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The consequences of inhibition of ergosterol biosynthesis in *Sclerotinia sclerotiorum* (Lib) de Bary by Hexaconazole

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Abstract

Sclerotinia sclerotiorum (Lib) de Bary is one of the most non-specific, and ubiquitous fungal plant pathogens affecting agricultural crops. This is known to cause some important diseases like white mold, *Sclerotinia* wilt or stalk rot, or *Sclerotinia* head rot on a wide variety of broadleaf crops. This pathogen infects about 408 species of plants. The brief study with *S. sclerotiorum* and its control with hexaconazole, (ergosterol biosynthesis inhibitor) revealed that the fungicide is a potential antifungal and that it affects the biology of the pathogen in a variety of ways. It directly affects the cell and can bring about growth inhibition by impairing one or the other of the vital phenomena. All the vital functions of the fungus appear to have been impeded. The study also revealed that the mode of action of fungicide is not only by inhibiting ergosterol biosynthesis but probably also by an ancillary mode by photo-oxidation of ergosterol wherein, hexaconazole acted as a photo-sensitizer. This study also reveals that hexaconazole, exerted an excellent fungistatic effect at 100 µg/mL concentration on *S. sclerotiorum.* Therefore, this fungicide could be effectively used for control of the notorious pathogen, *S. sclerotiorum* at a time when many pathogens are acquiring resistance to different classes of fungicides.

Key-Words: *Sclerotinia sclerotiorum,* Hexaconazole, Photo-sensitizer, Ergosterol biosynthesis inhibitor

Introduction

Sclerotinia sclerotiorum (Lib) de Bary is one of the most devastating pathogens and is of a cosmopolitan occurrence. More than 60 names have been used to refer to the diseases caused by this fungal pathogen (Purdy, 1979 and Le Tourneau, D. 1979) including cottony rot, watery soft rot, stem rot, drop, crown rot, blossom blight and, perhaps most common, white mould.(Chet and Henis, 1975; Adams and Ayers, 1979; Willets and Bullock, 1992). This pathogen is known to infect about 408 species of plants in 34 families (Purdy, 1979). *S. sclerotiorum* poses a threat to dicotyledonous crops such as sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry pea, lentils and various vegetables, besides monocotyledons such as onion and tulip (Boland and Hall, 1994). Annual losses caused by *S. sclerotiorum* in the United States, for example, have exceeded $200 million. In a 1999 Sclerotinia head rot epidemic on sunflower, the crop loss was estimated at $100 million (Anon, 2005).

Extensive crop damage, lack of high levels of host resistance and the general difficulty of controlling diseases caused by *S. sclerotiorum* have been the impetus for sustained research on this pathogen. Diseases are of common occurrence in plants, often exerting a significant economic impact on yield and quality, thus rendering managing the diseases an imperative for most crops (Willetts, H.J. and Wong, J.A. 1980).

Fungicides either kill the organism by damaging the cell membranes, inactivating enzymes or proteins, or by interfering with certain vital processes. Fungicides can either be contact or systemic. Others impact specific metabolic pathways such as the production of sterols or chitin. Many of these elicit a response from the host plant known as Systemic Acquired Resistance (SAR).

Hexaconazole (See fig-1) belongs to a group of triazole fungicides that has protective, curative and systemic activity.

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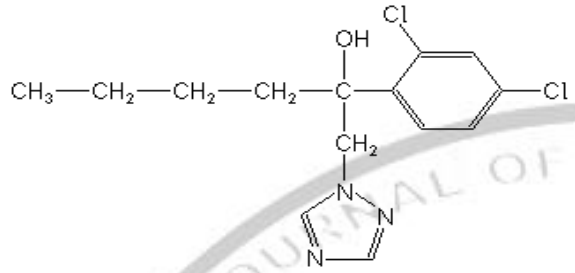

Fig. 1: Hexaconazole

This study was undertaken to evaluate the efficacy of hexaconazole against *Sclerotinia sclerotiorum*, a notorious fungus with regard to: Vegetative growth, sclerotium production and germination and the efficacy of the fungicide against certain environmental variables. The fungicide's effect on macromolecular synthesis and certain vital functions as respiration and antioxidant enzymes has also been studied.

Material and Methods

The test fungus was raised on Czapek-Dox agar containing Sodium nitrate (2.0 g), Potassium phosphate (1.0 g), Magnesium sulphate (0.5 g), Potassium chloride (0.5 g), Ferrous sulphate (0.01 g), Sucrose (30.0 g) and Distilled water (1000 mL). Discs were cut out with a cork borer of 7 mm diameter from the margin of a 5 day old culture and were transferred to 9 cm petridishes containing the experimental media. Whenever liquid medium was used, 30mL of it was taken in 100 mL Erlenmeyer flasks and the same inoculated with a 7 mm disc of the mycelium. A light chamber as described by Leach (1968) was used in the study. In experiments requiring light, two Philips cool white/day light (6500k) fluorescent lamps providing energy @ ~50µEm^{-2} s^{-1} were used. The cultures were exposed to light through the lids of the petridishes.

