barnacle, *Chthamalus fragilis* (Johnson, 1958). *Lagenidium giganteum* has been reported in mosquito larvae, the water flea, *Daphnia* and copepods (Couch, 1942). Undescribed species of *Lagenidium* have also been isolated from cultivated white shrimp, *Litopenaus setiferus* (Lightner and Fontaine, 1973), brown shrimp *Penaeus azteus* (Lightner, 1975), *Penaeus monodon* (Ramasamy *et al.,* 1996) and wild crustaceans such as the dungeness crab, *Cancer magister* (Armstrong *et al.,* 1976) and the American lobster, *Homarus americanus* (Nilson *et al.,* 1976). Fungal diseases that infect larval *Macrobrachium rosenbergii* remain to be investigated. The present study is an attempt to investigate *in vitro* growth characteristics, resistance to antifungal agents, pathogenicity and histopathology of *Lagenidium* sp. infection in the freshwater prawn *Macrobrachium rosenbergii.*

Materials and methods

Isolation of fungi

Samples of eggs, larvae and post-larvae of *M. rosenbergii* from larval rearing tanks were routinely observed under microscope for the presence of fungi. When fungi were observed, 100 mg wet samples of infected larvae were homogenised in a sterile tissue homogeniser, containing 1 ml of 12 ppt autoclaved water. A small aliquot (100 µl) of homogenised tissue samples was then inoculated on potato dextrose agar (PDA) plate. The inoculated plates were incubated for about 4-7 days at 30 ºC and the growth of fungi was monitored (Fuller *et al.,* 1964).

Identification of oomycete

A small portion of the sporulating fungal colony was removed with a sterile needle and placed on glass slides containing Amman's mounting medium (Onions *et al.,* 1981). The size, shape and structure of the fungal zoospores were examined using a binocular research microscope and the fungi were identified as per Karling (1981), Willoughby (1970) and Lilley *et al*. (1999).

Induction of zoospore formation of the fungal isolates

Fungal colony of 5.5 mm diameter (sterile no. 2 cork borer) was cut from an actively growing edge and placed

in a beaker containing 50 ml of sterile 12 ppt water and then incubated at 27-30 ºC for 24-48 h to induce zoospore formation. Zoospore development and morphology were studied using a light microscope following the criteria of Nakamura and Hatai (1995).

Effect of temperature on the growth of the fungi

The growth of fungal isolates in potato dextrose agar was monitored at four different temperatures at 10, 20, 30, and 40 ºC, in triplicate. A 5.5 mm diameter agar disc taken from the actively growing colony edge was placed in the centre of PDA Petriplates. By measuring the colony diameter (Nakamura *et al.,* 1994) the growth rate of each isolate was determined daily.

Effect of NaCl concentration on the growth of the fungi

Potato dextrose agar medium was prepared with different levels of NaCl *viz*., 1, 2, 3, 4 and 5% NaCl and twenty ml of the medium with each concentrations of NaCl was poured into petriplates. As described earlier, a 5.5 mm diameter agar disc taken from the actively growing colony edge was placed in the centre of PDA Petriplates and incubated at 27- 30 ºC. Growth rates were determined daily by measuring colony diameter of each triplicate preparation (Nakamura *et al.,* 1994).

Effect of antifungal agents on the growth of the fungi

Antifungal agents *viz*., clotrimazole, griseofulvin, micanozole, itracanozole and flucanozole were tested against *Lagenidium* sp. isolated from *M. rosenbergii*. Different concentrations (1 -10 g ml^{-1}) of antifungal agents were used to determine the minimum inhibitory concentration of the antifungal agents in effectively controlling the *in vitro* growth of *Lagenidium* sp. on PDA. The growth rate of the fungus was monitored in triplicate plates as described previously (Armstrong *et al.,* 1976).

