Evaluation Of Cytotoxic Activity Of Microbial Biofertilizer And Agrochemical Treated Curcuma Longa L. By Brine Shrimp Lethality Assay

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ABSTRACT

Cancer is a growing health problem around the world, particularly with the steady rise in life expectancy, increasing urbanization and the subsequent changes in environmental conditions, including lifestyle. The modern agricultural system goes in the way of chemical usage to improve crop production that is, leading to drastic health ailments. So, it was intended to use and compare the quality and activity of turmeric obtained through cultivation using agrochemical and biofertilizers inputs. The quality of agricultural product (turmeric rhizome powder) and soil was analyzed for the presence of three heavy metals such as Pb, Ni and Cr by atomic absorption spectroscopy. In addition, the cytotoxicity variation by agrochemical and biofertilizer treated turmeric was assessed by brine shrimp lethality and hatchability inhibition assay (Meyer et al. 1982). The microbial biofertilizers were highly effective in reducing the concentrations of Pb, Ni and Cr at cultivation level in both turmeric rhizomes and rhizosphere soils. While in agrochemical treatments, such high level reduction of heavy metals was not observed. Similarly, the brine shrimp mortality and hatchability inhibition assay showed that the phytochemical composition of turmeric was high under cultivation with microbial bioinoculant treatments. The mortality of brine shrimp occurred with 25µg/ml of turmeric extracts obtained through microbial bioinoculants.

Key words: Heavy metal contamination; turmeric; curcumin; brine shrimp cytotoxicity and hatchability inhibition

Introduction

The World Health Organization (WHO) reports that about 70-80% of the world’s population relies on non-conventional medicine, mainly of herbal sources, for their primary health care (Akerele 1993). However, the industrialization and modern agriculture is threatening the biological ecosystem and almost contaminated the environment that finally resulted in distribution of heavy metals in plant systems. Curcuma longa L., the genus that consist thousands of species belonging to the group of aromatic spices (Maheshwari et al. 2006). The biological activities attributed to turmeric include anticancer, anti-inflammatory, antioxidant, antimicrobial, antiangiogenic, neuroprotective, immunomodulatory and wound healing (Rapaka Rao and Coates 2006). But according to the report of Nirmal Kumar et al. (2007), the turmeric was found to be contaminated with heavy metals such as Pb, Ni, Cu, Co, Fe, Zn and Cd. In particular, the heavy metal contamination in turmeric makes it unsuitable for culinary and medicinal purposes. For mitigation of heavy metals, it is necessary to reduce heavy metals at cultivation level.

As far as the medicinal properties of turmeric and use by the human kind as food are concerned, it is indeed essential to assess the potential of microbial bioinoculants in increasing the qualitative aspects (i.e., high curcumin content and its activities). It is of prime importance to combine both agricultural and pharmaceutical aspects of turmeric to attain the sustainable agriculture and safe health care. The cultivation of turmeric was done with application of microbial biofertilizers as well as with agrochemicals. The harvested rhizome powder and rhizosphere soil were analyzed for the presence of heavy metals such as lead (Pb), nickel (Ni) and chromium (Cr). The efficiency of microbial biofertilizers in reducing the heavy metal contamination and increase in cytotoxic activity of turmeric was compared with agrochemical treatment.
Materials and Methods

Maintenance of the Experimental Plants:

The experiments were carried out at Elathur Chettipalayam village (11°23’8” N, 77°18’55” E), Erode District in Tamil Nadu, India. Turmeric rhizomes were inoculated with biofertilizers and biocontrol agents (*Azospirillum* sp., PSB, AMF, *Trichoderma* sp. and *Pseudomonas* sp.) individually or in various combinations and the control plants were raised in field conditions. Watering was done whenever necessary throughout the duration of the experiment.

Biofertilizer Application:

Liquid based *Azospirillum* sp. and PSB each 200ml was mixed in 1Kg of rhizome seeds along with 5% jaggery solution. The rhizome seeds were soaked in this slurry, shade dried for 30 minutes and sown within 24hrs. 10g of AMF inoculum were applied per rhizome seed at the depth of 2 to 3cm before sowing. 50ml of *Trichoderma* sp. and *Pseudomonas* sp. liquid culture along with 5% jaggery solution per Kg of rhizomes was mixed. The treated rhizomes were shade dried for 30 minutes and then sown.