Effect of fungicide on radial growth, intensity of hyphal branching, sclerotium formation and sclerotium germination

The test fungus was inoculated onto the CDA amended with different concentrations of hexaconazole (Analytical grade chemicals from sigma (St.Louis, USA)) and incubated for 5-7 days at 25°C.

The radial growth of the fungus was measured for 7 days at daily intervals.

The intensity of hyphal branching was determined after 5 days of incubation by counting the number of hyphal tips under the low power of the microscope and the same expressed as the number of tips/mm of colony margin. The average of 10 random counts was given as the number of hyphal tips.

Next, the effect of fungicide on the sclerotia production was studied. The degree of sclerotia production was assessed by enumerating the sclerotia as a function of the test inhibitor's strength after 10 days of growth.

To assess the germination potential of the sclerotia, 30 number sclerotia were inoculated on CDA. On the third day, the sclerotium germinating potential of the fungus under test conditions was assessed. Inoculated plates were kept in dark at 25°C. Percent inhibition of sclerotium germination was calculated and expressed as percent reduction over control.

Biomass measurement of *Sclerotinia sclerotiorum*

The biomass of *S. sclerotiorum* was measured by growing the fungus in Czapek-Dox broth. Two discs of 7mm diameter each were inoculated into 100 mL Erlenmeyer flasks containing 30mL medium and then the flasks were incubated over a period of 10 days. For estimating the dry weight, the mycelia were filtered on a pre-weighed Whatman No.1 filter paper and dried in an oven at 60°C to a constant weight.

Effect of light on the efficacy of the fungicide

For the effect of light on the efficacy of the fungicide, the test fungus was inoculated onto CDA amended with different concentrations of fungicide (pH 6.5) and was irradiated by visible light at a distance of 45cm. Two source of visible light namely; a white fluorescent light and a cool day light were used in the experiment. A dark control was maintained. Colony diameter of the test fungus was measured after 3 days.

In order to find out as to which of the visible wavelengths is more efficacious, the test fungus was inoculated onto CDA are amended with different concentrations of fungicide and the inoculated plates were wrapped with Green, Blue, Yellow and Red colour cellophane paper. Then the plates were kept under the light in the light chamber. The colony diameter was measured after 3 days of exposure to light. A dark brown culture served as the control.

Determination of optimum pH, optimum temperature and water activity for the efficacy of the fungicide

The test fungus was inoculated into Czapek-Dox broth amended with different concentrations of fungicide and adjusted to different pH values, temperatures and molar strength of glucose to yield the corresponding water activity levels and incubated for 10 days.

Effect of fungicide on membrane integrity

The conductivity of the culture filtrates of the culture grown in different concentrations of the fungicide was measured in a Conductivity Bridge and the conductivity given in terms of 'Siemens' to note the change in the conductivity, if any.

Effect of fungicide on respiration

Around 5 mL of the culture filtrate was taken in a boiling test tube, shaken well and oxygen consumption was measured in YSI oxygen monitor at 5 minute intervals.

Effect of fungicide on lipid and sterol biosynthesis

The lipid and sterol contents were estimated by Folch *et al*., (1957) method and Courchain *et al.,* (1959) method respectively.

Estimation of total protein

The protein was estimated by the Bradford method, (1976).

Effect of fungicide on anti-oxidant enzyme activity

Both the anti-oxidant enzyme (catalase & peroxidase) levels were estimated by Beers and Siezer, (1952) and Anonymous, (1972) methods.

Specific Activity of Catalase

$$\text{Specific activity} = \frac{\text{Change in absorption/minute X 1000 X dilution factor}}{\text{43.6 X Protein in mg/g}}$$

$$\text{O.D Change/minute} = \frac{\text{Final O.D – Initial O.D}}{3}$$

Specific Activity of Peroxidase

$$\text{Specific activity} = \frac{\text{Change in absorption/minute X 1000 X dilution factor}}{\text{11.3 X Protein in mg/g}}$$

$$\text{O.D Change/minute} = \frac{\text{Final O.D – Initial O.D}}{3}$$

Results and Discussion

In the present study, an attempt has been made to control the growth of *Sclerotinia sclerotiorum* with hexaconazole, an ergosterol biosynthesis inhibiting fungicide. The consequences of ergosterol biosynthetic inhibition were studied.

The effect of the fungicide on the biomass of the fungus against increasing doses of the fungicide was evaluated. It was observed that the biomass dwindled with increase in the strength of the fungicide. However, at 100µg/mL level, the fungicide was found to be fungistatic but not fungicidal. As for the hyphal morphology, the treated mycelium produced short, slender hyphae with profuse branching. This is a well-known effect of the fungicide on hyphal branching potential. The thickening of the hyphal walls (Plate1 & Fig 2, 3 & 4) was evident but hyphal tip swellings or tip bursting was observed not only at 100µg/mL of the fungicide. Next, the effect of the fungicide on sclerotium production and germination were studied (Haverkate, F., Tempel, A. and A. J. Den Held. 1969). As expected of any fungicide, these two important phenomena which are crucial for the proliferation and establishment of the fungus on the host tissue have been adversely affected confirming the sensitivity of the pathogen to the fungicide (Fig 5 & 6).