Experimental infection and pathogenicity of fungi

Pathogenicity of the isolated *Lagenidium* sp. from *M. rosenbergii* larvae was determined using first stage larvae of *M. rosenbergii*. First stage larvae of *M. rosenbergii* were collected from the Periyar Integrated Hatchery, Chennai and were kept in tanks containing 12 ppt sterile brackishwater under continuous aeration. A 5.5 mm diameter disc cut from an actively growing edge of a colony of each *Lagenidium* sp. was placed onto 50 ml of sterile brackishwater and then incubated at 27-30 ºC to induce zoospore formation. Zoospore densities were estimated with the aid of a haemocytometer. In a 1l beaker, 500 ml of 12 ppt sterile brackishwater containing zoospore suspension (10^3 spores ml^{-1}) was exposed to $1x10^3$ 1st stage larvae of *M. rosenbergii*. All the experimental containers were continuously aerated. After initial exposure to fungal zoospores, larvae from each beaker were collected randomly and were examined for fungal infection under a microscope. Fungal infected larvae were used for re-isolation of the fungi on PDA agar. Controls without inoculation of the fungal zoospores were also maintained in the experimental set up.

For scanning electron microscopy, experimentally infected and control larvae of *M. rosenbergii* were collected at 4 h intervals post-exposure over the next 24 h, washed repeatedly with 12 ppt sterile brackishwater and they were fixed for 24 h in 4% glutaraldehyde buffered to pH 7.2 with 0.1M sodium cacodylate-HCl containing 3% sucrose and 0.5% sodium chloride, and post-fixed in 1% osmium tetroxide for 1h. Following post-fixation in 1% osmium tetroxide, all larval samples were then buffer washed repeatedly at intervals of 2h using 0.1M sodium cacodylate-buffer for 24 h at 4 ^{0}C, dehydrated in a graded series of acetone, dried in a CPD 750 critical point dryer using liquid carbon dioxide, mounted on aluminium stubs, sputter-coated with gold-palladium and examined in a scanning electron microscope (Ramasamy *et al.,* 1996; 2000; Rajan *et al.,* 2000). *Lagenidium* exposed larvae were examined for severity and extent of fungal growth every 4 h post initial exposure.

Results and discussion

The present study has demonstrated the occurrence of *Lagenidium* sp. infections in zoea of the freshwater prawn *M. rosenbergii* in India. *Lagenidium* has been particularly troublesome in aquaculture of marine decapod crustaceans (Lightner, 1977). *Lagenidium callinectes* was first described by Couch (1942) on the eggs of blue crab, *Callinectes sapidus* and later discovered from the eggs and larvae of various crabs and shrimp (Crisp *et al.,* 1989). The fungus *Lagenidium* has been reported as a parasite of various marine crustaceans and was isolated from the eggs of the barnacle *Chelonibia patula* (Johnson and Bonner, 1960), and larvae of the white shrimp *Litopenaeus setiferus* (Lightner and Fontaine, 1973), brown shrimp *Penaeus azteus* (Lightner, 1975), the dungeness crab *Cancer magister* (Armstrong *et al.,* 1976) and tiger shrimp *Penaeus monodon* (Ramasamy *et al.,* 1996). *Lagenidium scyllae* is known as a parasite of the eggs and larvae of mangrove crab (Bian*et al.,* 1979), while *Lagenidium myophilium* has been reported from various stages of the shrimp of the genus *Pandalus* (Hatai and Lawhavinit, 1988; Nakamura *et al.,* 1994).

