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The Effect of Temperature and Soil Composition on the Infection and Transmission of Endophytes Isolated from *Curcuma longa*

Shaju Reema Thankam*[1] and Suba G. A. Manuel[2]

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A B S T R A C T

The continuous rise in demand for food has forced men to improve crop productivity. This is achieved by increasing amounts of fertilizers to boost the growth of the plants, invention of genetically modified plants and use of pesticides to reduce the attack of pests and pathogens. The use of these chemicals may have a negative impact on the microorganisms present in the soil and the plants, they also tend to reduce the fertility of the soil and increase the mineral content in the long run. The plants act as a host to different kinds of microorganisms known as endophytes which helps in developing resistance against biotic and abiotic stresses, promote plant growth, and also protect them against herbivores. In the present study, the endophytes isolated from C*urcuma longa* were introduced into a crop plant *Solanum lycopersicum* grown in three different field conditions. The effect of temperature and soil composition in the transmission and growth of the endophytes were observed. Maximum colonies were isolated from the samples collected from Field 3 and maximum diversity from Field 2. Hence the change in temperature and soil composition can influence the number and type of bacterial endophytic strain grown in the same host plant.

***Key words*:** Soil fertility, Plant growth, Pesticides, Crop productivity, Plant stresses, *Curcuma longa*

The soil is home to numerous Plant Growth Promoting (PGP) microorganisms that helps the plant to sustain and grow in varied conditions [1]. The continuous use of chemicals to improve the productivity of crops has led to its accumulation in the soil which in turn harms the soil microflora, affect its enzyme activities and physiological characteristics thus having an effect on the performance of the crops [2]. The use of chemical fertilizers, mainly the nitrogen fertilizers can also cause a temporary increase in the osmotic potential, pH and the level of ammonia in the soil thus affecting the microflora of the soil [3]. The global use of fertilizers has increased from approx. 27 to 170 million of nutrient tons over the past 50 years before 2010. This can reduce the fertility of the soil, increase the mineral contents, pH and other physical parameters thus making it unfit for the growth of microflora. The use of pesticides and fertilizers can have a negative impact on the micro ecosystem in the soil which in turn effect the endophytes present inside the plant.

The endophytes are microbes which occur within the plant tissue for at least part of their life cycle without causing any disease under the known circumstances. They are found across many phyla like the Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes [4-7]. They are present inside the plant tissues locally and systematically either latently or actively colonizing them [8]. The endophytes and the host plants have a non-pathogenic relationship that is developed by gene disruption or gene regulation. The use of endophytes as a bio control agent can be a sustainable and eco-friendly replacement to the chemically derived fertilizers and pesticides used in the present agricultural practices.

In the present study, the effect of temperature and the change in soil composition on the transmission and growth of the endophytic bacterial strains were observed in the same host plant *Solanum lycopersicum.* Several studies have shown that the rate of growth of endophytes inside the plant can vary according to the temperature. This can cause a change in the type of enzymes or chemicals that are released by them, thus causing a change in the plant growth and its resistance to stresses.

MATERIALS AND METHODS

* Shaju Reema Thankam
✉ reemashaju8@gmail.com

[1] Department of Life Sciences, Bangalore University, Bangalore - 560 056, Karnataka, India

[2] Department of Life Science, Mount Carmel College, No. 58, Palace Road, Bangalore - 560 052, Karnataka

Isolation of endophytes

The six endophytic isolates from *Curcuma longa* showing plant growth promoting activities were selected, identified for and inoculated into the crop plant - *Solanum lycopersicum*.

Preparation of bacterial suspension

The bacterial suspension was prepared by growing the 24-hour old cultures in the LB broth treated with Spectinomycin. They were loop inoculated into the broth and was incubated at 28±2°C for two days at 180 rpm in a shaker incubator. The cells were separated aseptically by centrifugation at 2500 rpm and the pallet was re-suspended in PBS (0.2 g/L KCl, 1.44 g/L Na2HPO4, and 0.24 g/L KH2PO4, in dH2O, pH 7.4) using a vortex. The suspension was further used for spraying into different plant parts [9]. Few drops of the suspension were also grown in nutrient agar and Tryptic soya agar to check the successful transmission of the colonies into the PBS solution.

