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Research Article

THE EFFECT OF MANURE AND FERTILIZER ON THE ENDOPHYTES ISOLATED FROM *CURCUMA LONGA*

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ABSTRACT

In the current scenario, chemical fertilizers are widely used to fuel plant growth. The use of chemical fertilisers affects the composition of the soil, its pH, salinity and the organic carbon content. This can be detrimental to the micro flora found in the soil, which can in turn affect the consistency of the soil and the number of endophytes that invade the plants and the plant health. The association of the endophytes with the plants allows them to cope with various functions, like plant growth promotion, yield enhancement and disease control, the microbes in turn receive shelter and nutrients from the host plants. The present study discusses the impact of chemical fertilizers and manures on the number of endophytes extracted from turmeric. The soil was divided into three sets. One set was treated with chemical fertilizer (NPK), one with manure and an untreated set of soil was used as control. The endophytes were isolated from the rhizomes and leaves of the plants grown in all the three sets and analyzed for the effect of the treatments on the bacteria isolated. The comparison showed that the number of endophytes were low in plants grown in soil treated with chemical fertilizers.

Keywords: Endophytes, Growth Promotion, Plant health, Medicinal plants, Diseases management, Chemical fertilizer.

1. INTRODUCTION

Endophytes are a diverse group of microbial species present in the plant endosphere that are said to make major contributions to plant health. According to the latest definition given by Cocq K L *et al.,* 2017, endophytes are microbes that live inside the plant tissues under the existing conditions for at least part of their life cycle without causing any disease [1]. A non- pathogenic relationship between the host and endophyte is formed either by gene destruction or by gene regulation [2]. Endophytes belong to various phyla such as Proteo-bacteria, Actinobacteria, Firmicutes and Bacteroidetes [3-5]. They are widespread and help enhance host growth, nutrient accumulation, improve plant capacity to withstand abiotic stress such as drought, increase insect and plant pathogen resistance and keep mammalian herbivores at bay. The medicinal plants are infected with a largest number of endophytes when compared to other plant species. These endophytes in the plant develop antibiotics against other fungi and bacteria [6]. They penetrate into the plant from the roots. Microbial groups in the rhizosphere are influenced by the release of photosynthates or exudates including organic acids, amino acids and proteins from the plant root [6].

Pesticides and chemical fertilizers are used to protect plants from pests and to boost the growth of the plants. In addition to the fighting against insect pests, it also affects the microbial ecosystem and hinders the activities of beneficial microbial species [7]. Chemical fertilizers and pesticides may have a long-term or short-term impact on the flora. Long-term application of pesticides affects the fungal activity, bacterial activity, microbial biomass and carbon content of the soil [8]. The indiscriminate application of both chemical fertilizers and pesticides can be harmful to the soil as it affects the pH and mineral content of the soil and can have a negative or positive effect on the micro-flora of the soil. The absorption of these pollutants into the soil and their persistence differs with the composition, pH and temperature of the soil.

2. MATERIAL AND METHOD

2.1. Preparation of soil

The soil was sieved and divided into three sets.

Control: Soil without any treatment.

Test 1: Soil treated with Neem cake and bone meal (1:1 added to the soil at a ratio of 1:4).

Test 2: Soil treated with chemical fertilizer (NPK water

soluble fertilizer-shivi products NPK 20-20-20) 15 ml/5-liter water.

The three sets of soils were further divided into 5 bags each holding 5kg of soil (5 replicates). The rhizomes of *Curcuma longa* (5 rhizomes each in 5 replicates per set of soil) were planted into each bag.

2.2. Sample collection and isolation

The collection of rhizomes, leaves and soil were done from month 3 after planting the rhizomes and endophytes were isolated at regular intervals (every month) till the 11th month. The isolation of endophytes was done within 8 hours after collecting the sample.

2.3. Surface sterilization of the samples from *Curcuma longa*

Rhizomes: The rhizomes were washed under running tap water and left to dry at room temperature for 30 minutes. It was weighed (1g) and was surface sterilized by treating it with 70% ethanol for 4 minutes, 4% sodium hypochlorite for 3 minutes and with 0.1% mercuric chloride for 3 minutes. The sample was rinsed four times with sterile distilled water [9].

