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Effect of Endosulfan on Indole acetic acid and Gibberellin secretion by *Azospirillum* SPP NCIM-2548 and *Azotobacter* SPP NCIM-2452

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Abstract

In this study an attempt has been made to study the effect of endosulfan, which has half life of 50 days. Certain microorganisms in soil like Azospirillum, which is an associative nitrogen fixer, also secrete Indole Acetic Acid. Like wise Azotobactor secrete Gibberellic acid. Both these are essential plant growth regulators. These substances play importance role in mineral binding to plant from soil and in seed germination. However it has been observed that residual endosulfan at a very low concentration of 50 ppm, is enough to inhibit the generation of these important chemicals.

Key words: Endosulfan**,** *Azospirillum spp, Azotobactor spp,* IAA, GA.

Introduction

Endosulfan is a nonsystemic organochlorine insecticide classified as cyclodiene subgroup of pesticides. Introduced in the 1950's, it is used extensively on many agricultural crops against pests like thrips, whiteflies, aphids, leaf hoppers and lepidopteron larvae. It is effective on pests particularly of vegetable crops, cotton, pulses, cashew, tea, coffee, and tobacco and timber crops[1]. It is a major pesticide among the 45% of the total pesticides used[2]. Chemically endosulfan is 6,7,8,9,10,10-hexa chloro- 1,5,5a,6,9,9a- hexahydro – 6,9- methano- 2,4,3-benzodioxathiepine-3-oxide[3]**.** Technically endosulfan is a mixture of two isomers – α and β endosulfan in the ratio 7:3, along with other related compounds like endosulfan alcohol, endosulfan ether and endosulfan sulfate[4]. Endosulfan is only very slightly soluble in water, but it dissolves readily in xylene, chloroform, kerosene and most organic solvents and is a noncombustible solid[5]. It has a half life of only 50 days, but it shows toxicity like fetotoxicity, teratogenicity, carcinogenicity and mutagenicity[6]. It is no more used in many developed nations. However, it is still used in India and in many developing countries of the tropical and subtropical regions[7].

However, it has been grossly misused resulting in high residual activity in environments like soil. Endosulfan affects carbon dioxide production and microbial biomass[8]. The persistence and degradation of endosulfan are affected by environmental conditions. Persistence is maximum in laterite soil followed by sandy loam and least in red loam soil. It was also observed that half life of α-isomers was lower than that of β-isomers in all three soils[9]. Endosulfan is transformed to endosulfan diol by hydrolysis in water whereas endosulfan sulfate is formed through oxidation. It is highly resistant to microbial bioremediation or biotransformation, i.e. it acts like a xenobiotic compound and that too in agricultural soil[10]. It was therefore considered necessary to study its effect on microorganism which affects the fertility of such soils. In this study the effect on two such important bacteria like *Azospirillum* and *Azotobacter* which produce Indole Acetic Acid (IAA) and Gibberelic Acid (GA), which are important plant hormones has been investigated as these two compounds help in seed germination as well as mineral binding to plant root, thus affecting crop yield. Also they are important in regulating agronomically important traits,like plant hight and flowering,by increasing cell division and elongation[11].

Azospirillum are found in the rhizosphere and root of a variety of plants including cereals and grasses like rice, maize, wheat and legumes. The success of *Azospirillum* plant interaction depends on the survival and persistence of these bacteria in soil and the effective colonization of the rhizosphere[12]. *Azotobacter* is a free-living nitrogen-fixing bacterium,these plant growth promoting rhizo bacteria which are the beneficial ones that stimulate plant growth by an array of mechanism[13] but its distribution is affected by soil characteristics and climate conditions[14]. It does not necessarily colonize rhizosphere of plants. However, it needs the presence of assimilable carbohydrates in soil for its growth.

Material and Methods

Microorganisms and Culture Medium: Pure cultures of *Azospirillum spp* NCIM 2548 and *Azotobacter spp* NCIM 2452 were taken from NCIM, NCL, Pune, India. The *Azotobacter spp* NCIM 2452 were maintained on medium having the following composition (Mannitol 0.15%, $MgSO_4$.7H2O 0.002 %, K_2HPO_4 0.002 %, Ferric chloride 0.0005%, Molybdenum trioxide 0.01%, Agar 0.15%). *Azospirillum spp* NCIM 2548 were

maintained on Nitrogen free Bromothymol Blue Medium having the following composition (Malic acid 0.05%,KOH 0.04%,K_2 HPO_4 0.005%, $FeSO_4.7H_2O$ 0.0005%, $MnSO_4.H_2O$ 0.0001%, $MgSO_4.7H_2O$ 0.001%, NaCl 0.0002%, Na_2MO_4 0.0002%, Bromothymol blue 0.02%, Agar-Agar 1.75%, $CaCl_2$ 0.01%). The organisms were grown in liquid medium having composition was (sucrose 0.03%, $NaNO_3$ 0.03%, K_2HPO_4 0.01%, $MgSO_4$.7H2O 0.005%, KCl 0.005%, $FeSO_4$ 0.0001%, tryptone 0.5%, starch (soluble) 0.5% and varying concentration of endosulfan) for all experimental purposes. The incubation was carried out at 28°C on a rotary shaker (120 r.p.m.) for 7days. All media components were of analytical reagent grade. Commercial preparation of endosulfan (35% EC) was used in all studies.