Introduction of the bacterial suspension to the crop plants

Solanum lycopersicum (Tomato) was taken as the sample crop plant. The seeds were sown in the month of July 2019 in Field 1, Field 2 and Field 3 with a temperature ranging from 28°C in Field 1, 31°C in Field 2 and 33°C in Field 3.

The crops were grown on soil treated with manure-neem cake and bone meal (local nursery) at a ratio of 1:1:4 (test 1), chemical fertilizer- NPK water soluble fertilizer (shiviproducts NPK 20-20-20) 15 ml of the chemical fertilizer was added to 5 litres of water (test 2) and untreated soil (control). The crop plants were further divided depending on the area of spray (flower, leaves, and soil). The plants were sprayed with the bacterial suspension, care was taken to make sure that the suspension does not fall on to the undesired areas. Extra suspension was wiped off with the help of a filter paper. The suspension was sprayed after every 7 days in the first month to the leaves and the soil and on every alternate day to the flowers.

Measurement of selected physical parameters of the soil

Soil samples collected from Field 1, Filed 2 and Field 3 were analysed for the following parameters namely pH, electrical conductivity, organic carbon content.

Measurement of pH of the soil

Soil samples (50g) were collected from control, Test 1(manure), Test 2(chemical fertilizer) from Fields 1,2,3, were mixed in 100ml of distilled water and left undisturbed for 30 minutes. The filtered suspension was used to measure the pH (Elico make pH meter) [10].

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 clear filtrate was used for measuring conductivity which was recorded in micro-ohms [10].

Estimation of organic carbon content of the soil

The organic carbon content was determined using a partial oxidation method suggested by Walkley and Black [11], 1934. Concentrated sulphuric acid was added dropwise to the soil samples (5g) that was kept on a water bath (80°C) till the emission of Hydrogen sulphide gas stopped. This soil was further washed several times with distilled water and was dried in an oven at 110°C for 30 minutes which was washed again with distilled water, to ensure the removal of chlorides and phosphates. The soil was dried in an oven at 110°C for 30 minutes. The soil (0.5g) was taken in a conical flask and potassium dichromate (10ml) and concentrated sulphuric acid (20 ml, 18.4M) were added and kept for 40 minutes at room temperature. Distilled water (200ml), 5ml of phosphoric acid and two drops of diphenylamine (indicator) was added to the sample and titrated against ferrous ammonium sulphate (1M). The end point was indicated by the colour changes from dark blue to green.

Estimation of enzyme activity of the soil

Urease and alkaline phosphatase activities of the soils collected from Field 1, Field 2 and Field 3 were estimated following the methods described by [12].

Urease activity of the soil

The soil samples (0.1 g) were mixed with 5ml of 5% aqueous Hydrochloric acid and incubated at 25°C for 24 hours. Urea solution (10%,1ml) was to the above solution and incubated at 37°C for 24 hours. 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 hydrolysed per gram of soil sample.

Alkaline phosphatase activity of the soil

Disodium phenyl phosphate (1ml,10mM) was added to the soil sample (1g) and incubated at 37°C for 1 hour on a shaker at 100 rpm. This was centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and sodium hydroxide (2 ml of 1 M) was added to it. PNP (p-nitrophenyl phosphate) produced was measured spectrophotometrically at a wavelength of 410 nm. The results were expressed as µg of p-nitrophenyl phosphate released per gram of dry soil.

Enumeration of microorganisms from the selected crop plant

The samples were collected from all the three parameters i.e., control, test 1 and test 2. The leaves, fruits and the soils were collected from the different types of the soil and were further assessed to understand the rate of infection and transmission of the endophytes into the plant.

