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Study of Optical Properties of *Macrophomina phaseolina* Impregnated Sol-gel Derived Silica Matrices

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Abstract In the present frame of work, *Macrophomina phaseolina* is encapsulated in silica matrices at various concentrations by low temperature sol-gel technique using tetraethylorthosilicate (TEOS) as precursor. The optical and photophysical properties of these samples have been studied by second harmonics of Nd:YAG laser at 532 nm. UV-visible absorption spectra of samples have been recorded and it is found that the absorption increases with increase in concentration of fungus. Further, a decrease in output transmission intensity of the laser has been observed with increase in fungus concentration. The temporal response of these samples has also been examined. The results show that the fungus concentration can be measured within ~15–20 min. This method of optical sensing of fungus in test sample is faster than other techniques, such as the conventional colorimetric method which takes about 1 h.

Keywords Sol-gel · Fungal biosensor · Porous silicon · Immobilization · *M. phaseolina*

Introduction

Filamentous fungi are an enormously important group of organisms, as pathogens of plants and animals, as the primary degraders in the ecosystem and commercially in the production

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of enzymes, antibiotics, and metabolites for use in the food and pharmaceutical industries. Fungal diseases are the major yield constraints in the semi-arid regions of India. Charcoal rot caused by *Macrophomina phaseolina* has been considered one of the most prevalent outbreaks in various crops of India, Brazil, Australia, North and South America, Africa, and some parts of Europe. *M. phaseolina*, is becoming increasingly more common and severe in west Kentucky. *M. phaseolina* is worldwide in distribution and populations of the fungus are quite high in Kentucky fields since both corn and soybean are natural hosts of the fungus. The estimated production of cluster bean seed in India was 640,000 MT in the year 2001–2002. Owing to unpredictable rains and heavy incidence of disease, the seed yield was reduced to 256,500 MT in the year 2002–2003. The fungus *M. phaseolina (Rhizoctonia batatiola)* is a soil-borne plant pathogen with an exceptionally broad host range belonging to the Phylum *Deuteromycetes* and Class *Coelomycetes* which thrives in soil and causes stem and root diseases in over 500 different species of plants. This fungus is primarily a soil-borne root-infecting fungus that infects all stages of the plant by rotting of vascular tissue in roots and lower stems or stalks and forms blackish lesions with outer brown area. Sclerotia produced in parasitized host tissue functions as units of long-term survival in soil and primary inoculum for root infection. *M. phaseolina* also forms pycnidia and conidia on certain hosts, which may enable potential aerial and soil borne transmission. Despite its wide host range, the genus *Macrophomina* contains only one species, *M. phaseolina*. Efforts to identify subspecies of *M. phaseolina* based on microsclerotia size, culture characteristics, changes in soil population in response to rotation, and differences in pathogenicity failed primarily because of the extreme variability within the species or difficulties in quantifying characteristics. The disease has been reported to cause loss of yields up to 15% [1].

For controlling the diseases caused by *M. phaseolina* it is important to detect its presence. Although various techniques have been used for entrapping the pathogen to be used in biosensors but we have chosen a method of low temperature sol-gel technique, porous silicon has received much interest because of its optical properties and tendency to use in chemical and biological sensors [2]. This method is widely been used for the making of sensors, catalyst supports, optical elements, coatings, and special polymers. Enzymes, catalytic antibodies, and other proteins may be entrapped in robust silica glasses under mild conditions. Because the resulting bioceramics can be fabricated as monoliths, thin films, powders, and fibers these achievements open many possibilities for research and application in bioanalytical chemistry, biocatalysis, biotechnology, and environmental technology [3, 4].