The environment has a profound influence on the growth and metabolism of fungi. In the following studies the efficacy of the fungicide in response to the environment was evaluated. This was done by manipulating the physical and chemical environment. The effect of light as an important environmental factor was studied. Generally, light was found to have exacerbated the effect of the fungicide (Fig 7a & b). Visible light enhanced the efficacy of the drug. Among the visible wavelengths, monochromatic blue wavelengths were more efficacious (Fig 8). Light generally has an adverse effect on fungal growth but in this case the adverse effect of the fungicide was more pronounced in the case of cultures exposed to blue wavelength of light than in those incubated in the other wave lengths and also darkness. This confirms that the deleterious effect of the fungicide worsened when it interacted with light. Perhaps, the fungicide acted as a photo-sensitizer leading to the photo-oxidation of ergosterol in the membranes thereby rendering the plasma membrane malformed and malfunctioning. This possibility was suggested by Miller, MR and Limberta EA. (1977) & Sideri M. and Georgiou DC (2000) in their study on selective destruction of microscopic fungi through photo-oxidation of ergosterol. Recently, it was shown that generation of Reactive Oxygen Species (ROS) is important for the antifungal activity of fungicides (Christos D. Georgiou, *et al.,* 2006). Perhaps, this served as an ancillary mode of action of the fungicide in this case. Besides, the fact that blue light enhanced the efficacy of the fungicide more than the other wavelengths lends credence to the hypothesis that blue light is involved in photoreception by fungi (Blue Light Photo Biology-Ninneman, 1995).

Rollins and Dickman (2001) observed that pH of the culture medium, exerts a significant influence on sclerotial development and that under neutral or alkaline pH, sclerotial formation is inhibited. In this study the important environmental factors such as temperature, pH and water activity were investigated for their effects on the efficacy of hexaconazole. At pH 4 and at 25°C and at a water activity of 0.95, the fungicide was most efficacious (Fig 9, 10 & 11). As for the permeability changes induced by the fungicide, if any, it was noticed that the membrane permeability did change appreciably suggesting that the fungicide inflicted damage on the membrane exteriorly. Whatever adverse effect the fungicide exerted on the fungal growth and metabolism was perhaps on account of the leakage of ions into the exterior thereby impairing metabolism and growth.

As for the influence of the fungicide on respiration, it was observed that initially the rate of oxygen

consumption increased but subsequently there was a fall in the rate of oxygen consumption. Perhaps, the initial increases were due to a stress induced by the drug. As the concentration of the drug increased, the toxicity increased and respiration was impaired perhaps by affecting the form and function of the mitochondria.

Next, the effect of fungicide on the macromolecular synthesis was studied. The fungicide in general, affected the overall physiology and biochemical make-up leading to impaired macromolecular synthesis. The cellular lipid and ergosterol contents dwindled with increase in the fungicide concentration (Fig 12). It is quite an expected trend for the fungicide is essentially an ergosterol biosynthesis inhibitor. Therefore, the ergosterol content dwindled. The total lipid and sterol contents could have decreased perhaps owing to the inhibition of the ergosterol precursor synthesis as well in *Sclerotinia rolfsii* (Waterfield, W.F. and H.D. Sisler, 1989). The overall protein synthesis was observed to have been inhibited in response to the fungicide treatment (Fig 13). An array of proteins and enzymes are synthesized during growth and metabolism. Inhibition of protein synthesis by the fungicides is a well-known effect and therefore, it is not surprising that the overall protein synthesis machinery was adversely affected (Sisler.H.D. 1969). But what is surprising is that the activity of two enzymes, catalase and peroxidase shot up in response to the action of the fungicide. This may be a stress response by the fungus to protect itself for, these two enzymes are protective enzymes and hence their enhanced activity (Fig 14 & 15). Alternatively, the rise in the catalase activity in response to the antifungal agent's presence could ultimately lead to the aquisition of resistance as observed by T. Hsiang and G. A Chastagner 1992 in *Botrytis sp*.

This brief study with *Sclerotinia sclerotiorum* and its control with hexaconazole, an ergosterol biosynthesis inhibitor thus revealed that the formulation is a potential antifungal and that it affects the biology of the pathogen in a variety of ways. It directly affects the cell and can bring about growth inhibition by impairing one or the other of the vital phenomena related to growth and metabolism. All the vital functions of the fungus appear to have been impeded. The study also revealed that the mode of action of fungicide is not only by inhibiting ergosterol biosynthesis but probably also by an ancillary mode by photo-oxidation of ergosterol wherein, hexaconazole acted as a photo-sensitizer. On the whole, it is clear that the pathogen has not acquried any resistance to hexaconazole and that it is sensitive to the same. Therefore, this fungicide could be effectively used for the control of the notorious pathogen, *S. sclerotiorum* at a time when many pathogens are acquiring resistance to different classes of fungicides. However, more intensive studies are needed to have a holistic approach towards the same goal.

