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Synthesis of halogenated benzylidene aryl amines as potent fungicides against plant pathogenic fungi *Rhizoctonia solani, R. bataticola* and *Sclerotium rolfsii*

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Abstract
Phytopathogenic fungi affect the economic yield of legumes, a key source of protein and so necessitate the development of new economically and environmentally viable products for protecting crops from diseases. Therefore, the current research programme was conducted for developing crop protection agents based on azomethine to save crop yield losses due to diseases caused by phytopathogenic fungi. Fourier Transform Infra Red and Nuclear Magnetic Resonance spectral data were used to establish the chemical structures of synthesized azomethines. *In-vivo* antifungal studies were carried out to test their antifungal potential against the test fungi. The respective best performers were 2,4,5-trichloro-N-(2,4-dichlorobenzylidene) aniline in managing *R. solani*, *N*-(2,4-dichlorobenzylidene)-4-fluoroaniline for *R. bataticola* and 3-Chloro-N-(2,6-dichlorobenzylidene)-4-fluoroaniline against *S. rolfsii*.

Keywords: Azomethines, ED_{50}, fungicide, pesticide, soil borne fungi, *Vigna radiata*

Introduction
Pulses are edible part of pod bearing plants being a rich source of protein ranging from 20-40 percent. This makes pulses a perfect accompaniment of the majority of the foods of India from the view point of human nutrition. Protein calorie malnutrition is a widespread issue in Indian subcontinent and pulses can be a gamechanger in bridging this gap. Phytopathogenic fungi affects economic yield of various crops including pulses to the tune of 20 percent or more [1]. Serious damage is caused by phytopathogens resulting in critical losses of quality, yield and profit. The crops are attacked by these fungi at various stages including sowing till harvest of the crop. As a result charcoal rot/dry root rot, collar rot, wilt, damping off diseases affect them [2-3].

Mung bean or green gram (*Vigna radiata)* (L.) is a main leguminous pulse crop in India. It is a short duration, annual, herbaceous and self pollinated crop. It has the capability to symbiotically fix atmospheric nitrogen [4] and is a rich source of high-quality proteins, folate and iron [5]. About 90 percent global production of mung bean is in Southeast Asia and South Asia, where India is the leading producer [6]. Major Mungbean producing states in India are Andhra Pradesh, Bihar, Karnataka, Maharashtra, Orissa and Rajasthan [7]. The important fungi which attack mung bean are *R. solani, S. rolfsii, R. bataticola.* Depending upon the crop variety, the losses in pulses because of disease causing fungi has been reported to be about 44 percent [8] and among biotic stresses, about 40– 60 percent yield is reduced due to fungal diseases in mungbean [9]. However, there are reports of up to 80 percent yield losses in the vulnerable mungbean varieties and there is substantial increase in severity and incidence of the disease in recent past [10].

The present fungicidal chemicals for managing these phyto-pathogenic fungi suffer from problems such as high cost, complex synthetic procedures, environmental and health hazards due to fungicide residues in food commodities and chemical resistance due to their injudicious use. To address this challenging task of designing and developing new crop protection products [3, 11-19], the present study was taken up to search novel fungicides which are host specific, selective, cheap and easy synthetic method for managing the diseases caused by these phytopathogenic fungi with an objective to develop suitable, easy, efficient and economically Stimulating method for evaluation of developed agrochemical under *in-vivo* pot culture experiments.

Materials and methods

Various aromatic aldehydes and anilines used for synthesizing azomethines were purchased from Avra, ChemsWorth, Fisher Scientific, Sigma-Aldrich, Sisco Research limited and Spectrochem, Methanol and dimethyl sulphoxide (DMSO) were purchased from Merck. The reference fungicide, hexaconazole (5% EC) was bought from Tata Rallis India Ltd. The reaction was monitored by thin layer chromatography (TLC) on pre-coated Merck silica gel $60F_{254}$. Electro thermal's melting point apparatus was used for determining the melting points (M.P.) of the title compounds. Perkin-Elmer model 2000 Fourier Transform Infrared spectrometer was used to record Infrared (IR) spectral data. Nuclear Magnetic Resonance (NMR) spectral data were obtained on Bruker spectrospin NMR spectrometers (Avance II, 400 MHz & Ascend 500 MHz) and Jeol (JNM-EXCP 400) NMR spectrometer using tetramethyl silane (TMS) as an internal standard.

