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Cellular destruction, phytohormones and growth modulating enzymes production by *Bacillus subtilis* strain BC8 impacted by fungicides

Mohammad Shahid*, Mohammad Saghir Khan

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India

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ABSTRACT

In vitro experiments were performed to ascertain the impact of kitazin, hexaconazole, metalaxyl and carbendazim on growth behaviour, enzymatic profile, ultrastructure, cell permeability and bioactive molecules of phosphate-solubilizing bacterium. Strain BC8 isolated from *Brassica oleracea* rhizosphere was characterized and identified as *Bacillus subtilis* by 16S rDNA sequencing (Accession no. MG028650) technique. Strain BC8 was unambiguously chosen due to its high tolerance capability to various fungicides and substantial production of plant growth regulators. The biomarker enzymatic assays (lipid peroxidation, lactate dehydrogenase) and oxidative stress (catalase) induced by fungicides exhibited significant ($p < 0.05$) toxicity of fungicides toward strain BC8. Fungicides caused the cellular/ultrastructural damage and reduced the viability of strain BC8 as clearly revealed under scanning (SEM), high resolution transmission (HR-TEM) and confocal laser scanning (CLSM) microscopy. As the concentration of fungicides increased, a gradual drop in the plant growth promoting traits of *B. subtilis* strain BC8 was observed. Kitazin at 2400 μg mL^{-1}, hexaconazole at 1500 μg mL^{-1}, metalaxyl at 1200 μg mL^{-1} and carbendazim at 1200 μg mL^{-1}decreased the IAA production by 35 (48.3 μg mL^{-1}), 27 (51.5 μg mL^{-1}), 39 (43.6 μg mL^{-1}) and 47% (37.3 μg mL^{-1}), respectively, over control (71.3 μg mL^{-1}), while, α-ketobutyrate was declined by 51 (29.6), 56 (26.2), 61 (22.8) and 68 (19)%, respectively, over untreated control (59.9 mg $protein^{-1}$ h^{-1}). Also, with increase in the concentration of fungicides there was a significant decrease in plant nutrient (P); the maximum being (19.6 μg mL^{-1}) observed at 1500 μg mL^{-1} hexaconazole with consequent drop in pH (from pH 6.4 to 4.2). The current findings clearly suggest that despite injury, *B. subtilis* maintained secreting active biomolecules and this property makes this organism truly indispensable for enhancing crop production under fungicide stressed conditions.

1. Introduction

Fungicides in intensive agricultural practices are frequently used to control/manage numerous soil borne phytopathogens concurrently optimizing crop production [1]. It is estimated that approximately 2.8 million tonnes of the pesticides are used worldwide for enhancing crop production each year which has threatened the very sustainability of agro-ecosystem [2,3]. Whereas, pesticides including fungicides are essential for plants to relieve the phytopathogens pressure [4]. The supply of active biomolecules like phytohormones [5] and plant nutrients (P and N) on the other hands are equally important to facilitate plant growth. However, the continued and hysterical application of the synthetic fungicides in crop production has led to the destruction of soil fertility [6] and decline both in the quantity and quality of foods [7] which in turn *via* food chain adversely affects the human health [8]. In this regard, fungicides have shown inhibitory effect on the composition and functions of beneficial soil bacteria [9] and edible crops [10]. As a result, the microbial community structure and associated soil chemistry generally referred to as soil fertility are lost [11]. For instance, hexaconazole, a broad spectrum and synthetic fungicide have been found to negatively affect the soil microbial biomass, microbial physiology, respiratory activity, bacterial abundance and community structure [12]. Also, other fungicides like, mancozeb and dimethomorph are reported to reduce massively the number of soil microorganisms [13], microbial ecology and enzyme activity [14]. While, considering these and other related available data, many scientists have attempted to assess the lethal impact of fungicides on survival and associated plant growth regulating activities of plant growth promoting rhizobacteria (PGPR). In this context, the effect of varying doses of organochlorine pesticides on plant growth promoting traits of phosphate solubilizing *Paenibacillus* sp. strain IITISM08 isolated from rhizosphere soil, has recently been reported [15].

Several xenobiotic compounds including agrochemicals have been found to generate numerous free radicals which increases the cellular

* Corresponding author.
E-mail address: shahidfaiz5@gmail.com (M. Shahid).

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oxidative stress [16]. Of the various stressor molecules, the enhanced production of reactive oxygen species (ROS) under stressed conditions causes cellular toxicity to plants [17]. To overcome the stressed related problems, plants have evolved several mechanisms involving generation of redox reducing enzymes under harsh conditions [18]. Additionally, aggressively colonizing rhizosphere beneficial microbiota often termed PGPR have been shown to poses the ability to produce antioxidant enzymes [19] for example, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD). These enzymes in effect degrade the detrimental effect of ROS generated in plants during abiotic stresses and hence, allow the plants to grow normally. Due to these, the antioxidant enzymes generated under pesticide stress are likely to play a self-protective role in protecting crops from damage caused due to stress.

Despite these, there is a critical gap in understanding the mechanistic basis of fungicidal impact on functional diversity of heterogeneously distributed PGPR. To fill these gaps and to better explore the possibility of developing fungicide tolerant bacteria capable of expressing multiple plant growth promoting activities even under harsher fungicide environment, the present study was aimed at- (i) identifying fungicide tolerant bacterial strain (ii) assessing the impact of fungicides on growth behaviour of bacterial strain (iii) evaluating the effect of fungicides on enzymatic profile of strain BC8 (iv) determining the influence of fungicides on cell morphology, ultrastructure and viability of bacterial strain and (v) assaying the inhibitory/inducing effect of fungicides on bacteria mediated plant growth promoting traits.