References

1. Adams, P.B. and W.A. Ayers. (1979). Ecology of *Sclerotinia* species. *Phytopathology* **69**:896-899.
2. Anonymous (1972). Worthington Enzyme Manual, Worthington Enzyme Biochemical Corporation, New Jersey: pp. 43-45.
3. Anonymous (2005) *Sclerotinia Initiative Brochure.* Fargo, ND: United States Department of Agriculture.
4. Beers, R.F. Jr. & I.W. Sizer. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem*. **195**: 133.
5. Boland, G.J. and Hall, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol*. **16**, 93–108.
6. Bradford.MM.A. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem.* **72**:248-54.
7. Chet. I and Henis Y. (1975). Sclerotial morphogenesis in fungi. *Annu Rev Phytopathol.***13***:*169-92.
8. Christos D. Georgiou, *et al.,* (2006).Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. Integrative and Comparative Biology. **46(6)**:691-712.
9. Courchaine AJ, Miller WH, Stein JRDB (1959). Determination of free and unesterified cholesterol. *Clin. Chem*. **5**: 609.
10. Folch, J., Lees, M. and G. H. S. Sloane-Stanley. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. **226**:497−509.
11. Haverkate, F., Tempel, A. and A. J. Den Held. (1969). Interaction of 2,4,5-trichlorophenylsulphonylmethyl thiocyanate with fungal spores. *Neth. J. P1. Path.* **75(5)**:308-315.
12. Helga Ninnemann.(1995). Some aspects of blue light research during the last decade. *Photochemistry and Photobiology*.**61(1)**:22–31.
13. Hsiang, T and G. A Chastagner. (1992).Production and viability of sclerotia from fungicide-resistant and sensitive isolates

of *Botrytis cinerea, B. elliptica and B. tulipae*. *Plant Pathology*.**41**:600-605.
14. Le Tourneau, D. (1979). Morphology, cytology and physiology of *Sclerotinia* species in culture. *Phytopathology*.**69**:887–890.
15. Leach, C.M. (1968). An action spectrum for light inhibition of the 'terminal phase' of photosporogeneis in the fungus *Stemphylium botryosum*. *Mycologia* **92**:1033–42.
16. Miller, MR and Limberta, EA. (1977). The effects of light and tyrosinase during sclerotium development in *Sclerotium rolfsii* Sacc.*Can J Microbiol.* **23**:278–87.
17. Purdy, L.H. (1979). *Sclerotinia sclerotiorum:* history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology 69:875-880.
18. Rollins, J.A. and Dickman, M.B. (2001) pH signaling in *Sclerotinia sclerotiorum*: identification of a *pacC/RIM1* homolog. *Appl. Environ. Microbiol*. **67**, 75–81.
19. Sideri M, Georgiou DC. (2000). Differentiation and hydrogen peroxide production in *Sclerotium rolfsii* are induced by the oxidizing growth factors, light and iron. Mycologia **92**:1033–42.
20. Sisler, H. D. (1969). Effect of fungicides on protein and nucleic acid synthesis. *Annual Review of Phytopathology*.**7:** 31 1-330**.**
21. Waterfield, W.F. and H.D. Sisler. (1989). Effect of propiconazole on growth and sterol biosynthesis by *Sclerotium rolfsii .Neth. J. P1. Path. 95 Suppl.***1:**187-195.
22. Willetts and Bullock, S. (1992). Developmental biololgy of sclerotia. *Mycol. Res.* **96(10)**: 801-816.
23. Willetts, H.J. and Wong, J.A. (1980). The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. *Bot. Rev*. **46**, 101–165.

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Fig. 1: Effect of Monochromatic Light on the Efficacy of Hexaconazole on *S. sclerotiorum*