A wide range of biological species such as antibodies and whole cells have been trapped within sol-gel matrices [5]. The efficient immobilization of biological molecules in solid hosts is of great importance to exploit their unique selectivity and their catalytic or recognition abilities in variety of applications. Sol-gel processes are carried out under aqueous conditions and at ambient temperatures, and hence are well suited for the entrapment of sensitive molecules in porous matrices [6]. Silica has always been a useful matrix for the immobilization of biological materials [7]. The use of aerogel as a matrix in the design of biosensors is an interesting proposition due to several unique characteristics of aerogels, primarily their high porous nature, adjustable pore size, and extremely large internal adjustable pore size and internal surface area.

Major objective was to employ microorganisms immobilized within this material and together facilitate the collection of aerosols and detection of chemicals and/or organisms within the environment. In the present study, an attempt has been made to optimize the protocol for fabrication of a biosensor for rapid detection of *M. phaseolina* from soil/plants/

seeds. An improved understanding of the levels of optimization parameters present in this fungal sensor would help researchers to group up isolates of the fungus, based on important traits such as the range of plant hosts on which a particular isolate of the fungus is capable of causing disease.

Materials and Methods

Fungal Isolation

Soil/tissue samples (quadruplicates) collected from semi-arid zones were analyzed for seclusion with slight amendments in the protocol made by Purkayastha et al., [8] by direct spreading and cavernous inoculation. Isolations were made on potato dextrose agar (PDA) medium with chloramphenicol (0.03%). After isolation, fungi were identified according to general principles of fungal classification.

Fungal Inoculum

M. phaseolina was isolated from cluster bean and maintained on PDA as shown in Fig. 1. Fungal inoculum was prepared by culturing the isolates in acidified potato dextrose agar for 15 days at 28 °C. Excess water and nutrients were removed from mycelium and thus dried on blotting sheets. Five milliliters of this suspension containing 1 g fungus was inoculated per plant for its pathogenicity trials and confirmed by Koch's postulates for their respective viability.

For the homogeneous growth, 20–30 PDA plates were inoculated with the mycelium of *M. phaseolina* for 7–10 days at 28 °C. Subsequently, growing hyphae from a single

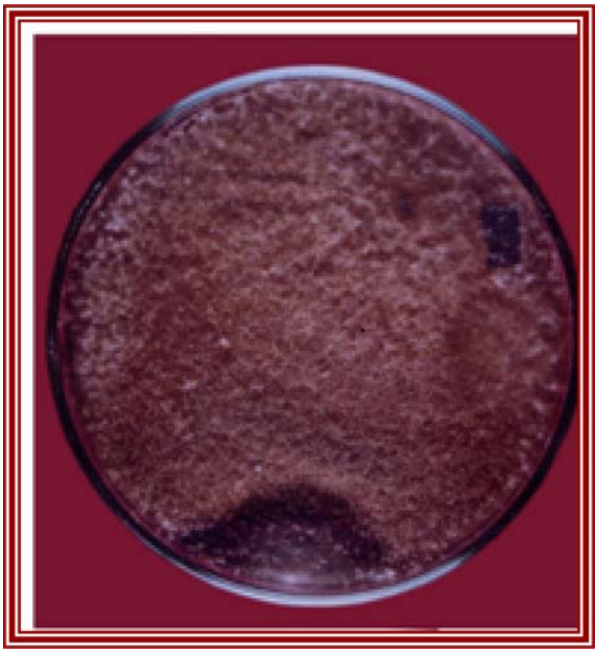

Fig. 1 Mycelial culture of *M. phaseolina* isolated from cluster bean on potato dextrose medium

sclerotium were taken from a pure culture and aseptically transferred to 250-ml flask, bearing 100 ml potato dextrose broth (Hi-media, India) with continuous agitation in dark. After 7 days homogenous suspension was prepared for further evaluation.