Microscopic examinations revealed that the fungus *Lagenidium* sp. infecting zoea of *M. rosenbergii*, appeared white in colour and hyphae were observed within the body of the larvae, invading and replacing nearly all tissues of the larvae. Fungal hyphae are irregularly branched, non-septate and generally develop vesicles at the apical end of the hyphae. The vesicles develop from the mass of sporoplasm from which zoospores develop. Zoospores were released by rupturing of the vesicles or through a small perforation in each vesicle (Fig. 1). Rupturing of the vesicle appears to be the primary method of zoospore release in *L. callinectes* (Nakamura and Hatai, 1995). In contrast, zoospores of *L. scyllae* are released one by one from small multiple perforations on vesicles, or simultaneously by rapid deliquescence of the vesicle wall (Bian *et al.,* 1979). Both modes of liberation are observed in *L. callinectes* isolated from the egg of the crab *Portunus pelagicus* (Nakamura and Hatai, 1995). In *L. myophilum*, the entire vesicle may separate from the discharge tube before the zoospores were released (Nakamura, 1994). In the present study zoospore liberation was found to occur after the vesicles got separated from the discharge tube. Bursting of the vesicle, discharges the zoospores of the fungus or zoospores are released one by one through a pore on the vesicle. The envelope of the vesicle is gelatinous in *L. myophilium* while non-gelatinous in *L. scyllae*. *L. myophilium* has a longer discharge tube, a smaller vesicle and a smaller encysted zoospore than *L. callinectes* (Hatai and Lawhavinit, 1988). Zoospore formation was observed to occur after 12h. Incubation temperature also affects zoospore production (Nakamura *et al.,* 1994). Rogers-Talbert (1948) reported that egg to egg transmission is extremely rapid, and the entire exposed group of eggs can be destroyed by *Lagenidium* sp. in three or four days. In aquaria, the eggs of healthy egg bearing crabs were infected when crabs with diseased eggs were introduced. Infection is brought about by zoospores, which settle on the eggs and encyst. Eggs thus infected are totally destroyed by the fungus within 48 h and are filled with a mycelium of branched, sparingly septate, thin walled hyphae. Zoospores mature in the vesicle and are released within an hour after infestation (Couch, 1942; Rogers-Talbert, 1948).

Scanning electron microscopy (SEM) revealed the progression of invading *Lagenidium* sp. in the larvae of *M. rosenbergii*. An increase in diameter (size) and number of hyphae of *Lagenidium* sp. within infected larvae occurs over time from exposure. SEM demonstrates fungal hyphae that appeared externally on infected larvae as early as 24 h post-exposure. Initially erupting hyphae were stout, irregularly branched and non-septate. After 32 h of infection, fungal vesicles were seen among the hyphae. After 40 h of the fungal infection, the zoospores were seen on the infected larvae (Fig. 2 a-f). The results of the present study have shown that the fungus *Lagenidium* sp. causes up to 100% mortality within four days of infection with 10^2 -10^3 spores ml^{-1} (Table 1). *L. callinectes* and *L. scyllae* are reported to cause 70% mortality with