The individual as well as multiple combinations of biofertilizers introduced were follow: T1 – Uninoculated Control, T2 - *Azospirillum* sp., T3 - *Trichoderma* sp., T4 - *Pseudomonas* sp., T5 - *Azospirillum* sp. + *Trichoderma* sp., T6 - *Azospirillum* sp. + PSB, T7 - *Pseudomonas* sp. + Arbuscular Mycorrhizal Fungi (AMF), T8 - *Trichoderma* sp. + PSB, T9 - *Azospirillum* sp. + *Trichoderma* sp. + PSB, T10 - *Trichoderma* sp. + PSB + AMF, T11 - *Azospirillum* sp. + PSB + AMF, T12 - *Azospirillum* sp. + *Trichoderma* sp. + *Pseudomonas* sp. + AMF.

Agrochemical Application:

As like usual agrochemical practices, the turmeric samples and rhizosphere soils were collected from three different places of Erode District, where the turmeric cultivation is prevalent namely Athani (11°31’18” N, 77°34’49” E), Elathur chettipalayam (11°23’8” N, 77°18’55” E) and Andipalayam (11°24’4” N, 77°16’57” E). The name of these sampling areas where designated as AS, CS and SS respectively.

Sample Collection:

The samples such as rhizosphere soil and rhizome were collected after 8 months interval. A pit was dug around the root zone of turmeric plant followed by the removal of surface soil. Rhizosphere soil samples were air dried whereas the rhizomes were washed thoroughly with tap water to remove adhering soil particles, rinsed with distilled water and stored at 4°C for further analysis. The turmeric samples were then dried and grind coarsely for heavy metal studies and cytotoxicity assays. All the *in vitro* experiments were carried out in the Rhizosphere Biology Laboratory, Bharathidasan University, Tiruchirappalli, India.

Estimation of curcumin content:

0.5g of turmeric powder is refluxed with 250ml absolute alcohol for 3-5h and after extraction, the volume is made to 250ml to compensate alcohol loss. 1ml of the aliquot is diluted to 10ml with absolute alcohol and the color intensity was measured at 425nm (ASTA 1997).

Heavy Metal Analysis:

(i). Heavy Metal Analysis in Turmeric Plant Rhizome:

To the 1g of turmeric rhizome powder, 15ml of the nitric acid was added and heated at 50°C for 20min. 3ml of the hydrogen peroxide solution was added, again heated for 5min and cooled. The acid digested sample was made up to 50ml using double distilled water and filtered. The filtrate was analyzed for the quantification of heavy metals such as Pb, Cr and Ni through atomic absorption spectrophotometer (AAS).

(ii). Heavy Metal Analysis in Rhizosphere Soil:

To the 0.5g rhizosphere soil, 15ml of the hydrochloric acid and nitric acid (3:1) was added. Then, it was heated at 50°C for 20min and cooled. The acid digested sample was made up to 50ml using double distiller...
water and filtered. The filtrate was analyzed for the quantification of heavy metals such as Pb, Cr and Ni through AAS.

**Artemia Brine Shrimp Cytotoxicity and Hatchability Inhibition Assay:**

The examinations were conducted in test tubes using sterilized sea water. A stock solution of the crude turmeric ethanolic extract (1mg/ml) was prepared by dissolving it with ethanol. Different concentrations of extracts ranging from 20µg-1mg were prepared from stock solution. Controls were also prepared and used with filtered sea water as negative control and potassium dichromate as positive control. The brine shrimp cytotoxicity and inhibition assay was performed by following the methods of Meyer et al. (1982).

