Ecotoxicology and Environmental Safety 84 (2012) 268–273

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Light induced degradation of phorbol esters

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ARTICLE INFO

Article history:
Received 25 April 2012
Received in revised form
18 July 2012
Accepted 20 July 2012
Available online 11 August 2012

Keywords:
Phorbol esters
Ecotoxicity
Bioassay
Light degradation
Detoxification
Jatropha

ABSTRACT

Jatropha curcas (Jatropha) is a tropical shrub that is gaining popularity as a biofuel feedstock plant. Phorbol esters (PEs) are tetracyclic tiglian diterpenoids that are present in Jatropha seeds and other parts of plant. Epidermal cell irritating and cancer promoting PEs not only reduce commercial values of Jatropha seed cake but also cause some safety and environment concerns on PE leaching to soil. A simple bioassay of PE toxicity was conducted by incubating 48 h old brine shrimp (*Artemia salina*) nauplii with Jatropha oil for 24 h. 1–4% of Jatropha oil (corresponding to PE concentration of 25–100 mg L^{-1}) had mortality rate of 5–95%, with LC_{50} estimated to be 2.7% of oil or 67 mg L^{-1} of PE. Jatropha oil was incubated with clay or black soil (autoclaved or non-autoclaved) in the darkness or under sunlight for different periods of time before oil was re-extracted and tested for PE content by HPLC and for remaining toxicity with the brine shrimp bioassay. Under sunlight, PE decreased to non-detectable level within six days. Toxicity reduced to less than 5% mortality rate that is comparable to rapeseed oil control within the same period. In contrast, PE level and toxicity remained little changed when Jatropha oil was incubated in the darkness. Such PE degradation/detoxification was also found independent of the presence of soil or soil microorganisms. We conclude that sunlight directly degrades and detoxifies PEs and this finding should alleviate the concern on long term environmental impact of PE leaching.

1. Introduction

Jatropha curcas (Jatropha) is a member of Euphorbiacaeae family. It is a shrub that has been used in tropical and subtropical regions of Central America, Asia and Africa as "living fences" around fields and settlements or folk medicine (Heller, 1996). It is gaining popularity as a biofuel feedstock plant due to its non-edible seed oil, drought tolerance and adaptability to almost any type of soil, including those in arid and marginal land that are not suitable for crops (Heller, 1996). It was projected that as much as 25.6 million tons of Jatropha oil could be produced yearly by 2015 (GEXSI, 2008). The seed contains kernel and shell with an average ratio of 62.2:37.7. The kernel has higher crude protein (22–28%) and oil contents (54–58%) (Devappa et al., 2010a). Production of Jatropha oil can also produce large amount of seed cake. The defatted Jatropha kernel meal (JKM) obtained after solvent extraction of kernels (free of shells) contains higher protein content ranging from 57.3% to 63.8%, higher than those in commercial soybean meal (46.5%) (Makkar et al., 1998). If seed cake can be used for livestock feeding, commercial value of Jatropha plantation will be greatly increased and its commercial viability will be more attractive. However, antinutrients like trypsin inhibitor, lectin and phytate (Martinez-Herrera et al., 2006), and two major toxic components—curcin and phorbol esters (PEs), are present in Jatropha seeds (Makkar and Becker, 1997).

Curcin belongs to the ribosome inactivating proteins (RIPs) group. It shows cell-free protein synthesis inhibition, but not under *in vivo* conditions. Also, it is heat labile and easily degraded in soil (Devappa et al., 2010a; Gubitz et al., 1999; Lin et al., 2003). On the other hand, PEs are tetracyclic tiglian diterpenoids that are present mostly in Jatropha seeds. Six different PEs have been characterized from Jatropha (Devappa et al., 2010b; Haas et al., 2002). Unlike curcin, PEs are amphiphillic molecules and present in both oil and seedcake (up to 8 mg/g in oil and 3 mg/g in defatted kernel meal) and are heat stable. Since PEs are epidermal cell irritating and cancer promoting (NIH, 1984), Jatropha seed cake cannot be used directly as an animal feed without detoxification. Currently, they are often used as an organic fertilizer and increase of Jatropha seed yield between 13% and 120% was reported after returning Jatropha seedcake to Jatropha plantation. Not only do PEs reduce commercial values of Jatropha seed meal, they also cause some safety and environment concerns. Ingestion or skin application of PE results in a burning sensation and pain in

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http://dx.doi.org/10.1016/j.ecoenv.2012.07.021

the mouth and stomach, watery or bloody diarrhea. Eye exposure results in conjunctivitis (also called pink eye) (NIH, 1984). Human safety concerns are mostly on accidental exposure or occupational exposure of operators involved in seed harvest and seed/oil processing. As PEs are heat stable, there are also concerns on their possible ecotoxicity to other plants and animals when seed meals are used as fertilizers, or when seeds remain in soil for a prolonged period of time, with possible PE leaching into soil and being carried to water bodies (Devappa et al., 2010b).