2. Materials and methods

2.1. Biochemical and molecular identification of bacterial strain

Bacterial strains were isolated from cabbage rhizospheres soil (sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay 143 g kg^{-1}, pH 7.2, electrical conductivity (EC) 0.972 mv cm^{-2}, organic carbon (OC) 0.72%, total available N, P and K are 0.077, 20.25, and 196.65 kg ha^{-1}, respectively. Sulphur (S) and Boron (B), Zn, Fe, Mn and Cu 12.5 and 5.2, 1.08, 9.14, 4.05 and 0.38 mg kg^{-1}, respectively) by serial dilution plate method on Pikovskaya (PVK) agar plates containing fixed amount of tricalcium phosphate (TCP: 5.0 g L^{-1}). The plates were incubated at 28 ± 2 °C until halos/zones appeared around the bacterial colonies. Then, the bacterial strains were repeatedly streaked, purified, and maintained on the same media until use. Among numerous bacterial strains appearing on solid PVK plates, BC8 showed a remarkable ability to dissolve inorganic TCP and was chosen for further studies. Strain BC8 was biochemically characterized and presumptively identified [20]. Further, strain BC8 was molecularly identified to species level using 16S rRNA partial gene sequence analysis which was done commercially by a DNA sequencing service provided by Macrogen Inc., Seoul, South Korea, using universal primers 785F (5′-GGATTAGATACCCTGGTA-3 and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The BLASTn program available online was employed to find similarities/variation with known taxonomic information accessible from the databank at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to precisely assign species name to strain BC8. The nucleotide sequence data received from Macrogen was deposited in the GenBank sequence database for Accession number. Sequences were aligned using bootstrapped neighbour-joining method and a phylogenetic tree was constructed using MEGA7.0 software.

2.2. Selection of maximum tolerant Bacteria

Bacterial strain BC8 was exposed to varying concentrations of kitazin, hexaconazole, metalaxyl and carbendazim (Table 1) in order to select the fungicide tolerant strain. After sterility check, the minimal salt agar (MSA) plates were amended with increasing concentrations (0–3200 μg mL^{-1}) of each fungicide and 10 μL of overnight grown bacterial strains were spot inoculated. Plates were incubated at 28 ± 2 °C for 48 h and the bacterial cultures surviving at the highest concentration of fungicides were picked up and designated as fungicide tolerant strains. Of the total of 25 phosphate solubilizing bacteria (PSB), strain BC8 showing maximum tolerance to each fungicide was selected for further studies.

2.3. Bacterial growth kinetics under fungicide stress

The growth kinetics was examined by growing bacterial strain into mineral salt medium (MSM) containing normal, double and three times more of normal rates of kitazin, hexaconazole, metalaxyl and carbendazim. The MSM without fungicides but having only BC8 strain served as control. The treated/untreated MSM was maintained at 28 ± 2 °C on rotary shaker and growth was measured spectrophotometrically at 600 nm at regular intervals, and growth curve was plotted.

2.4. Oxidative damage experiments

2.4.1. Catalase (CAT) assay

Catalase activity in fungicide untreated and treated bacterial cells was estimated following the method as previously described [21]. Of the tissue homogenate, 100 μL bacterial supernatant was mixed with 2 mL of phosphate buffer (50 mM, pH 7.0) and 100 μL of H_2O_2 (30 mM) was added to it. The reduction in the absorbance was measured at 240 nm for 180 s. using the UV–Vis spectrophotometer (Shimadzu, 2600, Japan). The results were interpreted as the nanomolar of H_2O_2 $minute^{-1}$ μg^{-1} protein.

2.5. Membrane integrity

2.5.1. Lactate dehydrogenase (LDH) assay

The extracellular lactate dehydrogenase (LDH), an indicator of the membrane penetration and cytotoxicity, was assessed in both fungicide-treated and untreated cells. The interacted bacterial cell suspensions were centrifuged for 10 min. at 7000 × *g*, and the LDH level in the supernatant was determined. To the 100 μL of supernatant, 100 μL of the sodium pyruvate (30 mM) and 2.8 mL of Tris–HCL (0.2 M) were added. A-100 μL of NADH (6.6 mM) was finally added and the rate of the decrease in the absorbance was determined at 340 nm using UV–Vis spectrophotometer (Shimadzu, 2600, Japan). The change in the absorbance of sample indicated positive LDH reaction.

2.5.2. Assay of total protein and lipid peroxidation (LPO)

The total protein in fungicide treated bacterial cells was estimated by the method of Bradford [22] using BSA (bovine serum albumin) as a standard and the enzyme activity was determined using different concentrations of fungicides. The untreated bacterial cells (without fungicide) served as control. The protein concentration in the bacterial cells was measured spectrophotometrically at 595 nm.

The toxicological impact of varying concentration of fungicides on the membrane damage was determined by estimating the LPO activity of bacterial cells [23]. The untreated bacterial cells served as control. For this, 100 μL of the sample was added to 2.5 mL of TBA solution and incubated at 95 °C for a duration of 60 min. After that, the tubes were allowed to cool at room temperature followed by centrifuged at 3000 rpm for 15 min. Finally, the supernatant so obtained was spectrophotometrically read at 532 nm for LPO activity. The absorbance of the experimental samples was compared to the standard graph obtained using the malondialdehyde (MDA) solution.