Synthetic procedure

Azomethines were synthesized following reported method [3]. Methanol was used for dissolving aromatic aldehyde (5 mmol) and subsequent addition of substituted aniline (5.5 mmol) was done to this methanolic solution. The resultant solution was subjected to continuously stirring for 3-4 hours on a magnetic stirrer. The product obtained as precipitate was filtered followed by washing with cold methanol. Purification of synthesized azomethines was carried out by recrystallization method using ethanol. The following analytical and spectral data of synthesized compounds was utilized for characterizing them.

2, 4, 5-Trichloro-N-(2, 6-dichlorobenzylidene) aniline (1)

Yield: 73%. Melting point: 132 °C. IR (Nujol) cm^{-1}: 1635(CH=N). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.20 (1H, s, H-6'), 7.39-7.47 (3H, m, H-3, H-4& H-5) 7.61 (1H, m, H-3') and 8.94 (1H, s, CH=N) ^{13}C NMR (125MHz, $CDCl_3$): 120.65(C6'), 122.63(C3'), 131.18(C4), 132.30(C2 and C6), 133.02(C3' and C5'), 142.28(C1), 151.25(C2'), 159.97(C5'), 163.37(C4'), 166.37(C1') and 167.88(CH=N).

3-Chloro-N-(2, 6-dichlorobenzylidene)-4-fluoroaniline (2)

Yield: 76%. Melting point: 104 °C. IR (Nujol) cm^{-1}: 1634 (CH=N). ^{1}H NMR (500 MHz, $CDCl_3$):^{1}H NMR: 7.15-7.17 (1H, m, H-6'), 7.29-7.33 (2H, m, H-2'& H-5'), 7.39-7.41 (3H, m, H-3, H-4& H-5) and 8.64 (1H, s, CH=N). ^{13}C NMR (125MHz, $CDCl_3$): 119.76 (C6'), 123.23 (C5'), 125.56 (C2'), 134.08 (C4), 135.40 (C2 and C6), 136.50 (C3 and C5), 137.9 (C1), 145.49 (C3'), 154.33 (C1'), 156.39 (CH=N) and 161.62 (C4').

N-(2, 4-dichlorobenzylidene)-4-fluoroaniline (3):

Yield: 63%. Melting point: 107 °C. IR (Nujol) cm^{-1}: 1618 (CH=N).^{1}H NMR (400 MHz, DMSO-D6) : 7.36-7.38 (2H, m,H-3' &H-5'), 7.46-7.49 (2H, m, H-2' &H-6'), 7.57-7.63 (2H, m, H-3 &H-5), 7.80-7.81 (1H, m, H-3), 8.12-8.14 (1H, d, J =8.5Hz, H-6) and 8.82 (1H, s, CH=N). ^{13}C NMR (125MHz, $CDCl_3$): 129.22 (C3'&C5'), 130.44 (C2'&C6'), 132.44 (C1), 133.64 (C6), 134.95 (C5), 134.96 (C3), 135.34 (C4), 136.29 (C2), 147.29 (C1'), 152.93 (CH=N) and 157.99 (C4').

2, 4, 5-Trichloro-N-(2, 4-dichlorobenzylidene) aniline (4)

Yield: 66%. Melting point: 120 °C. IR (Nujol) cm^{-1}: 1619 (CH=N). ^{1}H NMR (400 MHz, DMSO-D6): 7.60-7.63 (1H, m, H-5), 7.73 (1H, s, H-6'), 7.83-7.84 (1H, m, H-3), 7.95 (1H, s, H-3'), 8.15-8.18 (1H, m, H-6) and 8.81 (1H, s, CH=N). ^{13}C NMR (125MHz, $CDCl_3$): 116.35 (C6'), 117.95 (C3'), 121.08 (C5'), 125.68 (C4'), 128.01 (C2'), 130.04 (C1), 130.52 (C6), 131.05 (C3), 131.43 (C5), 138.61 (C4), 141.17 (C2), 142.50 (C1'), and 162.41 (CH=N).

4-Chloro-N-(2, 4-dichlorobenzylidene) aniline (5)

Yield: 88%. Melting point: 96 °C. IR (Nujol) cm^{-1}: 1611 (CH=N). ^{1}H NMR (400 MHz, $CDCl_3$): 7.11-7.21 (2H, m, H-3' &H-5'), 7.35-7.41 (2H, m, H-2' & H-6'), 7.47-7.51 (1H, m, H-3&H-5), 7.88-7.90(1H,m, H-3), 8.19-8.21(1H, d, J =8.0Hz, H-6) and 8.84 (1H, s, CH=N). ^{13}C NMR (125 MHz, $CDCl_3$): 122.11 (C3' & C5'), 123.21 (C2' & C6'), 126.72 (C4'), 131.11 (C1), 131.22 (C6), 132.21 (C5), 136.57 (C3), 138.27 (C4), 145.77 (C6), 156.21 (C1'), and 159.22 (CH=N).