Extraction Procedure: The medium after incubation from these flasks were centrifuged at 6000 x g, for 20 minutes. The cell free medium was extracted with diethyl ether in the ratio of 5:3 (v/v). The ether layer was dehydrated on anhydrous sodium sulfate and dried at 30°C. The residue was redissolved in pure dry methanol and used for analytical purposes.

The cell pellet was washed with distilled water to remove all media components. This was then homogenized using ultrasonic homogenizer. This was then extracted with diethyl ether in the ratio 5:3 (w/w). The rest of the procedure is as described for cell free medium.

Quantitation of IAA and GA by HPLC: Pure IAA and GA dissolved in methanol (20 µg, 50 µg and 100 µg), were used as standard for this analytical work. The instrument used in this study was 2 columns reverse phased HPLC (Waters 2690 system) using C8 column (4.6×250 mm). Elution was done with 70% acetonitrile at a flow rate of 1.0 ml min^{-1}, which was monitored by measuring UV A215 with a Waters Lambda- Max model LC Spectrophotometer.

Optimization studies for production of IAA and GA in absence of endosulfan: pH: The pH of the medium was changed as acidic, neutral and alkaline (6, 7, 8) to determine the pH value which was optimum for production of maximum GA and IAA.

Temperature: The microorganism was grown at different temperatures viz. 30°C, 37°C, 45°C to find the most suitable temperature of incubation for maximum yield of IAA and GA.

Effect of Endosulfan: Different concentrations of Endosulfan viz. 50 ppm, 100 ppm, 200 ppm, 500 ppm, and 1000 ppm were added in the growth medium of *Azotobacter spp* NCIM- 2452 and *Azospirillum spp* NCIM-2548. Endosulfan solution was added aseptically to sterile medium.

Statistical Analysis: Results obtained were the mean of three or more determinants. Analysis of the were carried out on all data at P< 0.05 using Graph Pad software (Graph Pad Instat version 3.00, 193 Graph Pad software, San Diego, CA, USA).

Observations:-

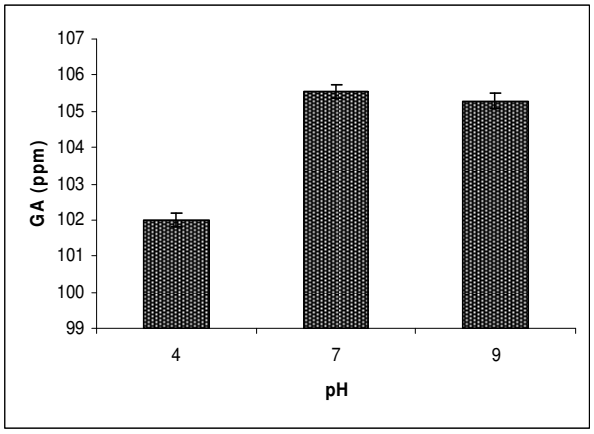

Figure-1
Figure showing the results of GA production by *Azotobacter spp* NCIM-2452 in medium free from endosulfan

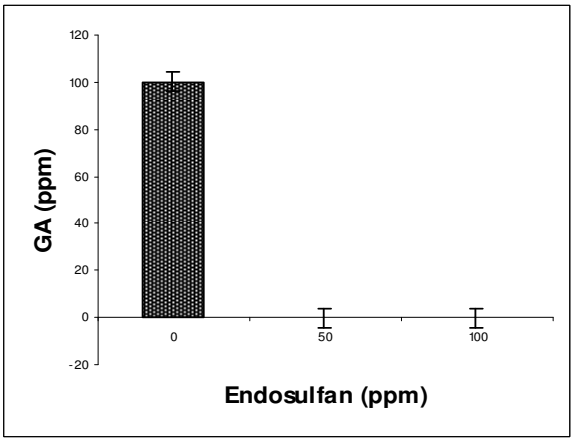

Figure-2
Effect of endosulfan on GA production by Growing cells of *Azotobacter spp* NCIM 2452