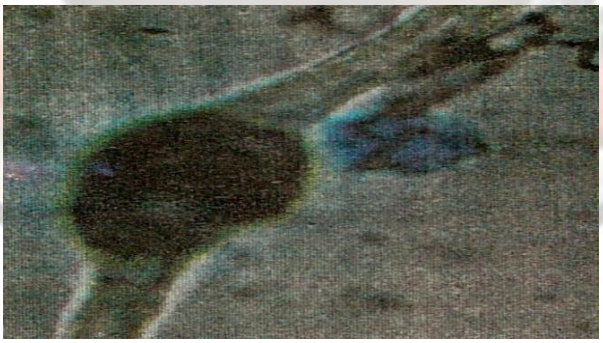

Fig. 2: Intercalary bulging of the mycelium

Fig. 3: Effect of hexaconazole on biomass

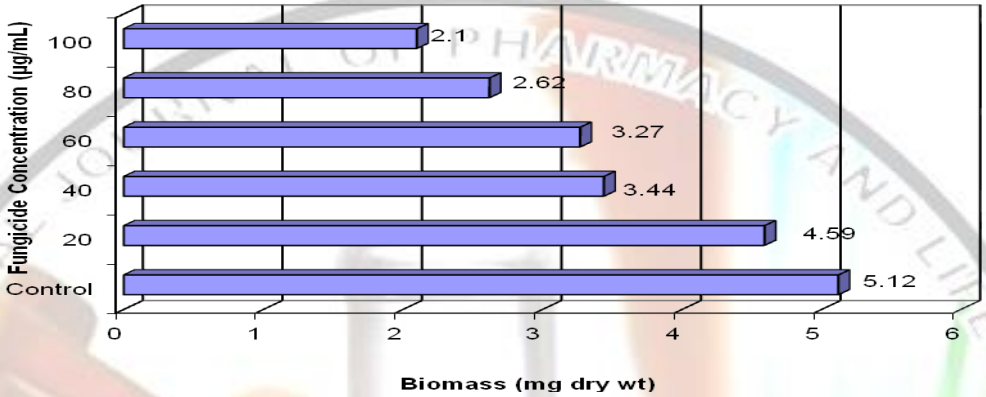

Fig. 4: Effect of hexaconazole on branching potential

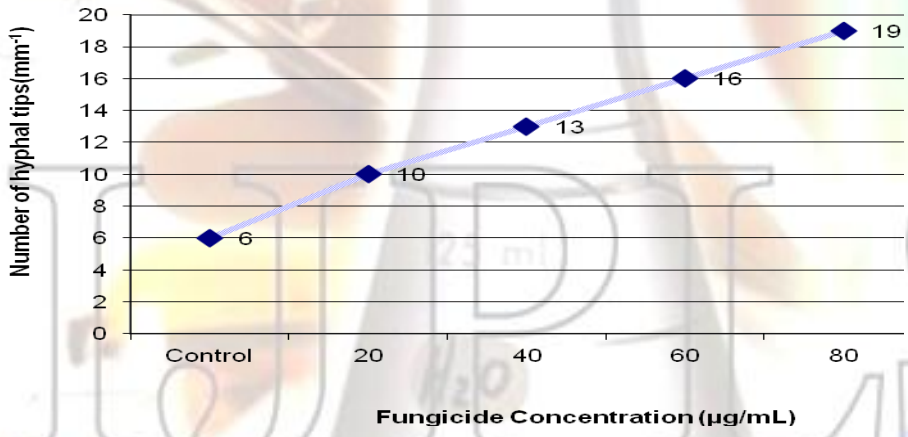

Fig. 5: Effect of hexaconazole on sclerotium formation