Leaf: A healthy leaf was collected from each set. The leaves were thoroughly washed under running water and were left at room temperature for 30 minutes to dry. The leaf samples (1g) were surface sterilized with 70% ethanol for 3-minute, 4% sodium hypochlorite for 4 minutes, 70% ethanol for 1 minute and rinsed with sterile distilled water at least for 4-5 times [10].

2.4. Determination of efficiency of surface sterilization

The efficacy of surface sterilization was checked using the imprint method. The uncut piece of the surface sterilized rhizomes and leaves were placed on the tryptic soya agar and nutrient agar media (treated with cycloheximide, 100mg/L) and pressed with a sterile glass rod to get a clear imprint of the rhizomes and incubated at 30°C for 24 to 48 hours [11].

The sterile distilled water (last rinse) was also inoculated on to the media using spread plate method. The plates were incubated at 30°C for 24 to 48 hours [12].

2.5. Isolation of endophytes from the rhizomes and leaves of *Curcuma longa*:

The surface sterilized rhizomes and leaves (1g) were ground using a sterile mortar and pestle and saline solution (10 ml of 0.85% saline) was added to it. This was left undisturbed for 30 minutes to allow the movement of the endophytes from the sample to the saline solution. After serial dilution, 0.1ml from each dilution of 10^{-4}, $10^{-5}$and 10^{-6} were taken for the spread plate method. Tryptic soya agar and nutrient agar were used for the process of isolation and cycloheximide (100mg/L) was added to the media to prevent the growth of any fungus. Five replicates for each concentration were maintained. The plates were incubated for 48-96 hrs at 30°C [12].

2.6. Isolation of bacterial colonies from the soil samples

The soil samples were dried at room temperature for an hour to remove any moisture. It was sieved and powdered. The sieved soil samples (10g) were mixed with 90 ml of sterile saline solution and left in a shaker incubator for an hour at 180 rpm. The solution was further filtered and 1 ml of this solution was used to perform serial dilution and 0.1ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions were taken and plated through spread plate method onto tryptic soy agar and nutrient agar. Cycloheximide (100mg/L) was added to the media to prevent the growth of fungus. The plates were incubated for 48-96 hours at 30°C [13]. The soil was also used to analyze the physical parameters and enzymatic parameters.

2.7. Measurement of Selected Physical Parameters

Soil samples were analyzed for the following parameters namely pH, electrical conductivity, organic carbon at regular intervals.

2.7.1. Measurement of pH of the soil

Soil samples (50g) were collected from control, Test 1 (manure), Test 2 (chemical fertilizer) and 100ml of distilled water was added to it. The solution was mixed vigorously using a glass rod and was kept undisturbed for 30 minutes. The suspension was filtered and the filtrate was used to measure the pH (Elico make pH meter) [14].

2.7.2. Measurement of electrical conductivity

Soil samples (50g) were mixed with 100ml of distilled water in a conical flask. The solution was filtered using Whatman filter paper (number 1) until a clear solution was obtained. The filtrate was used for measuring conductivity. Conductivity was recorded in micro-ohms [14].

2.7.3. Estimation of organic carbon content

The organic carbon content of the soil was determined using partial oxidation method [15]. Soil sample (5g) was

taken in a beaker and kept on a water bath (80°C). Concentrated sulfuric acid (18.4 M) was added dropwise till the emission of Hydrogen sulfide gas stopped. The soil was washed several times with distilled water and was dried in an oven at 110°C for 30 minutes. The dry soil was again washed with distilled water, to ensure the removal of chlorides and phosphates. The soil was again dried in an oven at110°C for 30 minutes. The soil (0.5g) was taken in a conical flask and potassium dichromate (10ml) and concentrated sulfuric acid (20 ml, 18.4M) were added and kept for 40 minutes at room temperature. To the above sample, distilled water (200ml), 5ml of phosphoric acid and two drops of diphenylamine (indicator) was added and titrated against ferrous ammonium sulphate (1M). The end point was indicated by the color changes from dark blue to green.

2.8. Estimation of Enzyme Activity

Urease and alkaline phosphatase activities were estimated following the methods described by Tabatabai and Bremner. The soil samples were collected from all the three sets of soil treatment (control, Test 1, Test 2) [16].

2.8.1. Urease activity

Soil (0.1 g) was mixed with 5% aqueous Hydrochloric acid (5ml) and incubated at 25°C for 24 hours. To the above sample 10% urea solution (1ml) was added and incubated at 37°C for 24 hours. After incubation, Nessler's reagent was added (colour changes to brown), and the absorbance was read at 410nm. The urease activity was expressed as the amount of urea hydrolyzed per gram of soil sample. A blank was used.