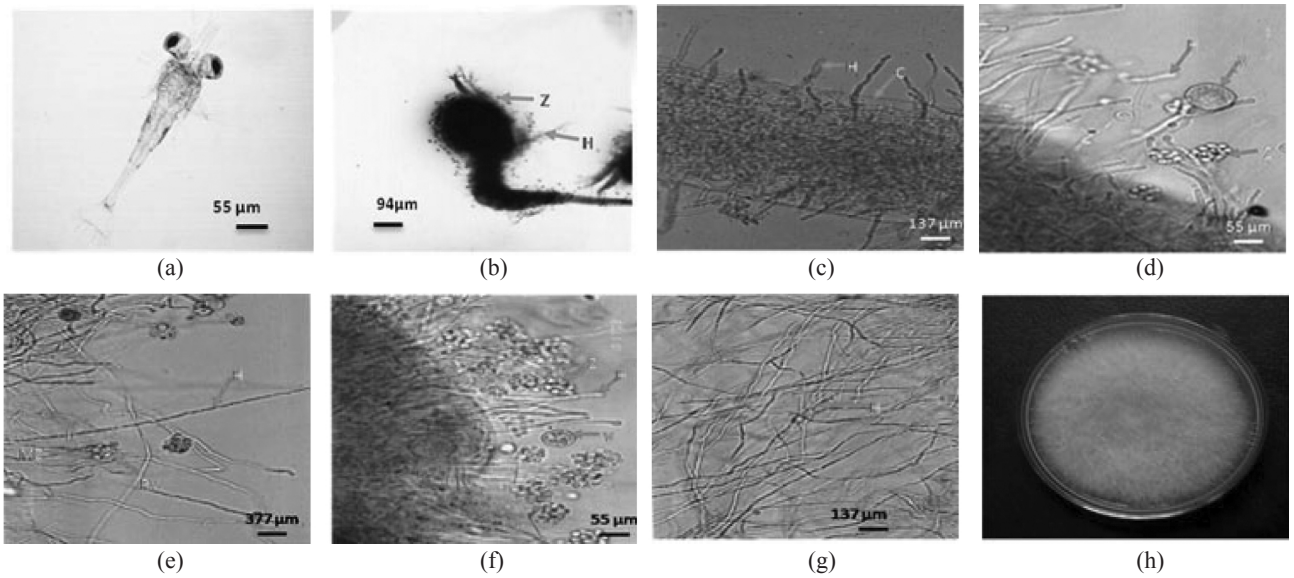

Fig. 1. *Lagenidium* sp. infection in *Macrobrachium rosenbergii* Light micrograph showing: (a) Healthy uninfected larva, (b) *Lagenidium* sp. infected larva, (b) – (g) Light micrographs of growing fungal hyphae protruding through a portion of the larval cuticle, Young vesicle [V] formed at the tip of the fungal hyphae [H] and Mature vesicle with zoospores [Z], (h), Colony of *Lagenidium* sp. grown on potato dextrose agar plate

10^3 spores ml^{-1} (Bian *et al.,* 1979; Crisp *et al.,* 1989). *Lagenidium* infection was reported to be severe with mortality rates exceeding 90% in laboratory reared lobster larval populations (Nilson *et al.,* 1976). Armstrong *et al*. (1976) found the fungus *Lagenidium* to cause 40% mortality in second stage zoea of the dungeness crab *C. magister*. In penaeid shrimp, the mortality can be up to 100% (Lightner, 1977). Incubation temperature is known to affect zoospore production (Nakamura *et al.,* 1994).

The fungus *Lagenidium* sp. isolated from larval *M. rosenbergii* showed growth at all experimental temperatures (10 °C, 20 °C, 30 °C and 40 °C) (Fig. 3) but exhibited maximum growth at 30 °C, with the mean colony size of 88.66 ± 1.24 mm after 5 days of incubation. Thus the growth temperature of the fungus seems to be consistent with that of the host habitat. Similarly *L. myophilium* from *Pandalus* was reported to grow at 5 °C and not at 30 °C (Nakamura *et al.,* 1994). In contrast, thermotolerant growth was reported in *L. scyllae* (Bian *et al.,* 1979) and *L. callinectes* isolated from *P. pelagicus* (Nakamura and Hatai, 1995).Growth of the fungus *Lagenidium* sp. isolated from the larval *M. rosenbergii*

Table 1. Mortality rate zoea of freshwater prawn *M. rosenbergii* experimentally infected with *Lagenidium* sp. (Total no. of zoea = 100)

Total no. of zoospores inoculated	Percentage mortality (Days post-infection)			
	Day 1	Day 2	Day 3	Day 4
10^2 Zoospores ml^{-1}	0	5	15	42
	0	7	19	43
	0	8	21	45
10^3 Zoospores ml^{-1}	0	9	29	52
	0	12	35	52
	0	15	32	51
10^4 Zoospores ml^{-1}	0	15	30	53
	0	13	30	54
	0	19	35	50