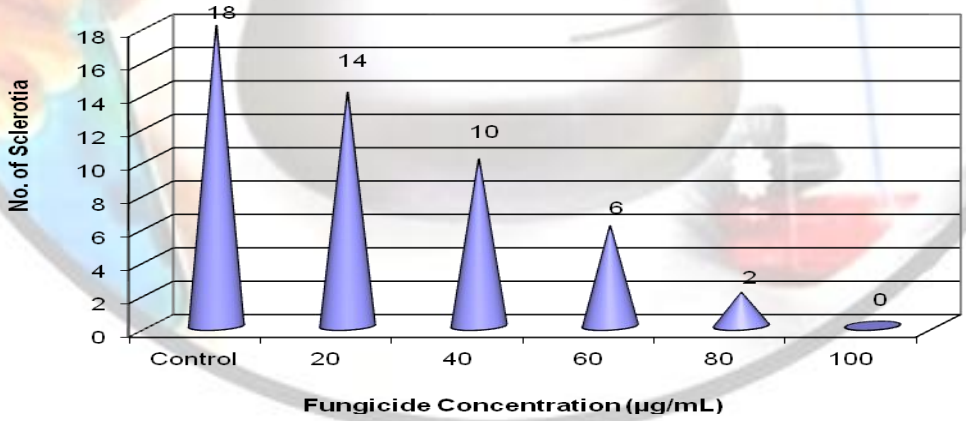

Fig. 6: Effect of Hexaconazole on sclerotium germination

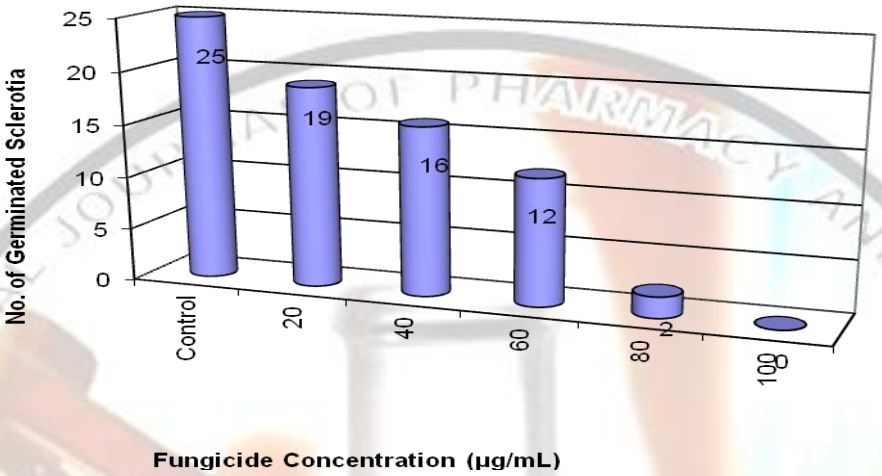

Fig. 7 (a) & (b): Effect of light on the efficacy of hexaconazole

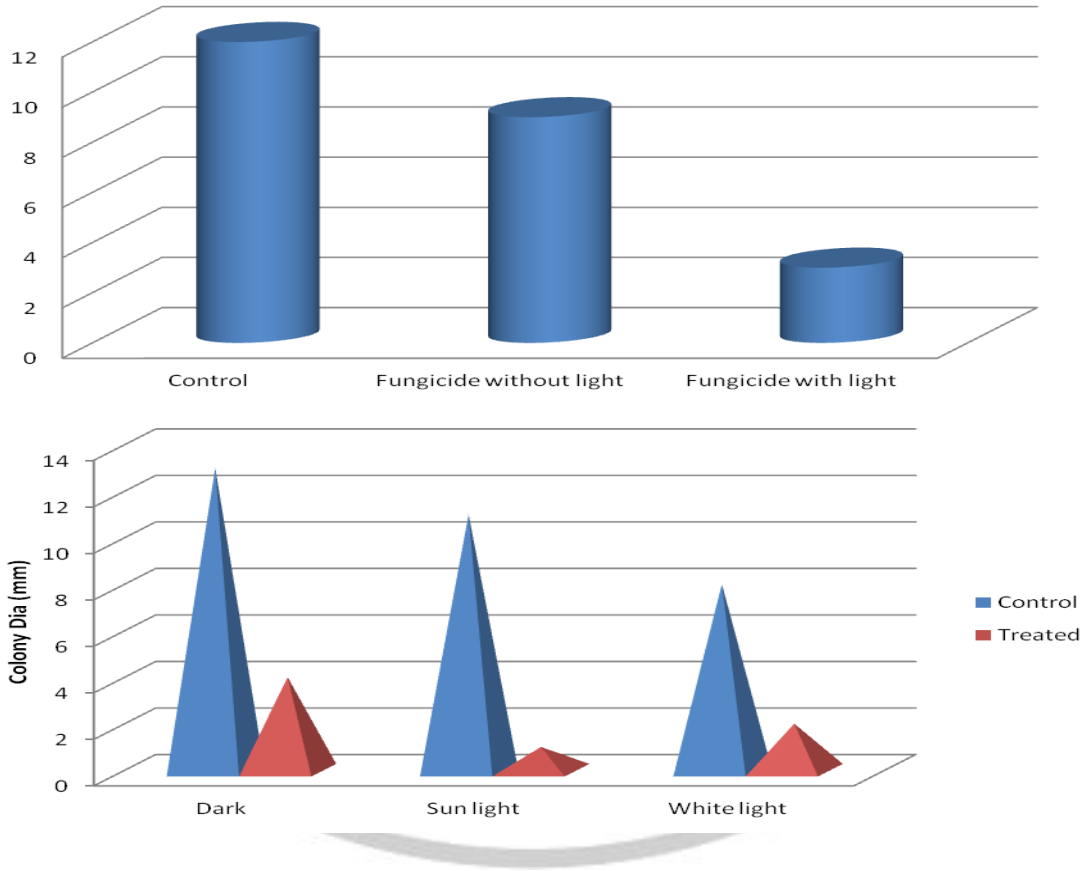

Fig. 8: Determination of effective monochromatic light for the efficacy of hexaconazole