To address these concerns, one important research topic is to follow the fate of PEs under field conditions for both structure integrity and overall toxicity. A recent study evaluated PE degradation in soil. Silica enriched with PEs extracted with methanol from oil or seed cake were incubated with soil sample in the dark before PEs were re-extracted and quantified with High Performance Liquid Chromatography (HPLC) and toxicity evaluated by snail (*P. fontinalis*) bioassay (Devappa et al., 2010b). Authors concluded that degradation of PEs can happen in soil over a period of time, and that such PE degradation in the darkness depends on the presence of microorganisms, and is enhanced by higher temperature and higher moisture levels (Devappa et al., 2010b).

In the present study, we simulate PE leaching to the environment by incubating PE containing Jatropha oil with various types of soil for a period between zero and nine days, in the presence or absence of microorganisms and keeping them in the darkness or exposure to sunlight. HPLC was used to assess structure integrity of the toxic PEs in oil, Brine shrimp (*Artemia salina*) was used for our evaluation of PE toxicity in test samples. Brine shrimp filter feeds non-selectively and is a primary consumer in the aquatic food chain. It is a potential vector for bioaccumulation and biomagnification of contamination (Sorgeloos et al., 1978). It is also readily available due to its common use as a live feed for both freshwater and salt water fish larvae. We aim to find out if PE can be detoxified or degraded under natural conditions with sunlight, and identify key factors that affect such process. A key finding is that sunlight degrades and detoxifies PEs effectively in few days, and such light-dependent detoxification is independent of soil types, microorganisms and even the presence of oil.

2. Materials and methods

2.1. Materials

Jatropha seeds were collected from a Jatropha Research Farm (JOil Pte Ltd., Singapore) and two different soil types were used in the experiment—local red clay soil collected from the National University of Singapore campus (1.2941°N, 103.778°E), and imported universal potting soil (Jiffy, Netherlands), referred to as black soil because of its major constituent of black peat. Soils were dried and homogenized with a pestle and mortar. A portion was subjected to normal autoclaving treatment at 121 °C for 15 min to kill all microorganisms. *Artemia salina* (brine shrimp, INVE of Belgium) cysts were purchased from a local aquatic shop. The natural seawater was filtered using Nalgene 90 mm filter unit (0.45 μm) and its salinity (30–34 parts per thousand, ppt) was measured by a hydrometer (Aquarium Systems, USA). Dimethylsulfoxide (DMSO) and phorbol-12-myristate 13-acetate (PMA) were obtained from Sigma (St Louis, USA). Other chemicals, such as hexane, ethyl acetate, methanol, tetrahydrofuran and acetic acid were obtained from Fisher Scientific.

2.2. Crude oil extraction from Jatropha seed kernels

Jatropha seeds were manually processed. Kernels were separated from seed shell and ground into fine power in mortar with pestle. Kernel powder was mixed with diatomaceous earth (DE) and the mixture pressed into a 15 ml extract cell. Oil extraction was conducted at 105 °C at 1200 psi by hexane solvent with an Accelerated Solvent Extractor 200 (ASE 200, Dionex) for 60 min. Re-extraction was conducted in the same method by ethyl acetate with 10% methanol. Solvents were evaporated by nitrogen gas flow at 45 °C. Crude Jatropha oil extracts were then combined.