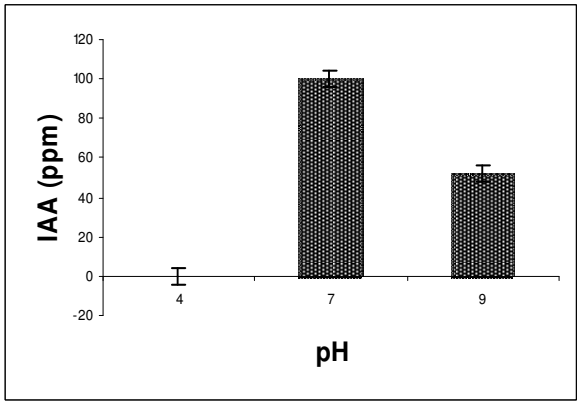

Figure-3

Effect of pH on IAA production by *Azospirillum spp* when grown in absence of endosulfan

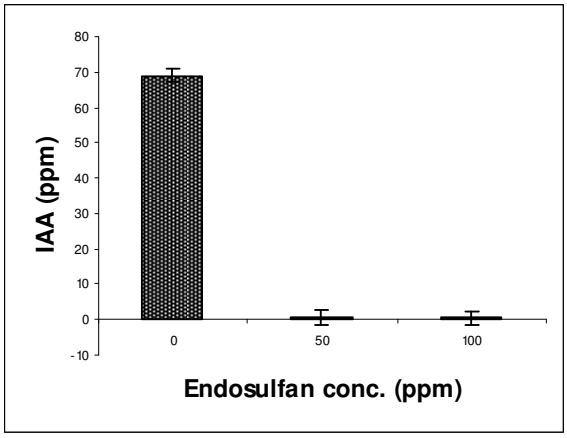

Figure-4
Effect of endosulfan concentration on IAA synthesis by *Azospirillum spp*.

Results and Discussion

Production of GA by Azotobacter spp NCIM 2452: It can be seen from figure 1 that the optimum pH for production of GA by *Azotobacter* NCIM-2452 is 7, when grown in the above medium free from endosulfan. Similarly the temperature optimum for GA production under these conditions is 30°C (as GA produced, was not detectable at temperature 37°C and 45°C, the results are not shown).

It has been so far reported that *Azospirillum spp* are known to produce GA at pH 7 in the rhizosphere of many plants[15], and it is linked only to nitrogen fixation. There are very few reports that *Azotobacter spp* are capable of producing GA 4. However, it can be assumed that in *Azotobacter*, GA production is not only linked to N_2 fixation but primarily to high amylase activity[3]. This is not the case with IAA production by *Azotobacter*[16].

It is known that free living N_2 fixation occurs well in agricultural soils. The temperatures of such soils are usually between 28°C to 30°C. Hence it is not surprising to see that *Azotobacter* is producing optimum GA, though in very small amount as compared to IAA, at 30°C.

It has been noted in this investigation, that in the soils where there have been indiscriminate use of endosulfan, the residual level of this pesticide, is between 50 to 100 ppm. It is because of this reason that the concentration of endosulfan used in this study, are 50 and 100 ppm. It is evident from figure 2 that though there was visible growth of the organism in the medium, but there was no GA production in the media containing 50 and 100 ppm of endosulfan in spite of presence of 0.5% starch in the medium. This clearly indicates that GA production by *Azotobacter* is linked to amylase activity.

Production of IAA by Azospirillum spp NCIM 2548: It can be seen from Figure 3, that the optimum pH of IAA production by this organism was 7. It has been reported that *Azospirillum* is pH independent and is related to tryptophan and malate concentrations only[17].beside the nitrogenase activity. Again since these organisms are highly metabolically active in agricultural soil, the temperature optimum for IAA production is found to be 30°C (results not shown).

It is evident from figure 4 that though there was some growth of the organism at 50 ppm concentration of endosulfan, IAA production was completely inhibited.

Conclusion

It is known that the production of both GA and IAA beside dinitrogen fixation, play important roles in agronomy of crops, however, it is not always affordable to add these important compounds to soil from external sources. Since, agricultural soil is supposed to be rich in soil conditioning agents like organic manure in different forms; presence of *Azotobacter* and *Azospirillum spp,* which bring about nonsymbiotic dinitrogen fixation, are capable of producing plant growth regulators like GA and IAA too. It is known that the nitrogenase system is highly anaerobic, though the organisms may be highly aerobic. Conversion of endosulfan to endosulfan ether[15] could be detrimental to this system as the etheral derivative is a strong oxidizing agent capable of total inhibition of the nitrogenase system. The organisms might be showing growth due to the presence of tryptone in the medium. The dioxygenases that are required in the GA synthesis is also required in attempting to detoxify endosulfan by converting it to its ether form and finally to the diol compound, hence can not be utilized in GA synthesis.

The nitrogenase system of *Azospirillum spp* is completely inhibited by endosulfan and hence IAA synthesis is also inhibited as it is directly linked to the nitrogenase system. Here too the growth of the organism was due to the presence of tryptone in the medium.

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