2.8.2. Alkaline Phosphatase activity

The soil sample (1 g) was mixed with 1 ml disodium phenyl phosphate (1ml,10mM) incubated at 37°C for 1 hour on a shaker at 100 rpm, centrifuged the sample at 10,000 rpm for 5 minutes. To the supernatant, added (2 ml of 1 M) Sodium hydroxide. PNP (p-nitrophenyl phosphate) produced was measured spectro-photo-metrically at a wavelength of 410 nm. The results were expressed as μg of p-nitrophenyl phosphate released per gram of dry soil.

3. RESULTS AND DISCUSSION

3.1. Determination of Efficiency of Surface Sterilization of rhizomes

The imprints of rhizomes and the leaves did not show any growth of bacterial cultures. The addition of mercuric chloride to the surface sterilization procedure increases the efficiency of surface sterilization. A similar result was observed by Srivastava, N., *et al.,* where the addition of mercuric chloride to the surface sterilization procedure helped increase the efficiency of surface sterilization of rhizomes [17]. The distilled water used for the last rinse of the sample also showed no growth. Similar results were seen in the tests conducted by Ramalashmi K, *et al.,* No contamination was seen on the inoculated plates [18].

3.2. Isolation of endophytes from the rhizome of *Curcuma longa*

The number of colonies isolated from the different soil treatments varied from the 8th month to the 11th month (table 1). Maximum number of bacterial colonies isolated during the 8th month was 2.57 as seen in control and the minimum colonies were isolated from the rhizomes grown in soil treated with chemical fertilizer with an average of 1.71. It was seen that there was a steady raise in the colony forming units (CFU) from month 8 to month 11.

3.3. Isolation of endophytes from the leaves of *Curcuma longa*

The isolation of endophytes during the 8th month was maximum in the leaves collected from Test 2. The leaves collected from Test 1 did not show any growth. However, the number of endophytes isolated from the leaves of test 1 increased from 9th month to 11th. The control showed the maximum isolation during 11th month. There was a significant increase in the bacterial colonies isolated from the leaves of Test 1 (table 2).

The use of chemicals has a negative impact on the endophytic bacteria that are considered to be in symbiotic association with the plants which also help in controlling phytopathogens [19-21]. Thus, the diversity and the number of bacterial colonies isolated from the rhizomes grown in the soil treated with chemical fertilizer is comparatively lesser than the colonies isolated from Test 1 and control.

3.4. Comparison using ANOVA

An ANOVA test was conducted to understand the relationship between the effect of the different soil treatments (chemical fertilizer, manure, untreated soil) on the number of endophytes isolated from the plant samples (leaf, rhizomes) *Null hypothesis*: There is no effect of the treatment (fertilizer, manure) on the number of bacterial endophytes isolated.

Alternate hypothesis: There is an effect of the addition of fertilizer and manure on the number of bacteria isolated.

The F value obtained was 22.37 which was higher than the F crit value 9.55 and the P value (0.01) was lesser than the value of alpha (0.05) 13.03>9.55 (F>F crit), 0.01<0.05(P<α) Hence the null hypothesis can be rejected and the alternate hypothesis that the number of bacterial colonies isolated depends up on the type of soil treatments, it's pH and other factors can be considered as true.

3.5. Enumeration of Bacteria from the Soil

There was an increase in the number of colonies isolated from month 8 to month 11. The least number of bacteria were isolated from the soils treated with chemical fertilizer (table 3). The soils treated with manure (test 1) showed maximum growth of the bacterial colonies. As observed by Pratibha Prashar *et al.*, the presence of chemical fertilizer in soil changes it's physical and enzymatic parameters like the pH, soil salinity etc [22]. Thus, the use of chemical fertilizers reduced the soil microbial growth, disturbed the pH and enzymatic activities, which in turn affects the growth of the microbial population. There was a gradual increase in the number of bacterial colonies isolated from the soil treated with chemical fertilizer (Test 2) after the application of the fertilizer was stopped from the 8th month.