2.3. Oil/soil mixture and treatments

Each 10 g soil sample (clay soil or black soil, autoclaved or non-autoclaved) was mixed with 5 ml of crude Jatropha oil. Some samples were exposed to sunlight in a greenhouse (with thin polycarbonate cover that reduces about 30% of sunlight intensity, to shield samples from rain. There were about 6 h of sunshine per day during our test period, reaching a maximum light intensity of 80,000 lx) and some were put in a dark box at room temperature (25 °C) for zero–nine days. After each treatment, the oil/soil mixture was mixed with diatomaceous earth and the oil was re-extracted with hexane followed by ethyl acetate/methanol by the Accelerated Solvent Extractor (ACS200, Dionex) at 105 °C, pressure 1200 psi, 60 min for each extraction. Hexane or ethyl acetate was evaporated by nitrogen gas flow at 45 °C and re-extracted oil was mixed with DMSO (1:1 v/v) and stored at −20°C for subsequent toxicity tests (section 2.5). Oil re-extraction efficiency was found to be >95% with this setup.

2.4. Phorbol esters analysis by HPLC

Our HPLC method was a modification to Makkar's protocol (Makkar et al., 1997). 50 μl oil was dissolved in 200 μl Tetrahydrofuran/Methanol (4:1) and 10 μl of the mixture was injected to a HPLC system (Agilent 1200) with Symmetry® C18, 4.6 × 250 mm, 5 μm column (Waters). Mobile phase of water (A) and methanol plus 1% acetic acid (B) was applied. PEs were eluted by a gradient of 60–80% B (25 min) 80–85% B (15 min) then held at 95% B (10 min) and detected at 280 nm by a DAD detector. Pure PMA (Sigma) was diluted with methanol to concentrations between 0.031 and 0.5 mg/ml (to give 5 μg, 2.5 μg, 1.25 μg, 0.63 μg and 0.31 μg of PMA in an injection volume of 10 μl), analyzed as the external control, which appeared between 33 and 34 min. Average peak areas of three replicates were used to build a standard curve of good linearity ($R^2 > 99\%$) that was used to quantify total PE content in samples, which appeared in 5 peaks between 31 and 35 min. Repetitiveness of analysis was also evaluated by injection of the same sample (1.25 μg of PMA consequently 5 times and non-consequently (separated by other samples) 5 times). Limit of quantitation was also tested with serial dilutions of PMA (1, 0.5, 0.25, 0.125, 0.063, 0.031 and 0.015 μg of PMA in an injection volume of 10 μl).

2.5. Brine shrimp bioassay

A modification to a previous protocol was used (Kinghorn et al., 1977). 100 mg of brine shrimp cysts were incubated in natural seawater in a Petri dish at 25 °C, with indoor lighting for 24 h. Newly hatched nauplii were transferred into another Petri dish with fresh seawater and maintained for another 24 h. After a total incubation time of 48 h, 50 μl seawater containing ten 48 h old live brine shrimps were pipetted to each well in a 24-well tissue culture plate, flat bottom with low evaporation lid (Falcon) and topped up with 400 μl of fresh seawater, making up to a volume of 450 μl. Six wells with 60 nauplii in total were used for each toxicity test. 50 μl equal volume mixture of DMSO with re-extracted oil after each treatment was then added into each well making final volumes of 500 μl. These plates were then further incubated for another 24 h without feeding. 50 μl equal volume mixture of rapeseed oil with DMSO was used as the negative control. Number of surviving (free moving or moving upon stimulation) nauplii was counted at the end of 24 h using a stereo microscope (Leica). Mortality ratio was calculated as percentage of death in 60 nauplii. Toxicity bioassay for each treatment was repeated four more times. Different concentrations of PMA (from 20 to 250 mg L^{-1}) were also tested for toxicity.

2.6. Data analysis: LC_{50}

Various volumes (10–40 μl, corresponding to 1–4% of Jatropha oil, or 24.9–99.5 mg L^{-1} PE in the final volume) of oil mixed with non-autoclaved clay soil were incubated with brine shrimp to compute LC_{50}, the effective lethal concentration at which 50% of the brine shrimp die. Seven different concentrations (1%, 1.5%, 2%, 2.5%, 3%, 3.5% and 4%) of Jatropha oil had their mortality rate ranged from 5% to 95%. A linear regression graph was then plotted and analyzed by GraphPad Prism 5 (GraphPad software, San Diego). The goodness-of-fit to the linear regression line was evaluated based on the R^2 value. The concentration of Jatropha oil correlating to 50% of mortality is taken as LC_{50}. Student *T*-test and ANOVA tests were conducted by Microsoft Excel 2007.

3. Results

3.1. Validation of HPLC analysis methodology

Our HPLC method was a modification to Makkar et al. (1997). Using pure PMA compound (Sigma), we firstly built a standard curve based on various dilutions (to give 5, 2.5, 1.25, 0.63 and