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SYNTHESIS OF 4-HYDROXYAZOBENZENE, A PROMISING AZO DYE FOR ANTIFUNGAL ACTIVITY AGAINST *MACROPHOMINA PHASEOLINA*

[a]Aqsa Zafar, [a]Arshad Javaid, [a]Iqra Haider Khan, [b]Ejaz Ahmed, [b]Hamza Shehzad, [c]Aneela Anwar
[a] Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan.
[b] Centre of Organic Chemistry, School of Chemistry, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Lahore, Pakistan.
[c] Department of Basic Sciences, University of Engineering and Technology, KSK Campus, Lahore, Pakistan.

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ABSTRACT

In the present study, an azo dye 4-hydroxyazobenzene was synthesized and its antifungal activity against a notorious soil-borne plant pathogen *Macrophomina phaseolina* was evaluated. The 4-hydroxyazobenzene was synthesized by azo coupling reaction between aniline diazonium salt and activated phenol. The azo coupling preferably occurred at para position of the phenol ring since the charge density get reinforced at this position and to minimize the steric hindrance between ortho positioned hydroxyl group. Azo coupling involved an electrophilic substitution reaction of phenyl diazonium cation with phenolate ion, the coupling partner. *In vitro* antifungal activity of the compound was assessed by dissolving the compound in dimethyl sulfoxide (DMSO) and preparing its different concentrations (ranging from 0.78 to 100 mg mL^{-1}) in malt extract broth. All the concentrations of synthesized compound significantly ($P \leq 0.05$) reduced biomass of *M. phaseolina* by 31–49%. This study concludes that 4-hydroxyazobenzene can be used for control of *M. phaseolina.*

Corresponding Author: Arshad Javaid
Email: arshad.iags@pu.edu.pk

INTRODUCTION

Macrophomina phaseolina occurs in the soil with a host range of more than 500 plant species (Iqbal and Mukhtar, 2014; Javed et al., 2021; Lodha and Mawar, 2020). It has a wide geographical distribution and occurs in subtropical and tropical countries with semi-arid to arid climates in Europe, Asia, Africa, and South and North America (Tancic et al., 2019). It is seed- as well as a soil-borne pathogen having varied distribution in different soils (Khan and Javaid, 2020; Marquez et al., 2021). It is responsible for seedling blight, stem canker, dry root rot, stem rot, charcoal rot, stem blight, leaf blight, wilt and damping off diseases in several economically important crops including vegetables and legumes (Banaras et al., 2021; Hyder et al., 2018; Javaid and Saddique, 2012; Lodha and Mawar, 2020). The production of sclerotial structures is responsible for its prolonged survival in the soil (Short et al., 1980). The pathogen becomes more severe under warm (28–35°C) and dry growing conditions (Ko et al., 2020). In recent decades, several management strategies such as agronomic practices, genetic resistance, chemical control, biological control, plant metabolites and elicitors of plant defense have been evaluated against *M. phaseolina* with varying degrees of success (Banaras et al., 2020; Iqbal and Mukhtar, 2020a; Iqbal et al., 2014;

Iqbal et al., 2010; Javaid et al., 2018; Khan et al., 2021; Marquez et al., 2021; Shahjahan et al., 2018).

Till now, there is no registered fungicide that has been found to be effective against the pathogen (Tonin et al., 2013). Therefore, it is often difficult to control the growth of *M. phaseolina* through chemicals. However, many systemic and non-systemic fungicides such as dazome, azoxystrobin, benomyl, difenoconazole and carbendazim were found to be effective in inhibiting the mycelial growth and sclerotial formation of *M. phaseolina* (Iqbal and Mukhtar, 2020b; Lokesh et al., 2020; Parmar et al., 2017). There is need to search for new chemicals for the control of this pathogen. Aromatic azo molecules are an important group of compounds that possess many biological activities including antifungal (Adu et al., 2020; Węglarz-Tomczak and Gorecki, 2012). Keeping this in view, the present study was undertaken to synthesize and evaluate the antifungal potential of an azo dye, 4-hydroxyazobenzene against *M. phaseolina.*

MATERIALS AND METHODS

Aniline (93.13 g mol^{-1}, pure yellowish liquid, b.p. 184°C, flash point 158°F), Phenol/carbolic acid (colorless-to-white solid, purity ≥ 99.99 %), sodium nitrite (≥ 99.99 %), hydrochloric acid, sodium hydroxide were procured from central chemicals. All reagents were of analytical grade.