Table1: Enumeration of Bacteria Collected from the Rhizome of *Curcuma Longa*

Sample	Month 8 (CFU/ml)	Month 9 (CFU/ml)	Month 10 (CFU/ml)	Month 11 (CFU/ml)
Control	4.6±0.69	4.2±0.52	5.0±0.94	6.0±1.09
Test1	8.33±0.65	7.80±1.10	9.4±0.39	11.6±1.27
Test 2	0.4±0.14	2.4±0.23	3.6±0.67	8.0±0.54

Table 2: Enumeration of Bacteria from the Leaves of *Curcuma longa*

Sample	Month 8 (CFU/ml)	Month 9 (CFU/ml)	Month 10 (CFU/ml)	Month 11 (CFU/ml)
Control	1.2±0.46	1.8±0.21	3.0±0.4	5.8±0.9
Test 1	4.6±0.53	8.4±0.73	9.8±1.45	11.2±0.82
Test 2	0.4±0.1	2.6±0.84	4.2±0.52	5.6±0.78

Table 3: Enumeration of Bacteria from the Soil

Sample	Month 8 (CFU/ml)	Month 9 (CFU/ml)	Month 10 (CFU/ml)	Month 11 (CFU/ml)
Control	27.2±6.19	23.8±2.84	52.4±7.59	60.8±9.56
Test 1	21.2±3.07	31.2±4.60	47.8±5.35	62.2±6.99
Test 2	10.2±1.38	18.6±3.60	43.2±9.54	45.6±8.17

3.6. Estimation of Physical parameters of the soil

The physical and enzymatic parameters were checked for all the three different treatments.

The pH, electrical conductivity and the organic carbon content of the soils were considered for analyses of the physical parameters of the soil. The estimation of the pH, organic carbon content and the electrical conductivity was done at regular intervals from the 8th month after planting the rhizomes of the *Curcuma longa*.

The pH of the soil samples varied from 7.20 to 8.0. The least was seen in control with a pH ranging from 7.20 to 7.21. The soil treated with chemical fertilizer had a change in the pH range. The highest value was seen in the 8th month with a pH of 8.0. The pH of the soil collected from test 2 decreased when application of the fertilizer was stopped. The control and the soil treated with manure (test 1) did not show much fluctuation in pH. This result coincided with the results proposed by Pierre WH, who stated that the addition of chemical fertilizers can affect the soil pH that changing the acidity of the soil [23] (table 4).

The value of electrical conductivity was the highest in the soils treated with chemical fertilizer, with the values shooting up to 422 ds/cm in the 11th month. The values of electrical conductivity were least in the untreated soil (control). The addition of chemical fertilizers increases the dissolved minerals in the soil. It was observed by Atafar *et al.,* that the addition of chemical fertilizer to the soil can result in increase of the metal content of the soil [24]. Similar results were observed in the current study. The electrical conductivity of the soil increased in the soil treated with chemical fertilizers (table 4).

The organic carbon contents in the soil treated with the manure (test 1) was the highest. The least was seen in the soil that was treated with chemical fertilizers. The

addition of the chemical fertilizers results in deterioration of the organic carbon in the soil. Similar results were obtained by Kuntal M. Hati *et al.,* who stated that the addition of chemical fertilizer can reduce the carbon content of the soil [25]. The addition of fertilizer which is a mix of chemical fertilizer and manure in turn can retain the carbon content of the soil (fig. 1).

Table 4: Estimation of Physical Parameters of the Soil

Soil Type		pH	Electrical Conductivity (ds/cm)	Organic Carbon (g/kg)
Control	Month 8	7.22	288	4.22
	Month 9	7.20	279	5.09
	Month 10	7.21	271	4.97
	Month 11	7.21	277	5.32
Test 1	Month 8	7.52	290	5.77
	Month 9	7.52	310	5.64
	Month 10	7.49	286	4.98
	Month 11	7.51	299	5.66
Test 2	Month 8	8.0	411	2.36
	Month 9	7.68	421	2.39
	Month 10	7.18	420	3.10
	Month 11	7.7	422	2.39

