Biocatalysis and Agricultural Biotechnology 14 (2018) 96–108

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Evaluation of microbiological management strategy of herbicide toxicity to greengram plants

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ARTICLE INFO

Keywords:
Greengram
Herbicide toxicity
PGPR
Proline
Bioremediation
Growth promotion

ABSTRACT

In order to circumvent the problems of herbicidal toxicity, tolerant N_2 fixing and phosphate solubilizing bacterial strains were isolated and identified. Among 20 bacterial isolates, *Azotobacter* sp. strain AZ1 survived 2400, 3200, and 1600 μg/mL while *Bacillus* sp. strain PSB2 tolerated up to 1600, 2400 and 1600 μg/mL glyphosate, quizalofop and metribuzin, respectively. Under in vitro conditions, these bacterial strains secreted IAA, siderophores, exopolysaccharides, ammonia and transformed inorganic P into organic P even under herbicides stress. SEM and CLSM images revealed a clear toxic impact of herbicides on bacterial cells above tolerance limit. Phytotoxicity to greengram plants increased with increasing concentrations of herbicides. Herbicide tolerant *Azotobacter* strain AZ1 and *Bacillus* sp. strain PSB2 when used as inoculant, substantially reduced the herbicidal toxicity to greengram. For instance, strain AZ1 increased the length of roots (10%) and shoot (6%), dry biomass of root (28%) and shoot (6%), different symbiotic parameters like nodule number (6%), nodule dry biomass (8%), LHb (15%), photosynthetic pigments and seed yield (39%), whereas, *Bacillus* sp. strain PSB2 enhanced the measured parameters by 12%, 13%, 23%, 21%, 4%, 6%, 22%, 5% and 27%, respectively relative to positive control (1444 μg/kg glyphosate). Additionally, proline in shoot tissues declined rapidly in bio-inoculated plants. Conclusively, the microbial cultures resulted in better management of herbicidal toxicity to greengram plants. And hence, *Azotobacter* sp. strain AZ1 and *Bacillus* sp. strain PSB2 could be recommended for use as an effective and inexpensive microbial inoculant/biofertilizer to augment the production of greengram in herbicide contaminated soils.

1. Introduction

Pesticides application for protecting crops from pest's damage has been increased alarmingly in recent times. The pesticides however when accumulates beyond certain threshold level, alter soil microbial composition (Zaller et al., 2016), disrupts soil fertility (Mukherjee et al., 2016), and other physicochemical properties of soil (Usman et al., 2017). On the contrary, due to weed competition, the production of legumes suffers heavily. Henceforth, to control weeds and subsequently to augment crop production, herbicides are applied regularly (Jiddimani, 2017). Amongst legumes, greengram is a highly nutritious grain legume cultivated in tropics and provides proteins (19–28%), minerals (0.18–0.21%) and vitamins. It is largely used as human staple food in many countries including India (Hari et al., 2017). Also, when applied, herbicides had variable effects on greengram production (Gupta et al., 2017). These toxic problems associated with herbicide usage, however, can be circumvented by applying naturally occurring soil microflora. Among miscellaneous microbial communities, plant growth promoting rhizobacteria (PGPR) have been found to reduce the agrochemical toxicity by different mechanisms (Azubuike et al., 2016) and has therefore, received considerable attention by the agronomists than the microbiologists. To this end, the PGPR offers a greatly viable and economically inexpensive option for immensely safe detoxification/removal of toxic chemicals from contaminated sites (Akbar and Sultan, 2016). In this regard, *Azotobacter* (Gram negative), an aerobic and free-living, nitrogen-fixing-bacteria distributed over a range of agro-ecological niches have been reported to degrade /detoxify the agrochemicals leading eventually to a safe and viable solution to contamination problems (Gauri et al., 2012). *Bacillus* (Gram positive and aerobic) is yet another agronomically important soil bacterium which has the ability to solubilize inorganic form of P into organic form and is capable of degrading many toxic pesticides including herbicides to non-toxic forms (Geed et al., 2017). Apart from these, *Azotobacter* (Gothandapani et al., 2017) and *Bacillus* (Lastochkina et al., 2017) inoculation have shown beneficial effects against many field crops by supplying N to plants and through secretion/production of growth