**Results:**

The concentrations of heavy metals found in the agrochemical and biofertilizer treated turmeric rhizome powder and rhizosphere soils are presented in the table 1. The levels of Pb were lower in biofertilizer treated turmeric rhizosphere soil than in the uninoculated control (T1). Several treatments such as T5, T6, T8, T10 and T13 were showing least concentrations of Pb. In turmeric rhizomes, the Pb concentration was higher in the uninoculated control (T1 -0.410ppm), when treated with *Azospirillum* sp., the Pb concentration had reduced nearly more than two times (0.183ppm). All other treatments inoculated with microbial biofertilizers were showing reduced concentrations of Pb when compared to uninoculated control (T1). The performance of the treatments such as T4 (*Azospirillum* sp. & PSB), T9 (*Arthrobacter* sp. & PSB) and T10 (Azospirillum sp., Trichoderma sp. & PSB) were statistically indifferent. In agrochemical treatments, the samples of AS had highest Pb concentrations (0.376ppm) in turmeric rhizomes and least concentration were shown by SS samples (0.324ppm).

The concentrations of Ni in the rhizosphere soils of uninoculated control (T1) lead all other microbial biofertilizer treatments. The performance of T1 and T13 in the reduction of Ni content in turmeric rhizosphere soil was significantly indifferent. The single inoculation of *Trichoderma* sp. has lead to the ultimate reduction of Ni content in turmeric rhizosphere soils. In agrochemical treatments, the AS and CS samples showed similar level of Ni concentrations that were significantly indifferent. Likewise the concentrations of Ni were found reduced based on the combinations of microbial fertilizers applied starting from T2-T13. As well as the agrochemical treatments in AS, CS and SS sampling areas were also showing reduced concentrations in turmeric rhizomes.

Except in the treatment of T4, all other biofertilizer treatments showed the values to the least extend and they were found to be significantly indifferent. In the agrochemical practices, CS samples showed highest concentrations of Cr than the other two sampling areas. In this case, the multiple combinations of biofertilizer performance were better than single inoculations in reducing the Cr contamination in turmeric rhizomes.

The quality aspects of turmeric product by biofertilizer induction was assessed and compared with turmeric cultivation by agrochemical practices (Table 2). The brine shrimp cytotoxic activity of turmeric extracts was observed for 24hrs exposure. All the treatments showed mortality against *A. salina* ranging from 25 to 200µg/ml concentration. The least mortality percentage was expressed by the treatment of T3 (*Trichoderma* sp.) and maximum by AS (35%), followed by SS (32%) at the concentration of 25µg/ml. Among the biofertilizer induced turmeric samples, the treatments T12 (Azospirillum sp., Trichoderma sp., PSB & Pseudomonas sp.) and T13 (Azospirillum sp., Trichoderma sp., PSB, Pseudomonas sp. & AMF) found to have higher mortality rate at 25µg/ml concentration. About 50% of the analyzed samples were showed LD50 at 50µg concentration of the extracts. Starting from 75 µg/ml concentration, the control turmeric extracts were showing least values compared to agrochemical as well as biofertilizer treatments. But, the samples belonging to T1 (uninoculated control), T2 (Azospirillum sp.) and CS showed least mortality rate when extending the concentration up to 150µg/ml. The variations in the activities was due the phytochemical (cytotoxic compounds) strength of the turmeric i.e., mainly the curcumin. Nearly 85% of LD50 was obtained when the dosage increased up to 75µg/ml. The 100% of mortality was reached only in the biofertilizer treated turmeric extracts rather than the agrochemical treatments. Thus, the best mortality rate was shown by turmeric cultivated when induced with microbial bioinoculants.

The hatchability inhibition of brine shrimp by turmeric extracts is shown in table 3. The very high hatchability inhibition was exhibited by turmeric samples cultivated with inoculation of microbial biofertilizers at least turmeric extract concentration (25µg). Most of the tested samples showed IC50 at 50µg/ml dosage. All the samples reached IC100 when the concentration was increased to 75µg/ml. The treatments of T3 turmeric extracts had shown less potential in killing *A. salina*, whereas they were effective in hatchability inhibition.
Discussion:

The presence of heavy metals in rhizosphere soils and turmeric rhizomes proves that, due to industrialization, heavy metals have been a regular and deliberate constituent if agricultural products and environment. Rhizome containing spices were found to be most susceptible food groups contaminated with heavy metals. Usually, the problem arises when the concentration of these heavy metals increased than the permissible limits. But in our study, the concentrations present in all the samples occurred in permissible limits.