Sample Preparation

The metrology in silica gels can be customized as similar to the step for forming gel network. The present study is limited to the alkoxide sol-gel method, in which the inorganic polymerization occurs in two steps: hydrolysis and condensation reactions. In case of aloxysilanes, the general equations are:

$$\equiv \mathrm{Si} - \mathrm{OR} + \mathrm{H_2O} \equiv\!\rightarrow \mathrm{Si} - \mathrm{OH} + \mathrm{ROH} \quad \text{(hydrolysis)}$$
$$\equiv \mathrm{Si} - \mathrm{OH} + \mathrm{HO} - \mathrm{Si} \equiv\!\rightarrow \mathrm{Si} - \mathrm{O} - \mathrm{Si} \equiv + \mathrm{H_2O} \ \text{(condensation)}$$
$$\equiv \mathrm{Si} - \mathrm{OH} + \mathrm{RO} - \mathrm{Si} \equiv\!\rightarrow \mathrm{Si} - \mathrm{O} - \mathrm{Si} \equiv + \mathrm{ROH} \ \text{(alcoholisation)}$$

Hydrolysis gives reactive silanol groups, whereas, condensation leads to the formation of bridging oxygen. Doped samples of various concentrations ranging from ~0 μg/ml to ~16 μg/ml have been synthesized using TEOS. During the process, 5 ml of TEOS, 0.5 ml of formamide with few drops of dilute HCl hydrolysis catalyst is added. Formamide acts as drying control chemical additive (DCCA) and is added in order to obtain crack-free monoliths. The chemicals are taken in the following proportion:

$$[\mathrm{TEOS} + \textit{M.phaseolina} \text{ containing Rhodamine6G} + \mathrm{HCl}] : [\mathrm{H_2O}] : [\mathrm{Fomamide}] : : 05 : \ 03 : 01$$

The solution is kept under vigorous stirring at room temperature for about 3–4 h to yield clear and stable sol with subsequent gelation form. This is put for a week for drying and the glassy matrices were formed as the blank sample. Liquid *M. phaseolina,* co-doped with 1.2×10^2 μg/ml of Rhodamine6G (Rh6G), of varying concentration is added to the solution for its entrapment in the resulting silica matrices. The silica samples typically measures 15× 6×6 mm (length by breadth by height) and visually appeared to have a good surface finish, with the end faces seeming to be in parallel plane.

Figure 2 shows the schematic representation of the experimental set up employed in this study. Second harmonic of Nd:YAG laser was used as light source, which is focused by cylindrical lens (L1) of focal length 10 cm and spherical lens (L2) of focal length 10 cm before and after the sample respectively. L1 is used for line focusing of the light within the samples whereas L2 is used to focus the light onto the photodetector. A rectangular slot of

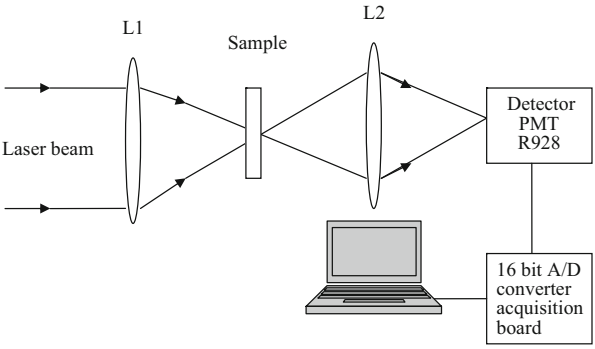

Fig. 2 Schematic transmission set up

dimensions 7×7×4 mm (length by breadth by height) was used as container using plastic wedges. It is done in order to fix the position of the samples under study to enhance the accuracy of the measurement. The transmittance through the *M. phasoelina* encapsulated silica aerogels is recorded as a function of concentration and time.

Results and Discussion

In order to confirm the entrapment of fungus in the samples, a comparative study of the UV-VIS absorption spectra of dye solution and fungus containing dye solution has been made and a blue shift is found in the latter case as shown in Fig. 3. A similar trend has been found in case of bulk silica samples of Rh6G doped alone (Sample 1) and fungus containing Rh6G doped samples (Sample 2). This further show that the absorption increases with the increase in concentration of the dopant impregnated in silica matrices. It may be due to aggregate formation at high concentrations of *M. phaseolina*. It is worth mentioning that for further studies only solid samples are considered as the main aim of the present study is to establish some of the optimization parameters for the design and development of fungus based sensors for which the liquid samples are not desirable because of bulky volume, flammable and toxic solvents, and difficulty of operation. Also the possibility of aggregate formation at higher concentration has already established [9] because of the similar environment in solutions and in silica cages that contain −OH group.