Enumeration of micro flora from the leaves sprayed with bacterial suspension

The leaves were washed under running water and were left at room temperature for 30 minutes for it to dry. They were surface sterilised by washing 1g of the leaf with 70% ethanol for 3minutes, 4% sodium hypochlorite for 4 minutes. The sample was again treated with 70% ethanol for 1 minute and rinsed with sterile distilled water at least for 5 times [13]. The bacterial endophytes were isolated by serial dilution followed by spread plate method.

RESULTS AND DISCUSSION

The crop plants were grown in different climatic conditions to check the effect of temperature and pH on the transmission and infection rate of the endophytic bacteria.

Isolation of endophytes

A total of 14 bacterial isolates were identified from *Curcuma longa*. The number of colonies isolated from the

plants grown in manure treated soil were maximum whereas the plants grown in chemical fertilizer showed minimum bacterial isolates. A similar pattern was observed for the soil micro flora isolated from the soils. The leaves showed a varied raise and fall in the endophytes isolated. The analysis of the physical and enzymatic parameters prove that the addition of chemical fertilizer and manure has in turn changed the soil composition and the effect of the change in the soil composition can be observed from the number of colonies isolated from the samples (Fig 1).

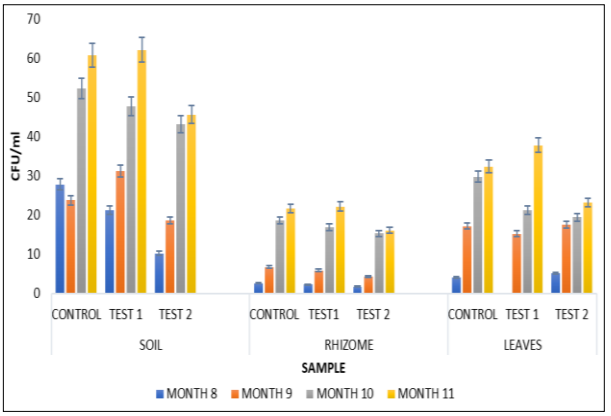

Fig 1 Enumeration of micro flora from *Curcuma longa*

Out of the 14 bacterial isolates from *curcuma longa*, six endophytic bacteria that showed Plant Growth Promoting activities were selected and were inoculated into the selected crop plant. The six endophytes were identified using 16s rRNA sequencing and analysis and they showed similarities to the following bacterial strains (Table 1).

The bacterial colonies were suspended in Phosphate Buffer Solution (PBS) and were sprayed to different parts of the selected crop plant *solanum lycopersicum* (Tomato) and the rate of transmission and infection was checked. The pot experiments with the crop plants were conducted in different regions of south India namely Bangalore (Field 1), Kottayam (Field 2) and Hyderabad (Field 3), the seeds were sown in the month of July.

Table 1 Identification of endophytic bacterial isolates by 16 s rRNA sequencing and analysis

Colony number	Identification	Accession number
C5	*Kocuria rocea*,	MT317205.1
C2	*Bacillus subtilis*	MT068199.1
C1	*Brevibacterium casei*	GQ365205.1
C8	*Actinobacterium* JS14 strain	AY372899.1
C7	*Bacillus Amyloliquefaciens*	MT131178.1
C11	*Bacillus velezensis*	CP028204.1

Physical and enzymatic parameters of the soil

The physical and the enzymatic parameters of the soil collected from Field 1, Field 2 and Field 3 were checked and compared to see the changes in the soil composition. The physical parameters taken into consideration were the pH, organic carbon content and electrical conductivity. The enzymatic parameters taken into consideration were the urease activity and the Alkaline phosphatase activity. The physical and enzymatic parameters for soil from control, Test 1 and Test 2 were accessed.