Certainly, there occurred reduction in Pb concentrations when treated with microbial biofertilizers. Since the microorganisms have the potency to reduce the Pb content in the rhizosphere soils. To the greater extent, the Pb concentrations were less when treated with Azospirillum sp. in turmeric. The biofertilizer treatments have the potential to reduce the Pb accumulation inside the turmeric rhizome when compared to untreated control. Similar type of report was given by Pramod and Khadka that in the rhizome containing spices ranges from 0.37 to 3.82ppm of Pb concentration. This is in accordance with our results.

Nickel accumulates in soil primarily through the disposal of industrial effluents, sewage sludge and fertilizers. Even moderate concentrations of this metal can severely limit the growth of plants (Belimov et al. 2005). Metals like nickel decrease protein content (Alvarez et al. 2006). Likewise, the Ni level in the control soils were higher, the Azospirillum sp. treatment was remarkably effective to reduce Ni concentration in both rhizosphere soil and turmeric rhizomes. Additionally, the combination of Azospirillum sp., Trichoderma sp., PSB and Pseudomonas sp. was highly active to reduce Ni level in turmeric rhizomes up to 0.0236ppm over the control (0.0635ppm). Up to 50% reduction of heavy metals was seen in biofertilizer treatments.

Most of all the treatments were showing noticeable reduction in Cr contents in the rhizosphere soils. All these treatments were showing values significantly indifferent at 5% level whereas the treatment of T; also found to be effective. Similar type of study was done by Wu et al. (2008) that estimated the concentrations of heavy metals in soils and turmeric rhizomes.

Here the multiple combination of biofertilizer could able to perform well when compared to single inoculations to reduce the accumulation of Cr inside the turmeric rhizomes. The co-inoculation of microbial bioinoculants was also one such favorable effect caused to out compete the heavy metal contaminations (Burd et al. 2000). The possible explanation for the reduction of Cr in the turmeric rhizomes and rhizosphere soil might be that the microbial biofertilizers involved may take up the Cr in their cells (Faisal and Hosnain 2006). Additionally, the reduced concentrations of Cr in the rhizomes of the turmeric were possible, because the microbial biofertilizers applied can able to resist or restrict the uptake of heavy metals by plant roots. Not only the soil bacteria, the cell wall components such as free amino, hydroxyl, carboxyl and other groups of soil fungi can bind to potentially toxic elements such as Cu, Pb, Cd, etc. (Kapoor and Viraraghavan 1995).

Neelam and Meena (2009) reported that the use of PGPR as biofertilizers can enhance the plant growth and remediate the Ni contaminated sites. Since biofertilizers have the potency to reduce heavy metals by involving in various direct or indirect mechanisms. Gonzalez-Chavez et al. (2004) emphasized the importance of AM fungi and its product named ‘glomalin’ could able to sequester potentially toxic elements such as Cu, Cd, Pb and Mn. Trichoderma sp. was also known to sorb certain heavy metals and it is used as commercial biosorbants of PTEs (Morley and Gadd 1995). Based on the above said results that, heavy metals reduction in rhizosphere soils and turmeric when treated with biofertilizer, the same principle can be applied to heavy metal contaminated sites for remediation.

In order to study the toxicity of these medicinal plants we performed brine shrimp lethality bioassay, which based on the ability to kill laboratory cultured brine shrimp nauplii. The assay was considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of plant extract toxicity (Krishnaraju et al. 2005). In the present study, there occurred dose dependent relationship that the mortality rate increases proportionally when the dosage increased. The results are in accordance with the findings of Khattak et al. (2005). According to their results, the ethanolic extracts of turmeric showed LD₀ at 33µg/ml dosage. Similar to mortality assay, hatchability inhibition also occurred just like dose dependent pattern. The results of this bioassay confirmed the previous uses and findings (Chuang et al. 2000). It is possible that, the extracts contained cytotoxic compounds that act individually or synergistically were responsible for the observed hatchability inhibition and mortality. From the pharmacological point of view, a good relationship has been found with the brine shrimp lethality assay to detect cytotoxic compounds in terrestrial plant extracts (Solis et al. 1993).

Conclusion:

The biofertilizers are also effective in the reduction of heavy metal contamination in soil and turmeric. The brine shrimp cytotoxicity test implies that turmeric possess the capability to inhibit and toxic to A. salina. The higher activity against A. salina was shown by biofertilizer induced turmeric than control.
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References