Formulations of synthesized azomethines

Method of preparation

Formulation (0.5%) of azomethines were developed by dissolving 0.3 gm dried crystals of azomethines in 60 g suitable solvent (dichloro methane or mixture of cyclohexanone and dichloromethane) and drop wise added to a mixture of 50 gm PEG 400 and 10 gm deionised water under continuous stirring. After complete addition of azomethine solution to PEG 400-water mixture, dichloromethane was completely evaporated on water bath.

Reagent Blank

It was prepared by using the above method except the addition of the azomethine.

Hexaconazole 5 EC as Standard

Hexaconazole 5 EC was used as a standard compound for the *in-vivo* pot culture analysis. This was serially diluted with deionised water to 100 ppm.

***In vivo* fungicidal activity**

Mycelia bits were used to inoculate the sterilized sorghum seeds followed by their incubation for fifteen days in BOD incubator at 26–28 degree Celsius for mass culturing of the test fungi (*S. rolfsii*, *R. solani and R. bataticola*). The mixing of fungal mass culture with steam sterilized soil was done at the rate of 1.5 percent (15 g/pot). The completely randomized design (CRD) was utilized for conducting the entire experiment with three replicates. The formulations of synthesized chemicals (Compounds 1–5) and standard fungicide, hexaconazole were evaluated at 100 ppm application dose with concomitant controls. The *in-vivo* antifungal bioassay of formulated products was investigated indirectly through their percent disease control in pots. The pot experiment was conducted in mung bean crop. In each inoculated pot, the seed sowing rate was ten seeds of mung bean (var. Pusa 0672) per pot. Two days after the sowing, the test products at 100 ppm dose were introduced into all the experimental pots using the soil drenching method. The formula used for calculating the germination percentage after ten days of sowing is given below:

Germination percentage = (number of seeds germinated / number of seeds sown)*100

Percent disease control was calculated by following formula:

Percent disease control = [C (Control) – T (test compound)] / C (Control)* 100

When pods were deep brown in colour, they were picked as per requirement.

Statistical analysis

One-way analysis of variance (ANOVA) was computed using online facility of OPSTAT, provided by Chaudhary Charan Singh Haryana Agricultural University for disease incidence data.

Results and discussion

Synthesis, characterization and formulation

All the test chemicals were synthesized by the reaction of aromatic aldehyde and substituted aniline in good yields (63-88%). These compounds were characterized using spectral techniques. A characteristic peak due to C=N absorption was observed in the range of 1611-1635 cm^{-1} in the IR spectra of test compounds. The 1H NMR spectra of azomethines (1-5) displayed a 1H-singlet due to proton of HC=N moiety at δ 8.64-8.94 ppm. The appearance of peaks in 1H NMR due to all other protons was in accordance with the structures. Moreover, the 13C NMR spectral data also confirmed the structures of the synthesized azomethines. These test chemicals were formulated using the polyethylene glycol-400 at the concentration of 0.5% for their *in-vivo* evaluation.

***In-vivo* fungicidal bioassay**

The formulated products based on azomethines were checked for their in-vivo antifungal bioactivity against all the three test fungi in a pot experiment. The bioassay results are depicted in the Figures 1-3.

All the test products displayed considerable antifungal activity against *R. solani* in comparison to inoculated control (Figure 1). The fairly high germination was observed with the values of 73.33 to 76.67 percent for synthesized imines and 90 percent in control uninoculated. The highest germination percentage was observed in compound 4 and 5 with 76.67 percent germination comparable to standard hexaconazole (83.33 percent). The percent disease control was in the range of 26.9 to 44.7 percent. The antifungal performance of product 4 was best with 44.7 percent disease control followed by compound 1 with 41.9 percent disease control.