Synthesis of 4-hydroxyazobenzene

Aniline (5 g) was dissolved in concentrated hydrochloric acid (20 mL) and 20 mL of water, contained in a conical flask. Diazotization step was done by the addition of a solution of 5 g of sodium nitrite in 20 mL of water. A solution of 5.8 g of phenol in 50 mL of 10% sodium hydroxide solution was prepared in a beaker. The solution was cooled to 5 °C by immersion in an ice bath. The phenol solution was strongly stirred along with slow addition of a cold diazonium salt solution. A color developed and the dark orange yellow crystals of 4-hydroxyazobenzene were soon appeared. The mixture was filtered through a funnel, spread on filter paper with a glass rod, let it dry in indirect sunlight, then put the powdery granules in a vial, and labeled the tag pasted on the vial.

Antifungal bioassays

First a stock solution of 100 mg mL^{-1} of the synthesized compound was prepared. For this purpose, 0.6 g of 4-hydroxyazobenzene was dissolved in 0.5 mL dimethyl sulfoxide (DMSO). Thereafter, 5.5 mL of autoclaved malt extract broth (MEB) was added to raise the volume up to 6.0 mL. It was serially double diluted to prepare lower concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg mL^{-1}. In a similar series of control treatments, the same amount of DMSO was added to prepare 6 mL of the growth media without the compound. To each 5-mL test tube, 1.0 mL of the growth media of different concentrations was poured for antifungal bioassays (Raza et al., 2021; Tanvir et al., 2021).

Culture of *M. phaseolina* was obtained from Biofertilizer and Biopesticide Lab, Department of Plant Pathology, Punjab University Lahore (Figure 1A). The fungus was sub-cultured on fresh autoclaved malt extract agar. After 7 days, 5 mL distilled water was added to the plate and scratched the fungal growth to prepare an inoculum. Test tubes were inoculated with 50 µL of this inoculum in triplicate and incubated at 28°C for 7 days in a completely randomized design. Fungal biomass was harvested on pre-weighed filter papers, dried and weighed (Javaid and Samad, 2012).

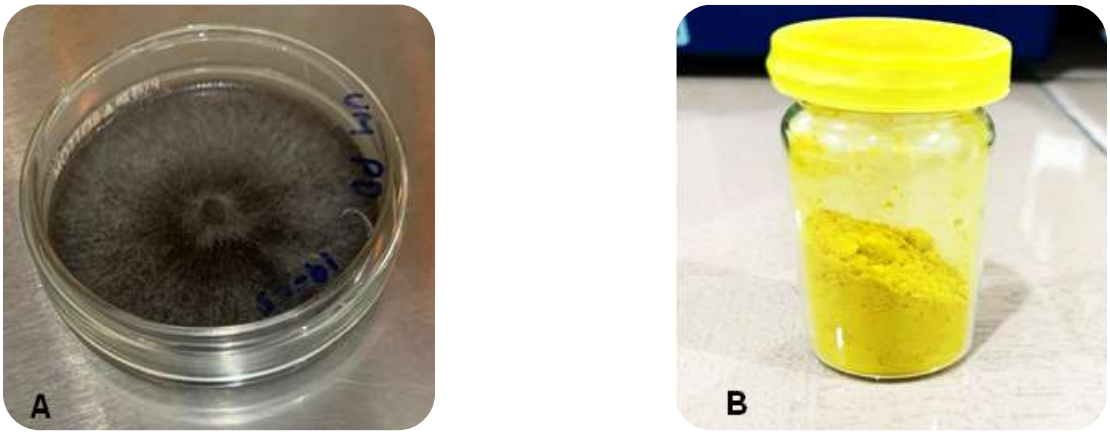

Figure 1: (**A**) Pure culture of *Macrophomina phaseolina*; (**B**) The synthesized compound 4- hydroxyazobenzene.

Statistical analysis

All the data were analyzed by ANOVA. Thereafter, LSD test was applied ($P \leq 0.05$) using Statistix 8.1 to separate treatment means. Inhibition in growth of *M. phaseolina* due to azo dye application over corresponding control was measured as follows;

$$\text{Inhibition } (\%) = \frac{\text{Biomass in control - Biomass in treatment}}{\text{Biomass in control}} \times 100$$

RESULTS AND DISCUSSION

Physical characteristics of the synthesized compound

Figure 1B shows the picture of synthesized azo dye 4-hydroxyazobenzene. The product appeared as orange yellow colored crystalline solid with sharp melting point at around 155°C.