Figure 4 illustrates the calibration curve of *M. phaseolina* with Rh6G doped silica matrices at 532 nm. There is a decrease in output intensity with the increase in concentration of the fungus, without changing the concentration of the dye, as the optimum concentration of the dye is selected on the basis of the performance obtained through absorption and fluorescence spectra. This curve is helpful to ensure accuracy during testing of the dopant concentration of the unknown sample, when the dopant concentration is within the dynamic range of the assay.

Figure 5 represents the temporal response of the samples when tested at 12 μg ml^{-1} of *M. phaseolina* at 532 nm of second harmonics of Nd:YAG laser. In the presence of high intensity LASER, total refractive index of the sample n is given by:

$$n = n_0 + n_2(I)$$

where n_0 is the static linear refractive index of the material and n_2 is the intensity dependent nonlinear refractive index of the material. Further, n_2 is contributed due to the thermal and electronic response of the sample as:

$$n_2 = n_2^{th} + n_2^{el}$$

where, $n_2^{th} = \left(\frac{\mathrm{dn}}{\mathrm{dT}}\right)\frac{\alpha R^2}{\kappa} n_2^{th}$

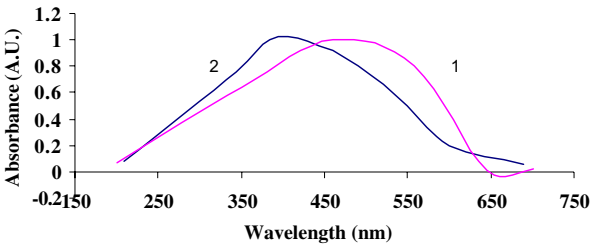

Fig. 3 Absorption spectra of sample 1 and sample 2

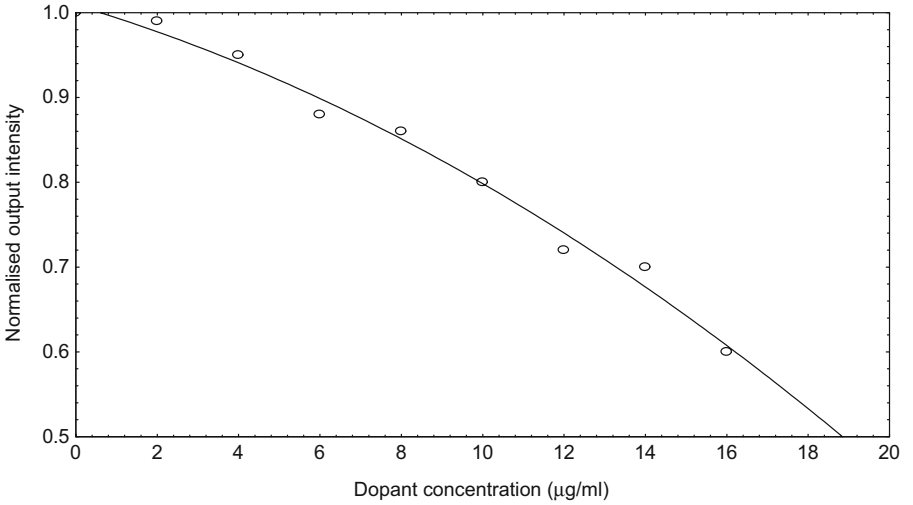

Fig. 4 Variation of output intensity with dopant concentration

Here dn/dT describes the temperature dependent refractive index of the sample, k denotes the thermal conductivity, alpha is the absorption coefficient of the material and R is the radius of the laser beam falling on the sample. The thermal contribution to the nonlinear refractive index (n_2^{th}) has been calculated in order to estimate the thermal effects caused by transmission of LASER through the sample. The calculated value of n_2^{th} is 0.171×10^{-7} cm^2/W which is negligible and hence the observed decrease in the output intensity with exposure time is not

