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Effect of malachite green toxicity on non target soil organisms

R. Gopinathan [a,1,3], J. Kanhere [a,2,3], J. Banerjee [b,*]

[a] Biology Department, Indian Institute of Science Education and Research (IISER), Pune, Dr. Homi Bhabha Road, Pune 411 008, India
[b] Biology Department, 900 NCL Innovation Park, Indian Institute of Science Education and Research (IISER), Pune, Dr. Homi Bhabha Road, Pune 411 008, India

HIGHLIGHTS

- Malachite green (MG) toxicity was tested on beneficial soil bacteria, fungi, earthworms and on seed germination of crop plants.
- Genotoxicity, cytotoxicity and scanning electron microscopy assays concluded malachite green induced toxicity in soil microorganisms.
- Filter paper and artificial soil test on earthworms demonstrated a LC 50 of 2.6 mg/cm^{-2} and 1.45 mg/kg respectively with evident morphological alterations.
- Seed germination of Mung bean, Wheat and Mustard is unaffected in presence of MG upto 100 ppm.
- MG negatively effects growth, physiology of tested soil borne micro organisms and earthworms raising concerns about its environmental hazard.

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ABSTRACT

Although malachite green (MG), is banned in Europe and US for its carcinogenic and teratogenic effect, the dye being cheap, is persistently used in various countries for fish farming, silk, dye, leather and textile industries. Current research, however, fails to elucidate adequate knowledge concerning the effects of MG in our ecosystem. In the present investigation, for the first time, an attempt has been made to study the effects of MG on soil biota by testing *Bacillus subtilis*, *Azotobacter chroococcum*, *Saccharomyces cerevisiae*, *Penicillium roqueforti*, *Eisenia fetida* and seeds of three crop plants of different families. Various tests were conducted for determining cytotoxicity, genotoxicity, acute toxicity, morphological and germination effect. Our data confirmed MG toxicity on fungi and bacteria (gram positive and gram negative organisms) showing elevated level of ROS. Genotoxicity caused in the microorganisms was detected by DNA polymorphism and fragmentation. Also, scanning electron microscopy data suggests that the inhibitory effect of MG to these beneficial microbes in the ecosystem might be due to pore formation in the cell and its eventual disruption. Filter paper and artificial soil test conducted on earthworms demonstrated a LC 50 of 2.6 mg cm^{-2} and 1.45 mg kg^{-1} respectively with severe morphological damage. However, seed germination of Mung bean, Wheat and Mustard was found to be unaffected in presence of MG up to 100 mg L^{-1} concentration. Thus, understanding MG toxicity in non target soil organisms and emphasis on its toxicological effects would potentially explicate its role as an environmental contaminant.

1. Introduction

Malachite green (MG), an N-methylated diaminotriphenylmethane basic dye is extensively used in aquaculture as it is highly efficient against fungal and protozoan infections (Sudova et al., 2007). It is also extensively used in food and textile industries. Due to its pervasive use and high solubility in water, MG can be released into the environment by various sources. Studies have reported its carcinogenic (Lee et al., 2006), teratogenic (Culp et al., 1999) and reproductive abnormalities (Cha et al., 2001) spanning its effect from various fish to mammals (Srivastava et al., 2004). Moreover, it is also demonstrated that MG is highly persistent in the environment (Xie et al., 2012). Due to its health hazard concerns, MG is banned in Europe, United States and Canada (Mitrowska et al., 2007). Being extremely economic and highly potent against infections, it is still used steadily in various countries. In a recent report from Germany, malachite green was detected in suspended particulate matter from German rivers with concentrations ranging between LOD (1 ng g^{-1} d.w.) and 543 ng g^{-1} (Ricking et al., 2013). As MG can be resistant to natural biological degradation (El Qada et al., 2008), its residues might cause environmental pollution

* Corresponding author. Tel.: +91 (20) 25908001
E-mail address: jayeetab@iiserpune.ac.in (J. Banerjee).
[1] Present address: 12 Atul Vihar, Lane 9, Dahanukar Colony, Kothrud, Pune 38, India.
[2] Present address: Flat 6, Chinar Apartment, 34 Shilavihar Colony, Erandwane, Pune 38, India.
[3] Equal contribution.

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(Jin et al., 2013). Furthermore, MG is speculated to be toxic to the food web and can disturb aquatic life (Chen et al., 2014; Kanhere et al., 2014). Contrary to several studies on fish, literature on effect of MG on non-target and beneficial soil organisms is extremely scanty (Sudova et al., 2007) in spite of all its hazardous information. Soil is one of the most important resources for food production. Its intricate structure and function depends on its physical properties as well as its biological components. This study investigates the ecotoxicity of MG on different non target soil organisms representing different trophic levels using a battery of test assays. Microorganisms in soil are essential players in carbon, nitrogen and phosphorous cycling including several elements indispensable to life. Organisms considered in this study were *Bacillus subtilis* (important as bio-fertilizers and bio-pesticides), *Azotobacter chroococcum* (nitrogen fixing bacteria), *Saccharomyces cerevisiae* (nutrient source for predatory soil yeast species and nematodes), *Penicillium roqueforti* (saprophytic fungi, producing various secondary metabolites and mycotoxins), *Eisenia fetida* (decomposers, maintaining soil structure and nutrient cycling) and seeds of three crop plants (plants, primary photosynthetic producers) from different families. Accumulation of chemical contaminants in the soil causes various problems such as alteration in physical and chemical properties, decrease in soil fertility, adverse effect on microbial fauna and flora and on soil invertebrates. Therefore, it is necessary to investigate the impact of toxic pollutants reaching soil ecosystem through various routes affecting soil biodiversity both qualitatively and quantitatively. Research on the potential environmental risks posed by unnecessary MG use is important, so as to make evidence-based policy decisions on the future management of its liberal use and to implement measures to protect environmental assets. Our study therefore, could potentially raise awareness of the long ignored ecotoxic effects of MG on soil quality and its environment.