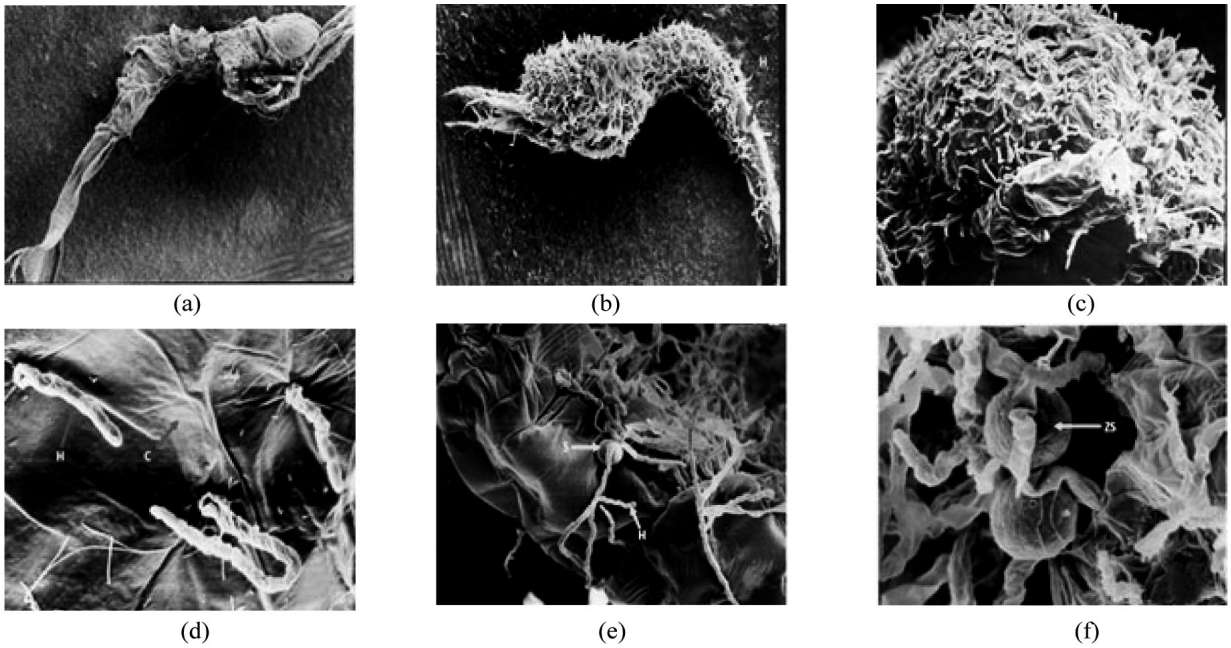

Fig. 2. Scanning electron micrograph showing *Lagenidium* sp. infection in larval *Macrobrachium rosenbergii,* (a) Healthy uninfected larva of *M. rosenbergii* (X 86), (b) After 40 h of infection with *Lagenidium* sp. (X78), (c) Hyphae (H) protruding throughout the cuticle (X 300), (d) *Lagenidium* sp. growing piercing through the cuticle (C) (X 1500), (e) Hyphae (H) of *Lagenidium* sp. filling the body of the larva and fungal spore (S) on the surface of the infected larva (X540), (f) Larvae with zoospore (ZS) (X 2000).

was observed at all (1-5%) NaCl concentrations, with a proportional increase of growth in size from day 1 to day 5. The current study has shown that the mean colony size of the *Lagenidium* sp. recorded was 76.66 ± 1.69 mm, 88.66 ± 1.24 mm, 66±0.8 mm and 47.66 ± 1.24 mm at NaCl concentrations of 1, 2, 3 and 4% respectively. Moreover the fungus exhibited optimal growth at a concentration of 2% NaCl, followed by 1%, 3%, 4% and the least growth at 5% NaCl indicating that the fungal strain *Lagenidium* sp. can tolerate and multiply both in freshwater and brakishwater where normally the host larval *M. rosenbergii* is maintained (Fig. 4). However, because the present fungal strain is known to grow well on media without seawater, it is not exclusively marine like *L. scyllae* and *L. myophilium* (Bian *et al.,* 1979; Hatai and Lawhavinit, 1988; Nakamura *et al.,* 1994). The present investigation has also shown that clotrimazole followed by griseofulvin were the most effective antifungal agents of *Lagenidium* sp. isolated from the larval *M. rosenbergii*. However, the fungal growth was inhibited by all five antifungal agents tested in the study. The minimum inhibitory concentration of the growth of *Lagenidium* sp. was found to be 1g ml^{-1} clotimazole, 3 g ml^{-1} griseofulvin, 4 g ml^{-1} miconazole, 8 gml^{-1} itraconazole, and 10 g ml^{-1} fluconazole (Fig. 5). Mycostatic and mycocidal doses for the larval zoea, mysis and post-larval prawn were identified *in vitro* (Lio-Po *et al.,* 1982; 1985). The mycostatic dose of 20 mg l^{-1} copper sulphate was reported to be destructive to zoea, mysis