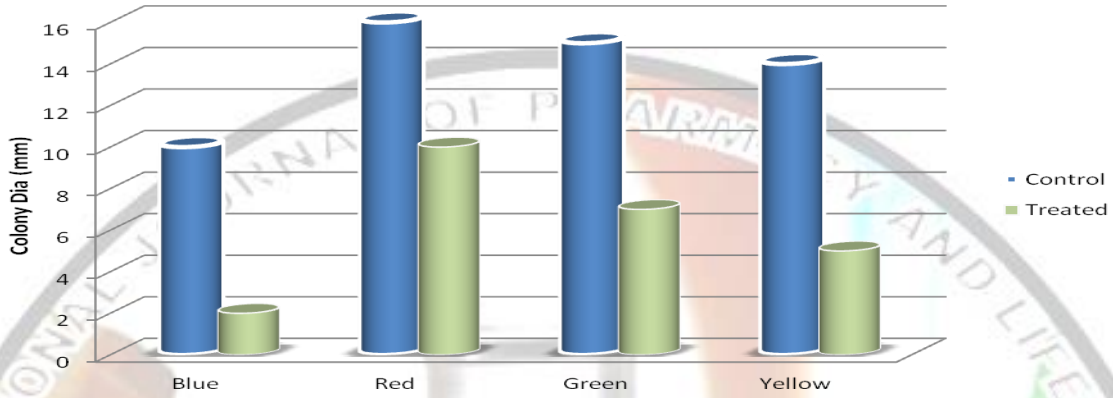

Fig. 9: Optimum pH for the efficacy of hexaconazole

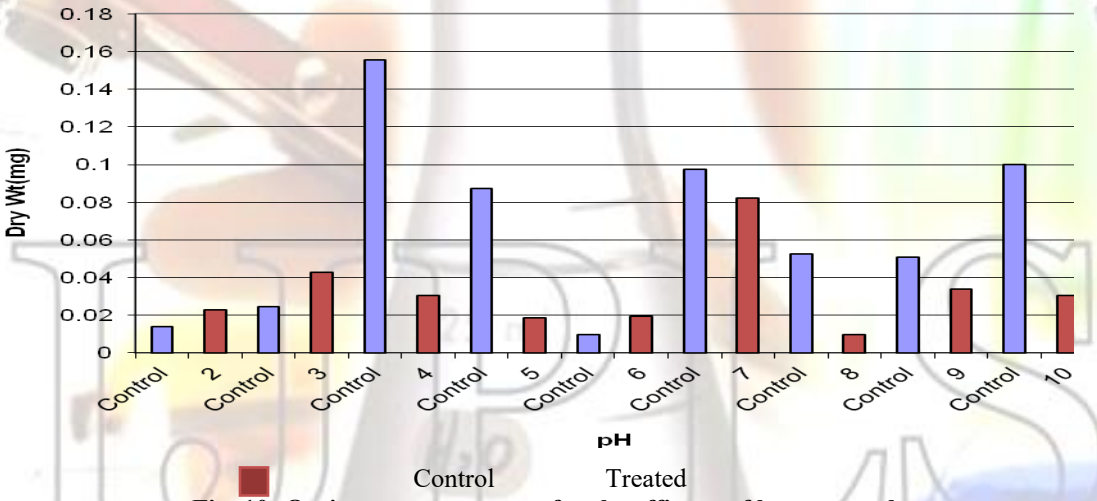

Fig. 10: Optimum temperature for the efficacy of hexaconazole

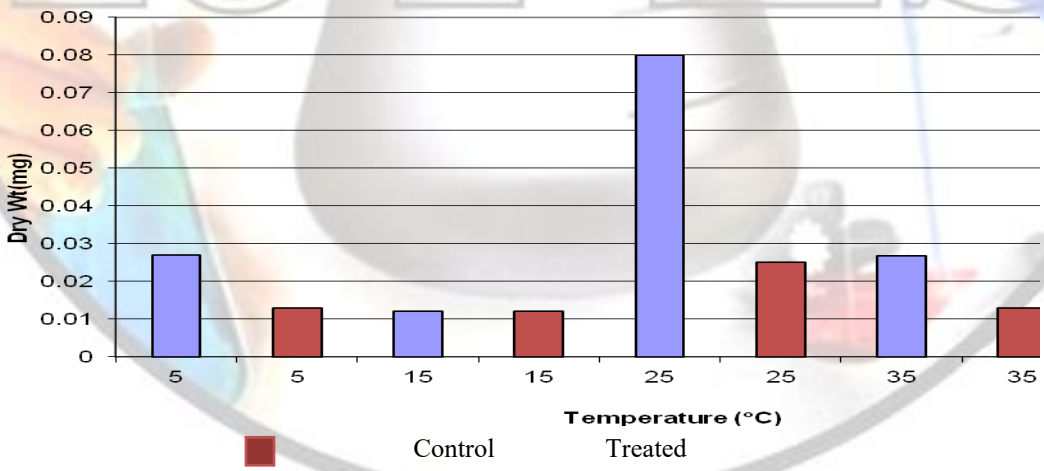

Fig. 11: Effective water activity for the efficacy of hexaconazole

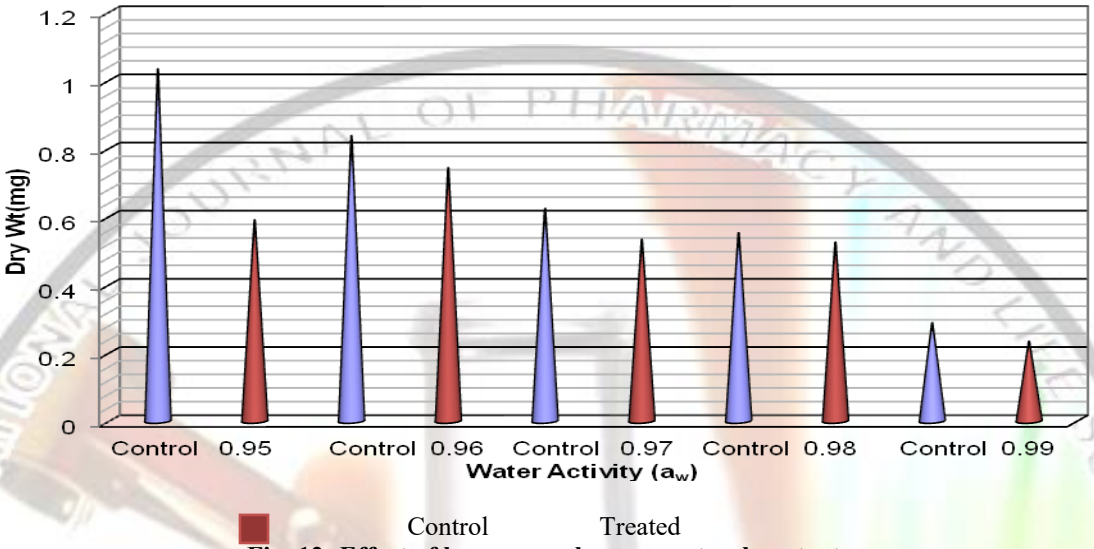

