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ORIGINAL ARTICLE

EFFECT OF NICOTINE ON SOIL NITROGEN FIXING BACTERIA

Sankar Jana, Swarnali Das, Gourab Mondal, Koushik Pal, Subhadip Samanta, Susmita Datta, Nazifa Afroze, Susovan Patra, Nandini Ghosh*

Department of Microbiology, Vidyasagar University, Paschim Medinipur, West Bengal -721102, India.

**Corresponding Author –nandini.ghosh14@gmail.com*

Abstract: *Nicotine is an alkaloid, which is present in different plants including Nicotiana tabacum (tobacco). The nitrogenous compound has the potential to act as mutagen by changing the DNA base sequences and by hydrocarbon group incorporation in the nucleotide resulting in incorrect base pairing. In the present study, the effect of nicotine on free living nitrogen-fixing bacteria of rice field was observed. Azotobacter, the free-living nitrogen fixing bacteria, was isolated from the soil of rice field, which was treated for prolonged duration of time with increasing concentration of nicotine. The culture of Azotobacter sp. was maintained in Ashby's media, added with nicotine at different concentrations. It was found that bacterial growth was decreased with the increase of nicotine concentration and duration of its exposure to nicotine. On the other hand, the nicotine-free culture media shows comparatively higher number of bacterial colonies. The genomic DNA content of Azotobacter sp., which was isolated from each of the sets were measured for comparison. The present observation depicts that nicotine can inhibit the growth of soil nitrogen fixing bacteria. The observation indicates the potential risk of nicotine to cause change in the ecological structure of soil.*

Key Words: Nicotine, free-living nitrogen fixing bacteria, growth, inhibition.

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1. INTRODUCTION

Nicotine ($C_{10}H_{14}N_2$) is the major pharmacologically active compound belonging to alkaloid group, produced in the genus *Nicotiana* (tobacco plant, family: Solanaceae). Among more than 70 species of *Nicotiana*, *N. tabacum* happens to be the most important commercial plant to produce tobacco, in the form of dried leaves. Tobacco is produced by different the countries like China, India, Brazil, United States of America, Indonesia and Cuba [1]. According to the world ranking, India is the second largest tobacco producer, as well as its consumer [2]. Nicotine is the major alkaloid accumulated the leaves in tobacco plants, contributing 0.6 - 5.0% of the dry weight.

Though Nicotine is oily in nature, it can form water-soluble ammonium salts after interaction with acids. Such water-soluble forms of nicotine can be transported from the solid wastes to the ground water. This can cause toxic effect to aquatic life, including microbes. It was found that, the drinking water of 30 cities of the world contains 1.9 ng × 10^{-3}mg/L of nicotine in average [3]. Nicotine or 3-2-(N-methylpyrrolidinyl) pyridine, is reported to has a broad spectrum of harmful effect on human cardiovascular and gastrointestinal systems. However, nicotine is found to has some beneficial

neuromuscular effects, in case of different neurodegenerative diseases (example: Alzheimer's and Parkinson's disease) and schizophrenia [4]. Such beneficial role of nicotine is supposed to be due to the recognition interaction of nicotinic acetylcholine receptors of human nervous system with nicotine molecule [5].

Regarding the structural aspect, there are the presence of a methylated pyrrolidine nitrogen (pKb1 = 6.16) and another pyridine nitrogen (pKb2 = 10.96) in nicotine molecule, which are connected with a chiral center. These two rings have nitrogen atoms, which are nucleophilic in nature. These Nitrogen atoms bind in a competitive manner, when they are reacting with electron rich species. Metal complex formation is another important character of nicotine reaction [6 -8]. It has been reported by different workers that a number of bioactive molecules attain high level of bacteriostatic as well as carcinostatic activity after the coordination with ions of metallic type [9, 10].

Nicotine is an allelochemical, which is produced by the tobacco plant to affect the growth of other plants and soil microorganisms to reduce competition and better survival in natural condition. This phenomenon is called Allelopathy [11]. When tobacco plant is grown, nicotine exudate comes out from the plants, mixes with soil environment, which often plays a supportive role in the growth of another crop plants [12]. The heterocyclic structure of nicotine helps it to enter at the different levels of sediments of soil environment. This phenomenon gradually influences the soil texture by regulating microbial degradation as well as toxicological influences with time [13,14]. The presence of jasmonic acid in the rhizosphere of tobacco field influences gene regulation expression, leading to synthesis of nicotine molecule and its capillary transport through xylary channels to the leaves of tobacco plants [15]. The amount of nicotine production becomes higher in case of damaged leaves, which is related to the defence mechanism of the plant [15]. Such defence mechanism results into the release as root exudates (RE) containing nicotine, which inhibits the growth of soil microbes and make a balance in the enzyme activities present in soil [16,17]. These microbial enzyme activities in the soil environment are basic trigger of the mineral decomposition process in the elemental form [18].