2. Materials and methods

2.1. Chemicals and glass wares

All the chemicals were obtained from Sigma–Aldrich, USA, unless otherwise mentioned. Malachite Green (CAT no-M6880), 2'7'-DCF diacetate (CAT no-35845), 4',6-diamidino-2-phenylindole (CAT no-D9542) were used in the study. Growth media were acquired from HiMedia, India. Glass wares from BOROSIL were used throughout the study.

2.2. Strains used and maintenance

2.2.1. Microorganisms (bacteria and fungi)

All micro-organisms (except for *S. cerevisiae*) were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. *B. subtilis* (NCIM no. 2063) was cultured and maintained in Nutrient Agar (catalogue no. M001) at 37 °C. *A. chroococcum* (NCIM 2452) was cultured and maintained on modified nitrogen-free Burks' media (catalogue no. M707) amended with 0.1% NH_4Cl at 28 °C. *S. cerevisiae* (W303d-kindly provided by Dr. M. Lahiri, IISER, Pune) was cultured and maintained on modified Yeast Peptic digest Dextrose (YPD) Agar (catalogue no. M670) supplemented with 0.1% Adenine at 30 °C. *P. roqueforti* (NCIM 710) strain was cultured and maintained on Potato Dextrose Agar (PDA) (catalogue no. M096) at 28 °C.

2.2.2. Invertebrates (earthworm)

E. fetida were obtained from National Toxicity Centre (NTC) Pune, India. They were maintained according to Organization for Economic Co-operation and Development (OECD) guidelines (OECD guidelines for Testing of Chemicals, 207, 1984).

2.2.3. Crop plants (seeds)

Seeds of Mung beans (*Vigna radiata* – Fabaceae), Mustard seeds (*Brassica nigra* – Brassicaceae) and Wheat grains (*Triticum aestivum* – Poaceae) were obtained from local market for germination assay.

2.3. Growth inhibition assay

The growth of each organism was analyzed by monitoring the cell division in presence of varying concentration of the dye. Samples of cultures were incubated at optimum temperature and periodically measured for cell density till stationary phase was reached. The OD (Optical Density) value was measured by Eppendorf Biophotometer in UVette having path length of 10 mm. Controls were kept for each concentration tested and all tests were done in triplicates.

2.3.1. Microbial growth inhibition test

Initial cell density used were of 0.2 OD_{600} for *B. subtilis*, 0.3 for *A. chroococcum*, 0.25 for *S. cerevisiae*. *P. roqueforti*, growth pattern in presence of MG was determined by using a 96-well flat bottom microtitre plate (Meletiadis et al., 2001) with initial spore count of approximately 15×10^4 spores ml^{-1}. Readings were taken after 24 h with absorbance at 405 nm on Scientific VarioSkan Flash Multimode reader. As most of the MG toxicity studies conducted earlier were from 0.2 to 2 mg L^{-1} (Arnold et al., 2009; Kanhere et al., 2014), we have chosen a similar concentration gradient (0–5 uM) for various organisms in the present study.

2.3.2. Toxicity test in earthworm

For *E. fetida*, filter contact test and artificial soil test were carried out according to OECD guidelines (OECD, 1984) to assess MG toxicity. Filter contact test till 48 h was conducted to determine concentration range in which 0–100% mortality of the earthworms was obtained. Ten replicates were used for each concentration. Artificial soil test for 14 d was conducted to assess acute toxicity. A range of concentrations, 0, 0.1, 1.0, 10, 100 and 1000 mg kg^{-1} dry soil were used to determine a concentration range that resulted in 0–100% mortality. To obtain LC50, test concentrations of 1.2, 1.3, 1.4, 1.5 and 1.75 mg kg^{-1} and a control were used. Determination of lethal values LC50 of MG was determined by probit analysis. For this purpose value of each concentration was plotted along *X*-axis and percent of mortality along *Y*-axis and the eye fitted curve was drawn. LC50 values were calculated from the obtained curve (Matsumura, 1975).

2.3.3. Seed germination assay

For germination assay, fifty seeds of each plant were tested on filter paper soaked with 10–100 mg L^{-1} of MG and water as control. Percent germination was noted after 48 h and root length in centimeter was measured after 96 h. All assays were performed in triplicates.

2.4. Cytotoxicity assays

2.4.1. Comparison of protein profiles

Total protein was extracted from *B. subtilis*, *S. cerevisiae* and *P. roqueforti* using TCA and acetone method (Damerval et al., 1986) and was further precipitated (Link and LaBaer, 2011). Isolated protein concentration was estimated by Bradford assay (Bradford, 1976). Whole cell protein was extracted from *A. chroococcum* using SDS method (Bhaduri and Demchick, 1983). Extracted proteins were re-suspended in Laemmli sample buffer (Cold Spring Harb Protoc, 2006) and loaded on a SDS-PAGE gel (Laemmli, 1970) for analysis. All protein isolations were carried out when the cells were in log phase of their growth.