Table 2 Physical and enzymatic parameters of the soil collected from Field 1, Field 2, Field 3

Soil type		Physical parameters			Enzymatic parameters	
		pH	EC (ds/cm)	OC (g/kg)	Urease (MG of urea hydrolyzed per gram)	Alkaline phosphatase (MG of P-Nitro phenyl phosphate released per gram)
Control	Field 1	7.02	293	4.95	32 ± 0.66	145 ± 0.66
	Field 2	7.0	288	4.99	39 ± 0	160 ± 0.66
	Field 3	7.21	271	5.01	65 ± 0.66	217 ± 0.66
Test 1	Field 1	7.22	300	6.01	162 ± 0.33	685 ± 0
	Field 2	7.34	310	5.92	163 ± 0	652 ± 0
	Field 3	7.39	301	5.42	164 ± 0	680 ± 0.66
Test 2	Field 1	8.0	410	3.2	118 ± 0	234 ± 0
	Field 2	7.9	419	2.39	111 ± 0.66	224 ± 0
	Field 3	7.8	425	3.10	114 ± 0	219 ± 0.66

The soil samples treated with chemical fertilizer which were collected from the field 1 had a pH of 8.0 which was the highest pH noted. The lowest pH was noted from the soil samples collected from untreated soil of field 2. The pH ranged from 7.0 to 7.21 in untreated soil, the soil treated with manure had pH range of 7.22 to 7.39 and the soil treated with chemical fertilizer showed the highest pH in all the three fields with a pH range of 7.8 to 8.0. These observations were in tune to the results obtained by [14], who in his study claimed that the addition of chemical fertilizer to the soil can alter the pH of the soil (Table 2).

The electrical conductivity of the soil helps understand the mineral content of the soil. The addition of chemical fertilizers to the soil can increase the mineral content of the soil thus altering the electrical conductivity of the soil. The value of electrical conductivity ranged from 425 ds/cm to 271 ds/cm. The highest value was noted from the soils treated with chemical fertilizer collected from field 3, with the values shooting up to 425 ds/cm and the least was recorded in the untreated soil (control) collected from field 3 with the value of 271 ds/cm. In a study conducted by [15] it was observed that the addition of chemical fertilizer to the soil has led to an increase in the metal content of the soil. These findings were similar to the current study. The soil treated with chemical fertilizer showed the highest electrical conductivity (Table 2).

The organic carbon content of the soil ranged from 6.01g/kg in the soil collected from field 1 which was treated with manure and the least was seen in the soil treated with chemical fertilizer collected from field 2 with 2.39 g/kg. The soils samples collected from Field 1, Field 2 and Field 3 which were treated with manure showed maximum organic content whereas the soil treated with chemical fertilizer had the least amount of organic carbon content in them. The

presence of excess chemical in the soil can reduce the organic content of the soil. In a study conducted by [16], a reduction in the organic carbon content was seen in the soil treated with chemical fertilizer, which stated that the addition of chemical fertilizer can cause a substantial reduction in the organic carbon content of the soil (Table 2).

The urease activity was seen maximum in the soil that were treated with manure which ranged from 162 mg to 164 mg of urea hydrolyzed per gram. The least was seen in the untreated soil with a range of 35mg/g to 65mg/g. The urease activity in the soil treated with chemical fertilizer was lesser than that of manure (Table 2). In a study conducted to identify the inhibition of the urease activity in the soil by [17], a similar observation was recorded. The chemical fertilizers add excess urea to the soil which in turn increases the ammonia in the soil. The excess ammonia in the soil can cause a change in the acidity, salinity and reduces the microbial diversity in the soil thus affecting the soil environment and the soil microflora.