Recently, several research works had been conducted with a focus on single factors acting behind soil fertility, especially in different tobacco growing areas of the world [19-21]. There are reports indicating the tobacco crop to either increase or decrease the levels of mineral macronutrients in soil environment [22-24]. Soil bacteria has important role on solubilisation of nutrients, and the diversity of soil bacterial flora related to tobacco cultivation has not been documented clearly. There are very few reports dealing with the effect of nicotine on soil microorganisms [25]. On the basis of this idea, the study was conducted to find the effect of nicotine at different concentrations on soil microbial community.

2. MATERIALS AND METHODS

Nicotine extraction and quantification

The nicotine content was determined according to standard protocol [26] with some modifications. Nicotine was extracted from the tobacco leaves by taking 10.0 g of the leaf sample and mixing it in 100 ml distilled water for 30 min in 10 mL of 5% (NaOH) sodium hydroxide solution using a vortex mixer. The whole mixture was then heated for 40 minutes. Then the sample was centrifuged for 10 min at 8000 rpm. To remove water, we used filter paper for collecting the nicotine extract.

Quantitative measurement of extracted nicotine

To undergo the qualitative and quantitative measurement, at first 20ml of kerosene oil was added to the extracted nicotine solution followed by centrifugation at 8000rpm for 10 min. Then the solution was poured in a separating funnel and it was kept in that position for 2 days. After two days two different layers were observed, one heavy layer in the bottom and the lighter layer in the top. The heavy layer from the bottom was discarded and lighter layer was remained in the funnel. Next, to the lighter layer

10ml concentrated H_2SO_4 was added followed by vigorous shaking. Then the mixture was left for some time and again two layers were formed, one heavy (dark brown) and another lighter (almost transparent) layer on the top. The valve of the separating funnel was opened and the heavy layer was collected in a test tube and heated for some time. Then the obtained mixture was sealed and stored intact at -20^{o}C. Finally, after two days of preservation the crystalline form of the nicotine was obtained.

Sampling and culture of microbes

Soil samples were collected from local rice field during mid-September to October. To culture the soil microflora, Ashby's Mannitol media was used, so that free living nitrogen fixing bacteria can use mannitol and atmospheric nitrogen as source of carbon and nitrogen respectively. Nicotine extract solutions prepared in four different concentrations was also added to the soil inoculated media. Along with that, one control was also prepared by adding only one pinch of the collected soil sample into the prepared Ashby's medium. The medias were incubated at 37^{o}C for 24-48 hours. After incubation growth condition was observed in different nicotine concentration.

To observe the growth of N_2 fixing soil bacteria, Ashby's agar plates were prepared by inoculating soil sample in different concentrations of nicotine added media, and incubated at 37^{o}C for 24-48 hours.

Extraction of genomic DNA

The total genomic DNA of soil samples was extracted by taking the samples from different concentration of nicotine containing media. At first, 2ml of 5%, 10%, 20%, 50% sub-cultures and media without nicotine (control) were taken in micro-centrifuge tubes and centrifuged. The pellet was collected and washed with phosphate buffer. TAE buffer was added to it, after mixing the solution with 10% SDS and Proteinase K and then incubated for 1 hour. Finally, DNA was extracted using a standard protocol [27]. The integrity of the DNA was determined by 1% agarose gel electrophoresis, while the purity and concentration of the DNA was assessed using nanodrop.

3. RESULTS

Effect of nicotine among soil bacteria

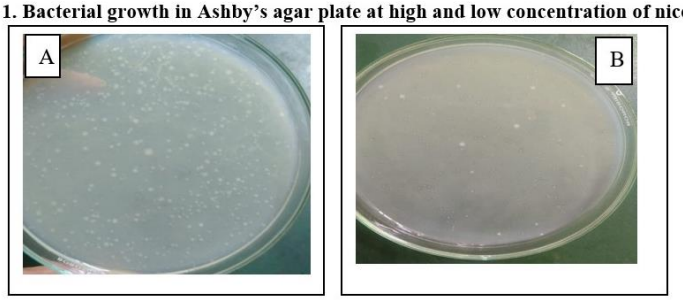
Growth turbidity was checked at different concentration of nicotine (Table 1). It was observed that with the increasing conc. of nicotine turbidity of growth decreases. Moreover, cultured Ashby's agar plates also showing dense colonies in the plate having less concentration of nicotine, while in the other plate the numbers of colonies are comparatively less.