Fig. 12: Effect of hexaconazole on ergosterol content

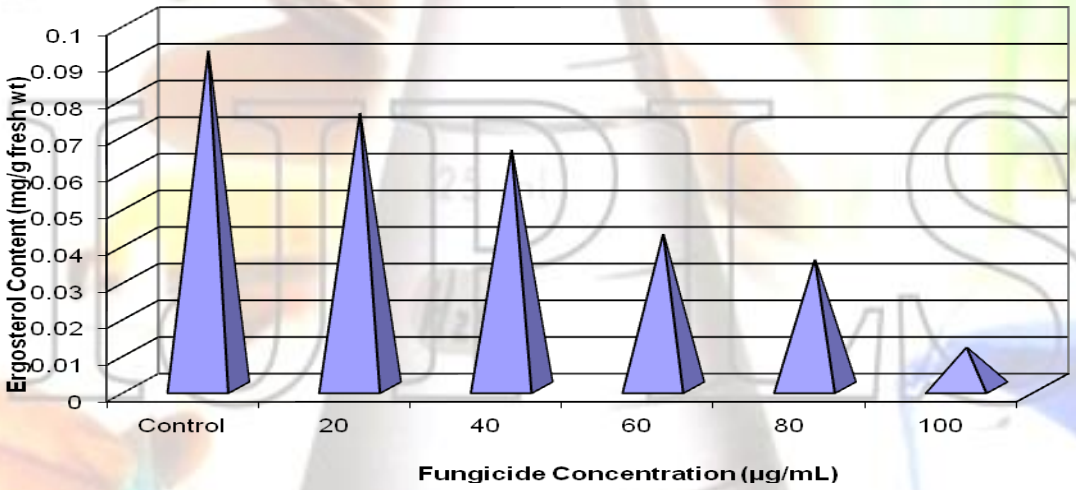

Fig. 13: Effect of hexaconazole on protein content

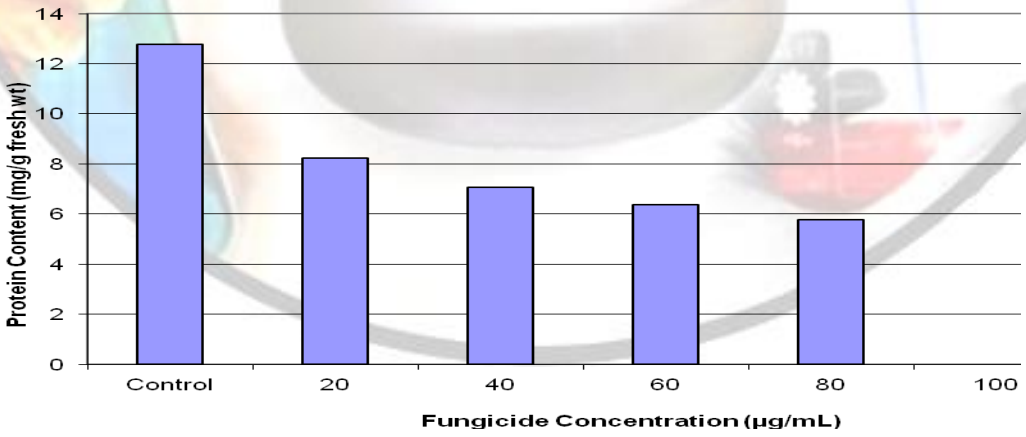

Fig. 14: Effect of hexaconazole on peroxidase activity

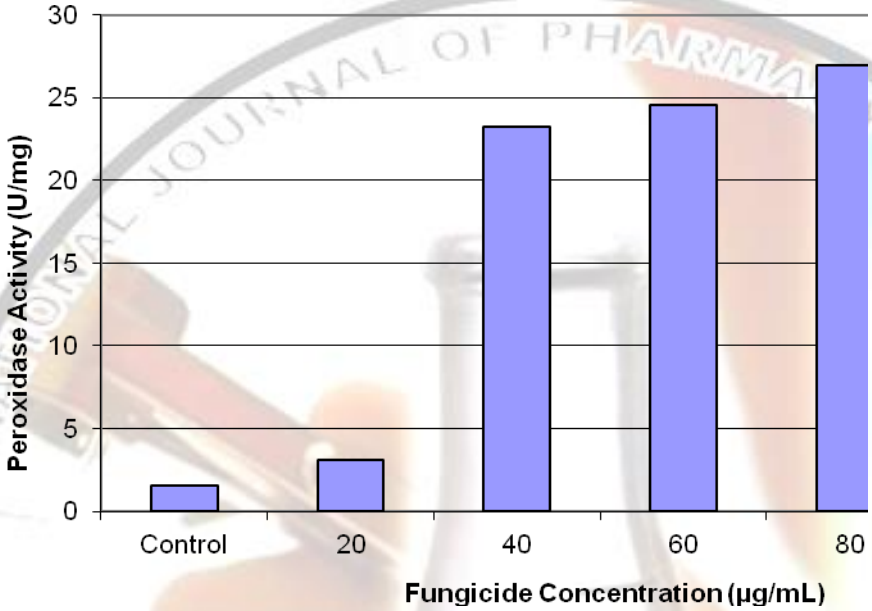

Fig. 15: Effect of fungicide on membrane permeability