Abbreviations: MIC, Minimum inhibitory concentration; IAA, Indole-3-acetic acid; HCN, Hydrogen cyanide; DAS, Days after sowing; LHb, Leghaemoglobin

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https://doi.org/10.1016/j.bcab.2018.02.009
Received 18 December 2017; Received in revised form 9 February 2018; Accepted 12 February 2018
Available online 19 February 2018
1878-8181/

stimulating substances like phytohormones (gibberellin, auxin and cytokinin) (Porte, 2017), siderophores (Kandel et al., 2017), exopolysaccharides and solubilize insoluble phosphate etc. (Alori et al., 2017) under adverse environmental conditions. However, the work on agronomic usefulness of *Azotobacter chroococcum* and phosphate solubilizing *Bacillus* in greengram cultivation is quite scarce. Thus, the toxicity of herbicide to greengram plants and the role of PGPR in the management of herbicide toxicity formed the very basis of the present investigation. And hence, the present study was undertaken with the following specific objectives-(i) isolation and identification of nitrogen fixing *Azotobacter* sp., and phosphate solubilizing *Bacillus* sp. strains (ii) selection of herbicides tolerant bacterial strains (iii) evaluation of plant growth regulating activity of herbicide tolerant strains exposed to herbicide stress (iv) assay of toxic impact of pre-emergence herbicides and bioremediation potential of herbicide tolerant PGPR strains against greengram [*Vigna radiata* (L.) Wilczek] plants and (v) determination of stress alleviator (proline) in inoculated plants grown under herbicide stress.

2. Materials and methods

2.1. Isolation and characterization of bacterial strains

A total of 20 rhizobacterial strains were recovered from the rhizosphere soils of some popularly grown vegetables raised in the experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29 latitude and 72° 29′longitude; western district of Uttar Pradesh, India) which had previous history of pesticide application for the last 7 years. For this, 10 g soil sample was serially diluted in sterile normal saline solution (NSS) shaken well by vortex and was allowed to stand for 30 min. For isolation of *Azotobacter* sp., Ashby's mannitol medium (g L^{-1}: mannitol 20, K_2HPO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCo_3$ 0.1, potassium sulphate 0.1, agar 20 pH 7.4) was used while phosphate solubilizing *Bacillus* sp., was isolated using Pikovskaya agar medium (Pikovskaya 1941). Soil suspension was streaked on plates containing approximately 30 mL of each melted (45ºC) medium and then incubated at 28 ± 2ºC for about 3–4 days. The colonies showing brown pigmentation on Ashby's medium were considered as *Azotobacter* while colonies expressing zone of solubilization (halo) on Pikovskaya agar plates represented phosphate solubilizers. A- single colony was picked and streaked 4 times on the same medium to confirm the purity of the cultures and the strains were maintained on their respective medium at 4 °C until use. The bacterial isolates were identified to genus level by numerous biochemical tests described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and were referred to as *Azotobacter* and phosphate solubilizing *Bacillus* throughout this study. The isolated bacterial strains were further identified to species level using 16S rDNA gene sequence analysis

2.2. Selection of herbicide tolerant bacterial strains

The tolerance level of rhizobacterial strains to glyphosate, metribuzin and quizalofop-p-ethyl herbicides (procured from Parijat Agrochemicals, New Delhi, India) was determined by agar plate dilution method using minimal salt agar medium (g L^{-1}: KH_2PO4 1.0, K_2HPO4 1.0, NH_4NO_3 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.02, $FeSO_4{\cdot}7H_2O$ 0.01, pH 6.5). The freshly prepared agar plates were amended individually with concentrations ranging from 0 to 6400 μg mL^{-1} at two-fold dilution intervals each for glyphosate, metribuzin and quizalofop-p-ethyl. The plates were spot (10 μL) inoculated with 10^8 cells mL^{-1} each of *Azotobacter* and *Bacillus* strains and incubated at 28 ± 2 °C for three days. The bacterial cultures surviving at the highest concentration each of glyphosate, metribuzin and quizalofop -p-ethyl were considered as the herbicide tolerant bacterial strains (HTBS).