This indicated that low concentrations of nicotine can stimulate the growth of microbes. However, as the concentration of nicotine increases the microbes couldn't tolerate it and it goes harmful to them.

Table.1. Growth turbidity with increasing concentration of nicotine

Concentration of Nicotine	OD value at 540 nm
5%	0.54
10%	0.41
20%	0.32
50%	0.15

Figure 1. Bacterial growth in Ashby's agar plate at high and low concentration of nicotine



Ashby's agar plate at (A) high conc. of nicotine and (B) low concentration of nicotine

Quantitative measurement of pure nicotine

Pure nicotine reacts with $HgCl_2$ and a flowery shape structure was observed under microscope. The amount of pure nicotine obtained was measured and it comes approximately 4% of the dry weight of the leaves according to Table 2.

Table 2. Quantitative measurement of the pure nicotine

Amount of *Nicotiana* leaves (gm)	Amount of pure nicotine(gm)	Average amount of pure nicotine (gm)
10	0.35	0.43
10	0.52	

DNA extraction

Extraction of DNA from the collected soil samples in different concentrations of nicotine was attempted. Concentration of DNA was measured in Nanodrop as shown in Table 3.

Table 3. Concentrations of DNA at different concentrations of nicotine

Concentration of Nicotine	**Concentration of DNA (mg/ml)**
Without Nicotine	600
5%	256
10%	65.3
20%	78.8
50%	11.8

4. DISCUSSION

Nicotiana or tobacco is a crop, which is well-known for its allelopathic defense against degrading fungi and bacteria (residing at both above and below the ground soil), due to the presence of the unique bioactive molecule called nicotine. As the nicotine is released by the tobacco plant in soil environment as root exudate, there is an importance to understand the implication of nicotine on the composition of soil microbes, fungal flora and crops growing in the same field [28-30]. There are some reports that microbial community of the soil have association with phenolic acids levels [31-33]. It was observed that there is a tendency of decreasing abundances of different groups of Proteobacteria, Chlorobia, Betaproteobacteria [34-36]. In the present study, the experimental sets were compared with control, and there was a notable reduction in DNA concentration from 256ng\ml to 11.8 ng/ml associated with the

increased concentration of nicotine. Based on the results from the series of experiments, it can be assumed that, contamination of nicotine can greatly impact the microbes. Even whene, the concentrations of DNA obtained in nanodrop are cheched, the soil microbes are found to be decreased with the increase of nicotine concentration.

It can be apprehended that, the presence of nicotine can has some effect on nitrogen fixing microbes/cyanobacteria. On the other hand, passive release of nicotine from meristematic root regions to the soil rhizosphere, plays a triggering role plant defence, by reducing pathogenic soil bacteria and fungi. As a result, the inter-specific competiton for nutrients betwwn plant and microbes is reduced. However, continuous cropping may often lead to the changes in the community composition of soil bacteria and reduce bacterial biodiversity as well.

The ability of nicotine to limit or interfere with the growth of selected microorganisms was a significant observation in this experiment. The experiment depicted a correlation between soil physiochemical properties and microbial diversity can be proposed .Thus, the present assesment will be helpful to provide knowledge about the changes in soil microbial population with the increasing toxicity of nicotine and also the need for remediation of the nicotine contaminated soil during tobacco cultivation.

5.CONCLUSION

The research was conducted to determine the effect of tobacco cultivation on soil microbes and to compare microbial population of tobacco based soil with non-tobacco based soil on randomly selected rice field. Nicotine is the unique secondary metabolite produced by tobacco plant which reaches to soil environments through root exudaton. This present study revealed the interactions of nicotine with soil microbes altering the microbial composition within soil environment. It was observed that, free living nitrogen fixing bacterial colony inoculated in media containing higher concentration of nicotine, gets decreased compared to the media containing low nicotine concentration. The result showed that nicotine stress has an inhibitory effect on the growth of microbes. It can be stated that tobacco nicotine allelopathy decreases the population of soil bacteria where tobacco is continuously cultivated for years.

The bacterial diversities are also found to be correlated with soil fertility. Thus, it can be said that nicotine has a negative impact on multiplication of some beneficial bacteria too. Beside this, tobacco is also associated with great uptake of soil macronutrient. Hence, understanding the relationship within microbial diversity in tobacco soil may also provide guidance for research on the interaction system of plants, microbes and soil as well. However, further studies are needed to define in detail the mechanisms by which nicotine suppress growth of bacteria.

6. CONFLICTS OF INTEREST

Authors have no conflicts of interest.

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