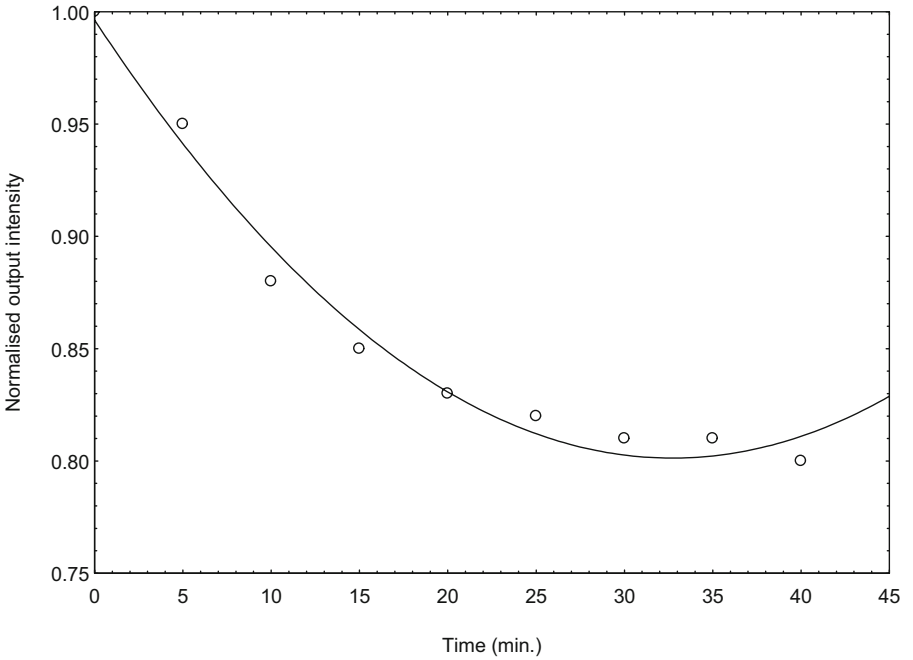

Fig. 5 Temporal response of the sample at 12 μg ml^{-1}

due to the thermal effects caused due to laser. The evaluation of thermal refractive index parameter suggests that thermal contribution is negligible and the electronic contribution is more. The figure depicts that output intensity decreases significantly with time up to about 25 min. For the first 20 min, there has been a significant reduction in output intensity, and from 10–30 min the gradient of output intensity reduction decreases. After 25 min the output intensity begin to stabilize. Therefore, the total fungus concentration could be measured in ~20 min. This is significantly faster than current techniques, such as conventional calorimetric method that takes about 1 h.

Conclusions

The technique based on spectroscopic method has been developed for the rapid and sensitive detection of *M. phaseolina*. It will enable single-step detection and quantification of microgram levels of *M. phaseolina* directly without destroying the sample. The response time of the dye containing *M. phaseolina* silica sample that will be the heart of the biosensor for determining the concentration of *M. phaseolina* is reduced to ~20 min in comparison to other conventional colorimetric method based on thermal detection.

This knowledge would help growers in the process of deciding which type of crop could be grown in particular fields in different eco-geographical regions of India. An easier method for screening out fungal infected seeds or planting material would enhance the rate of discovery and will lead to improvements in method available to farmers for controlling loses, due to soil-borne fungi such as *M. phaseolina.* The conventional screening and control procedures for the fungus are lengthy and tedious and often fail to provide tangible progress. Synergy between several approaches will thus offer cost effective, rapid and reliable tool for assessment of screening and to enhance selection efficiency of plant breeders. In convergence to Nano-Bio sciences, the challenge of proactive control for diagnostic and detection will no doubt provides important tools for evaluating and managing resources for better and concerted agricultural practices in future.

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