2.3. Impact of herbicides on active biomolecule secreting ability of PGPR

2.3.1. Indole acetic acid, siderophore and cyanogenic compound

The excretion of indole acetic acid (IAA) by PGPR was determined by the modified method of Bric et al. (1991). For this, *Azotobacter* strain AZ1 and *Bacillus* strain PSB2 were allowed to grow in Luria Bertani (LB) broth (g L^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5) supplemented with 0, 1X, 2X and 3X concentration of glyphosate, metribuzin quizalofop P ethyl. The 1× concentration of glyphosate, metribuzin and quizalofop P ethyl used in this experiment was 600,400 and 800 μg/mL, respectively. A 100 mL of LB broth supplemented with fixed quantity (100 μg mL^{-1}) of tryptophan was bacterized with one mL (10^8 cells mL^{-1}) of culture and incubated at 28 ± 2 °C for 4 days with shaking at 125 rpm. Rhizobacterial cultures removed at exponential growth phase were centrifuged (at 5433*g*) for 10 min. An aliquot of two mL supernatant was mixed with 100 μL orthophosphoric acid and four mL Salkowsky reagent (2% 0.5 M $FeCl_3$ prepared in 35% perchloric acid) was added to it and incubated at 28 ± 2 °C in dark for one hour. The resulting absorbance of pink color was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard. Siderophores released by the cultures (AZ1 and PSB2) were detected by $FeCl_3$ test (Neiland, 1981). For this, bacterial cultures were inoculated in nutrient broth (NB) treated with different concentrations of glyphosate, metribuzin and quizalofop P ethyl and maintained at 28 ± 2 °C for four days. After complete incubation, bacterial cultures were spun (3000 rpm) for 20 min and one mL supernatant was mixed with one mL of 2% ferric chloride ($FeCl_3$). Change in color from reddish brown to orange was recorded. Production of hydrogen cyanide (HCN) by bacterial strains was determined by the method of Bakker and Schipper (1987). Strains were grown in HCN induction medium (g l^{-1}: tryptic soy broth 30, glycine 4.4, agar 15) carrying 0, 1X, 2X and 3X concentrations of glyphosate, metribuzin and quizalofop p ethyl and incubated. A Whatman filter paper No. 1 soaked in 2% sodium carbonate (Na_2CO_3) prepared in 0.5% picric acid solution, was placed on the top of the plate and was sealed with parafilm and incubated. After successful incubation, change in filter paper color (from orange to red) represented HCN production.

2.3.2. Exopolysaccharide, phosphate solubilization and NH_3 production

The exopolysaccharide (EPS) secreted by the bacterial strains were also evaluated under herbicide stress. For this, the bacterial strains were grown in 100 mL capacity flasks containing- (i) basal medium supplemented with 5% sucrose (as C source) and (ii) recommended doses of glyphosate, metribuzin and quizalofop p- ethyl. The inoculated and herbicide containing flasks were incubated for 5 days at 28 ± 2 °C on rotary shaker. Culture broth was spun thereafter at 5433 × *g* for 20 min and EPS was extracted by adding 1:3 volume of chilled acetone to supernatant. The EPS so precipitated was washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying (Mody et al., 1989). The phosphate solubilization activity (PSA) was quantitatively estimated using liquid Pikovskaya medium amended with 0, 1X, 2X and 3X concentration of glyphosate, quizalofop and metribuzin. The amount of solubilized P was evaluated by chlorostannous reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1976). For ammonia production, AZ1 and PSB2 isolates (200 μL of 10^8 cells mL^{-1}) were inoculated in 10 mL peptone water supplemented separately with 0, 1X, 2X and 3X concentrations each of glyphosate metribuzin and quizalofop p ethyl and incubated at 28 ± 2 °C for 5 days. One mL of Nessler reagent was added to each tube. Development of yellow to orange color indicated a positive reaction for ammonia production (Dye, 1962). Each individual experiment was repeated three times at different time intervals.

2.4. Cellular damage induced by herbicides

Cellular damage to the bacterial strains AZ1 and PSB2 were

Forbidden

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