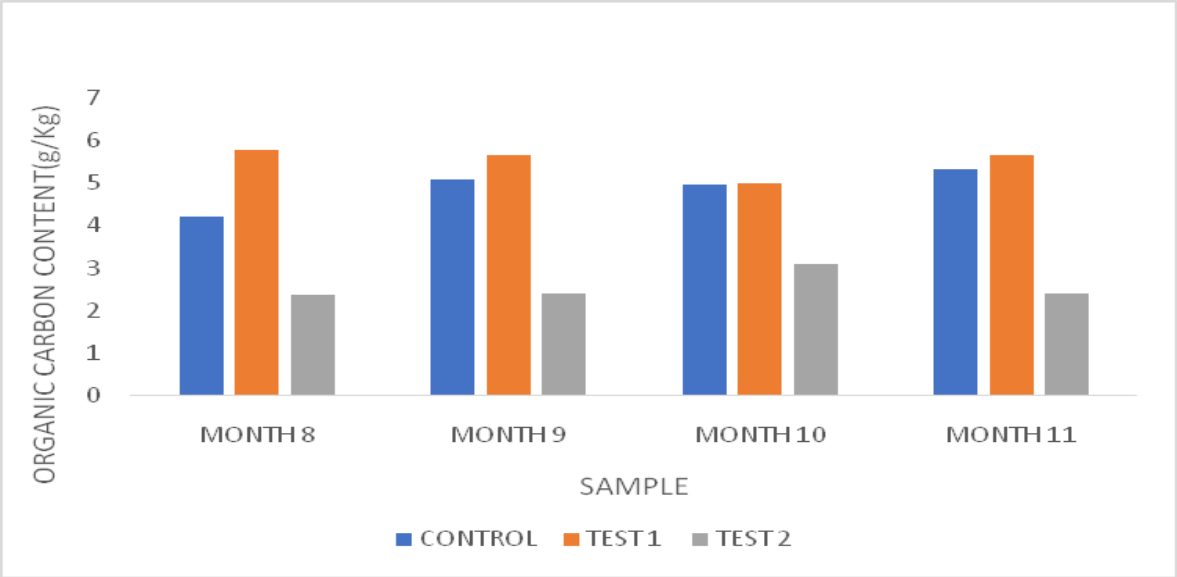

Fig. 1: Estimation of Organic Carbon Content of the Soil

3.7. Estimation of the enzymatic parameters of the soil:

The urease and alkaline phosphatase activity of the soil were estimated from month 8th to month 11th after planting of the rhizomes of *Curcuma longa*. The details are as given in table 5.

The urease activity was the maximum for the soils treated with manure and the least was seen in the soil treated with chemical fertilizer. A similar result was seen in experiments conducted by J. M. Bremner and L. A. Douglas in their studies on inhibition of urease activity in soil. They stated that the presence of excess urea in the soil, (from the chemical fertilizers) increases the amount of ammonia, which in turn can change the soil parameters (acidity, salinity), thus affecting the soil microflora. This results in decreased urease activity in the soil [26].

The alkaline phosphatase activity was seen highest in the soils treated with manure (test 1) control had the least activity during the 8th month. It gradually increased till month 11. The activity of alkaline phosphatase activity was seen least in Test 2 followed by control. A similar result was noted by Michihiko Sakurai *et al.,* who noted a decrease in the alkaline phosphatase activity in the soil

treated with chemical fertilizer when compared to organic manure. He stated that the reduction in the alkaline phosphatase activity can be due to the addition of chemical fertilizer which affected the microflora (alkaline phosphatase harboring bacteria) in the soil, which in turn results in the reduced activity [27].

Table 5: Estimation of Enzymatic Parameters of Soil

Soil Type		Urease (µg of urea hydrolyzed per gram)	Alkaline phosphatase (µg of p-nitro phenyl phosphate released per gram)
Control	Month 8	32±0.66	232±0.66
	Month 9	39±0	279±0.66
	Month 10	61±0.66	282±0.66
	Month 11	88±0	288±0.66
Test 1	Month 8	165±0.33	692±0
	Month 9	159±0	650±0
	Month 10	170±0	690±0.66
	Month 11	164±0.66	670±0.66
Test 2	Month 8	110±0	250±0
	Month 9	109±0.66	214±0
	Month 10	115±0	222±0.66
	Month 11	121±0	223±0.66

4. CONCLUSION

The use of chemical fertilizers not only triggered the soil's physical and enzymatic parameters but also had an effect on the soil microflora. The change in the pH, salinity and organic carbon content of the soil had an effect on the number and diversity of bacteria in the soil. The endophytes are useful bacteria that help the plant withstand both biotic and abiotic stresses. The presence of endophytes helps in developing a natural resistance in plants thus helping them to cope up with the changes in the environment and resist the attack of pathogens.

The reduction in the number of endophytes can make the plants susceptible to a number of plant pathogens and stresses that are harmful to the plant's health, reducing its productivity and life span.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publishing of this article.

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