FTIR analysis of 4-hydroxyazobenzene

The 4-hydroxyazobenzene was synthesized by azo coupling reaction between aniline diazonium salt and activated phenol. The azo coupling preferably occurred at para position of the phenol ring since the charge density get reinforced at this position and to minimize the steric hindrance between ortho positioned hydroxyl group. Azo coupling involves an electrophilic substitution reaction of phenyl diazonium cation with phenolate ion, the coupling partner. Figure 2 describes the proposed route for the synthesis of 4-hydroxyazobenzene. Figure 3 displays the FTIR spectrum of produced 4-hydroxyazobenzene. The broad peak at 3540-3215 cm^{-1} shows the presence of H-bonded hydroxyl (-OH) groups, peaks at 2965–2920 cm^{-1} correspond to sp2 hybridized methine (-CH) groups of Ph-CH. Sharp peak at the 1510-1500 cm^{-1} shows that the azo group (-N=N-) in 4-hydroxyazobenzene. Benzene ring was confirmed by the presence of different peaks around 1620-1680 cm^{-1} (C=C-H), The (C-O-C) stretching vibrations around 1016 cm^{-1}. The characteristic peak 1450–1400 cm^{-1} shows that bending frequency of –NH-group under the influence of azo coupling groups.

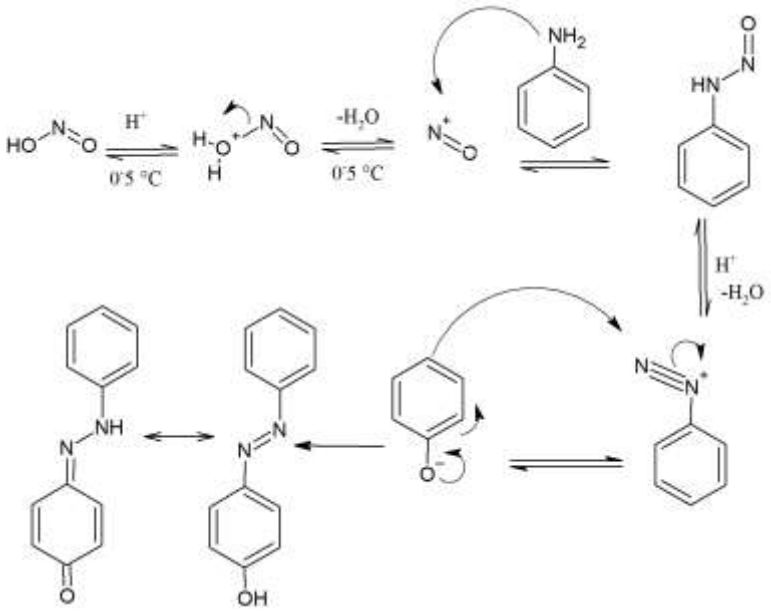

Figure 2: Proposed route for the synthesis of azo dye 1-phenylazo-2-naphthol.

Antifungal activity

The synthesized compound showed marked antifungal activity against *M. phaseolina.* Overall, all the concentrations (0.78 to 100 mg mL^{-1}) of this compound significantly ($P \leq 0.05$) suppressed biomass production of the target pathogen. Antifungal activity of the compound varied with change in concentration, generally increased by increasing the concentration. Various concentrations of this compound suppressed biomass of *M. phaseolina* by 31–49% (Figure 3). Results of this study are in agreement with various previous studies where both synthetic and natural azo dyes showed antifungal properties against various human and plant pathogenic fungi. In recent years, numerous researches have been carried out on the synthesis of novel azo dyes and to assess their antimicrobial

activities. For instance, three newly synthesized azo dyes exhibited effective antifungal properties against *Aspergillus niger, A. flavus* and *Candida albicans* (Keshavayya, 2019).

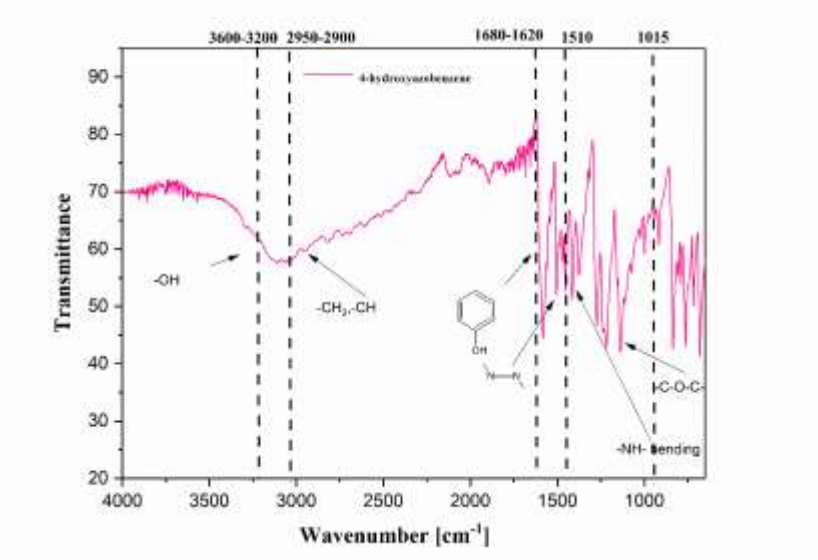

Figure 3: FTIR spectra of 4- hydroxyazobenzene.