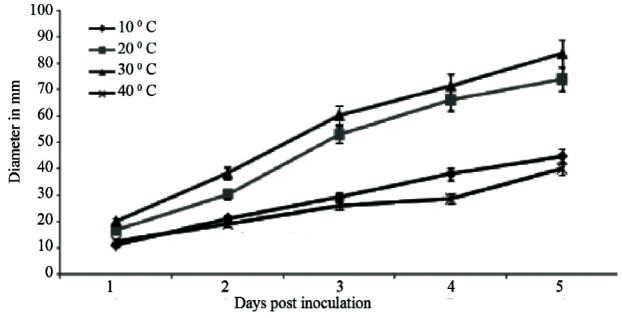

Fig. 3. Effect of temperature on the growth of *Lagenidium* sp.

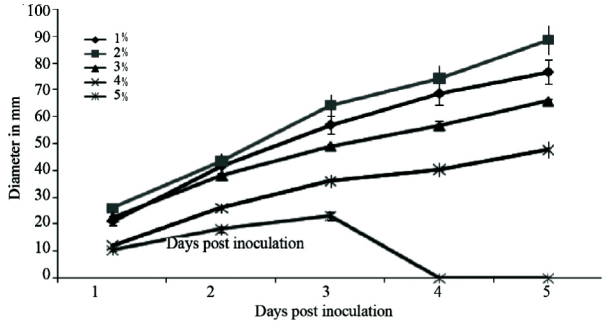

Fig. 4. Effect of salinity on the growth of *Lagenidium* sp.

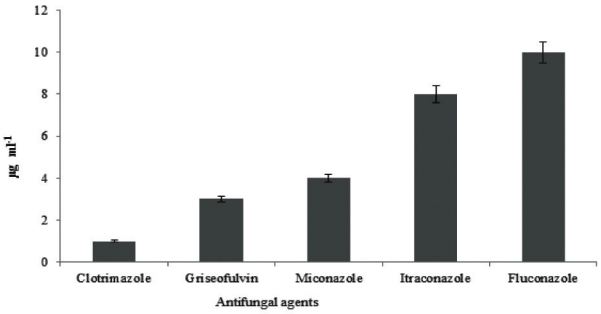

Fig. 5. Minimum inhibitory concentration for *Lagenidium* sp. against various anti-fungal agents.

and post-larvae since the 24 h LC50 level was less than 1 mg l^{-1} (Canto, 1977). LC50 levels of 1.6 mg Furanace l^{-1} and 2.2 mg Furanace l^{-1} for zoeae and mysis were reported (Gacutan and Llobrera, 1977). At 0.11 mg malachite green l^{-1} zoea can lead to larval death, while mysis and post-larvae could withstand the mycostatic dose of 0.4 mg l^{-1} (Lio-Po *et al.,* 1978). A 0.5 ppm treflon treatment inhibited the growth and spread of *Lagenidium*, while 0.1 ppm treflon treatment was less effective (Ramasamy *et al.,* 1996). Further investigations are needed to determine the *in vivo* effects of the most effective antifungal agents in inhibiting the growth of *Lagenidium* sp. in the larval zoea, mysis and post-larvae of *M. rosenbergii,* and their LC50 level and mycostatic doses of clotrimazole and griseofulvin to zoea, mysis and post-larvae.

Acknowledgement

The first author thank the University of Madras for the financial support through the award of Dr.A. Gnanam Endownment Award.

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Date of Receipt : 08-10-2012

Date of Acceptance : 23-11-2013