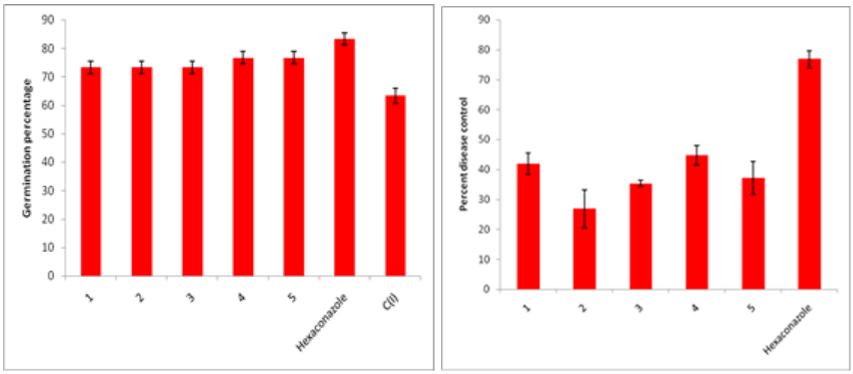

Fig 1: Germination percentage and percent disease control with respect to *R. solani*

As compared to inoculated control, all the test chemicals performed better in antifungal activity against *S. rolfsii*. The fairly high germination was observed with the values of 70.00 to 76.67 percent and in control (uninoculated) 86.67 percent. The highest germination percentage was observed in compound 2 with 76.67 percent germination comparable to standard hexaconazole (83.33 percent) as depicted in figure 2. The percent disease control was in the range of 18.75 to 33.88 percent. The percent disease control was highest with compound 2 (33.88 percent disease control) followed by compound 4 with 31.91 percent disease control. About fifty four percent disease control was observed with the application of hexaconazole.

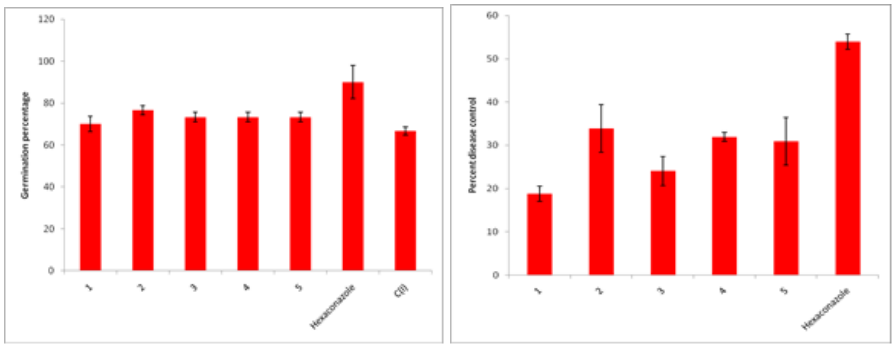

Fig 2: Germination percentage and percent disease control with respect to *S. rolfsii*

The results of antifungal activity of test chemicals revealed their moderate to high effect in controlling the disease caused by *R. bataticola*. The germination was moderately high (73.33 to 83.33) for synthesized imines and 86.67 percent in control uninoculated. The highest germination percentage was observed in compound 3 with 83.33 percent germination similar to standard hexaconazole (83.33 percent) .The percent disease control was in the range of 31.82 to 53.79 percent. The percent disease control was maximum in pots treated with compound 3 (53.79 percent) among the tested products whereas hexaconazole formulation demonstrated 66.67 percent disease control as depicted in figure 3.

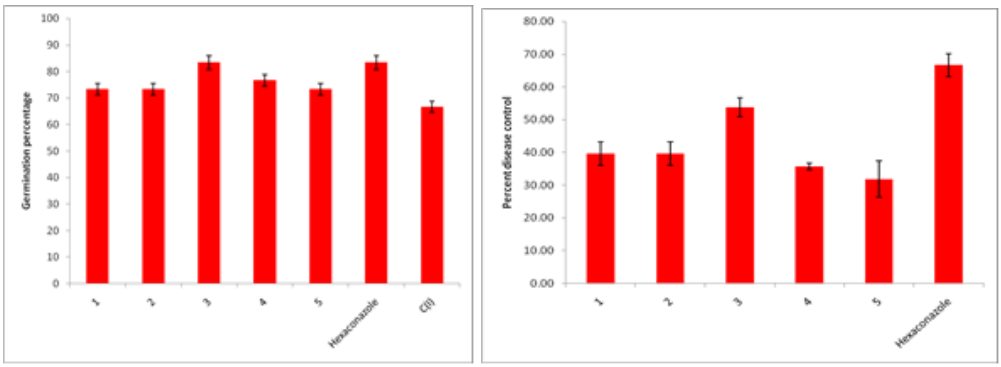

Fig 3: Germination percentage and percent disease control with respect to *R. bataticola*

The developed formulated products are able to effectively control the disease infestation caused by the test pathogens. Additionally, these products did not show any negative impact on the germination of mung bean seeds.

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