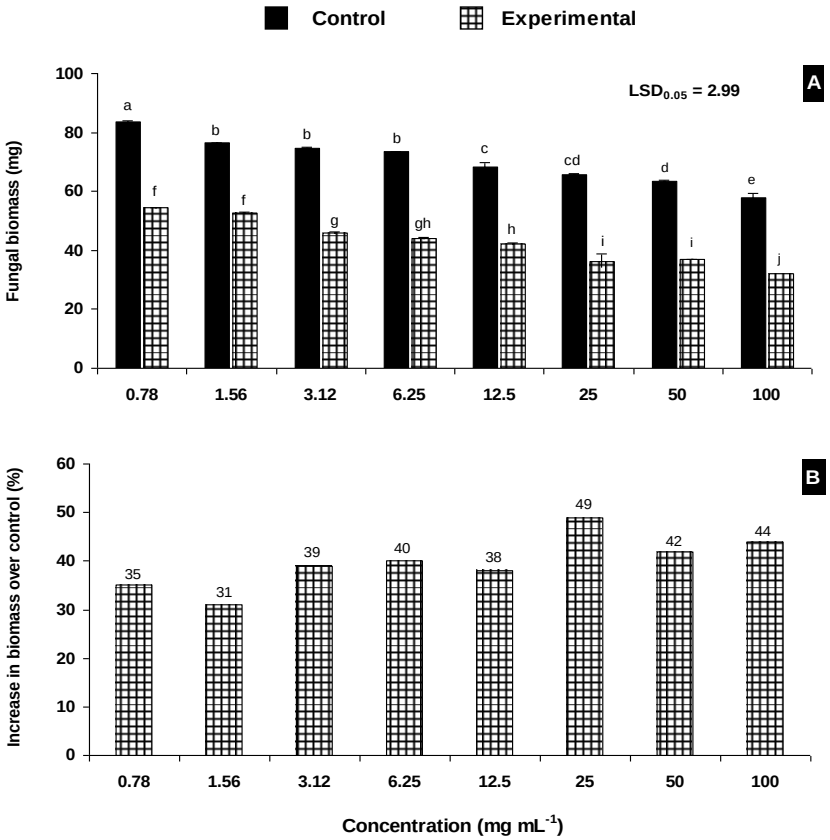

Figure 4: Effect of different concentrations of azo dye on growth of Macrophomina phaseolina. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by LSD Test.

Baginski and Czub (2009) reported the presence of multiple conjugated double bonds in azo dyes that potentially act as fungicide. This conjugated system functions after binding with fungal cell membranes and changes the transition temperature, resulting in ions leakage through membranes that lead to fungal cell death. Likewise, Raghavendra and Kumar (2013) prepared novel azo dyes from aminosalicylic acid and studied their antifungal potential against a variety of fungal species. The compounds significantly arrested the mycelial growth of *Alternaria alternata, Trichothecium roseum, Cladosporium herbarum, Aspergillus nidulans, Rhizopus nigricans, Aspergillus flavus* and *A. niger.* Similarly, an *in vitro* study carried out by Novak et al. (2005) that exhibited the fungistatic properties of organotin complexation of azo dyes against *Trichophyton mentagrophytes, Candida krusei, C. parapsilosis* and *C. albicans*. Novel coumarin-based and phenolic azo dyes were tested against *A. niger* where the compounds exhibited good to moderate antifungal activities (Bawa and Alzaraide, 2005; Jogi et al., 2013; Shahzaman et al., 2015). In addition to this, Bal et al. (2014) reported the antifungal potential of azo dyes and their complexes against *Candida albicans* and *Saccharomyces cerevisiae.* Sevastre and Hodorog (2021) also reported that azo dyes have strong antifungal, antibacterial, antiviral and cytotoxic activities.

CONCLUSION

This study concludes that 4-hydroxyazobenzene has pronounced activity against *M. phaseolina.* Its 0.78 to 100 mg mL^{-1} concentrations can reduce growth of this pathogen by 31–49%. Future studies are suggested to prepare derivatives of this compound in order to further enhance antifungal activity of this compound.

AUTHORS' CONTRIBUTION

AZ did experimental work, AJ and EA gave idea and supervised the work, AJ also prepared the graphs and did statistical analysis, IHK and HS contributed in write up, AA edited the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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