The alkaline phosphatase activity ranged from 145 to 685 mg of P-nitro phenyl phosphate released per gram in the soil. The least activity was observed on untreated soil and the maximum activity was recorded in the soil treated with manure in all the three fields (Table 2). In a study conducted by [18] it was observed that the soil treated with chemical fertilizer had a decreased alkaline phosphatase activity when compared to the soil treated with manure. The results obtained in this study coincided with the present study. This reduction can be due to the affect in growth and survival of the alkaline phosphatase harboring bacteria due to the addition of chemical fertilizer in the soil which in turn results in the reduction of the alkaline phosphatase activity. In a study conducted by [19], it was observed that the addition of manure in the soil had a positive impact on the soil health and increased the urease and alkaline phosphatase activity of the soil thus increasing the soil quality.

Enumeration of endophytic bacteria isolated from the crop plant

The samples from the three-month-old plants were collected during the month of November. Out of the 6 colonies that were sprayed onto the plants the number of colonies isolated from each field are as follows:

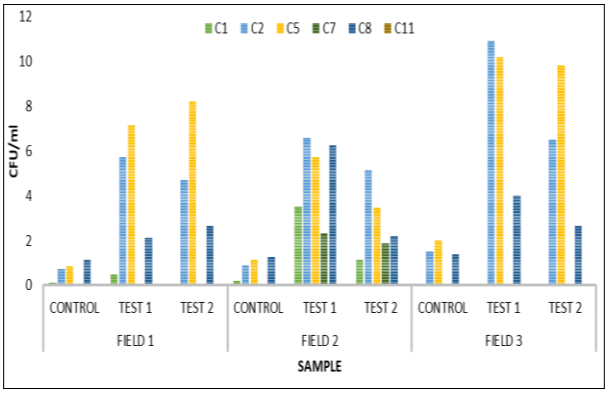

Fig 2 Enumeration of endophytic bacteria Isolated from the crop plant

Field 1 had minimum number of bacterial isolates whereas field 2 had the maximum. Among the three different soil parameters, plants grown in control and test 2 had the least number of bacterial isolates. Maximum number as well as diversity was observed in the plants grown in Test 1. With five bacterial endophytes isolated from Field 2 and four from field 1 (Fig 2). The bacterial endophytes thrived best in Field 3 as the number of colonies isolated in field 3 was greater than field 1 and 2. According to a study conducted by [20], chemicals in the soil can have a negative impact on the endophytic bacteria which are present in a symbiotic association with the plants.

Effect of temperature on the inoculation and the growth of endophytic isolates inside the crop plant

The climatic conditions while planting and collection of the samples were kept into consideration. The temperature was recorded during the period of the growth of the plant as well as while collecting the plant samples for analysis. The temperature range, humidity and the level of precipitation of the three fields during the period of growth of the plant are given (Table 3).

Table 3 Climatic conditions in the field during the growth of the plant

Place	Temperature (°C)	Humidity (%)	Precipitation (mm)
Bangalore	21-28	54-100	0.1mm-29.7mm
Kottayam	24-32	74-94	2.8mm-240mm
Hyderabad	30-33	78-90	0.5mm-40.1mm

The temperature while collecting the samples from the field for the analysis of microbial population were: Field 1- 28°C, Field 2- 31°C, Field 3- 33°C. The endophytes were isolated using spread plate method and the number of colonies isolated from different fields were analyzed.

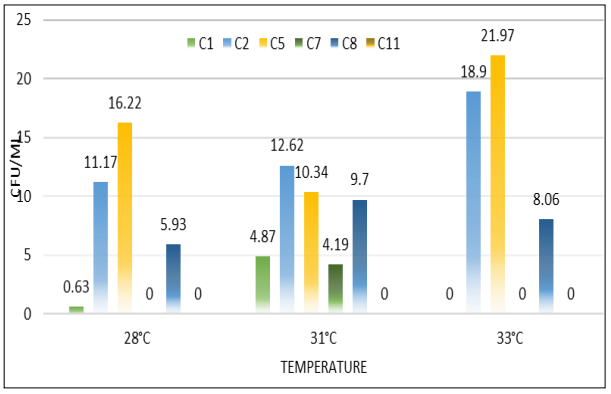

Fig 3 Effect of temperature on the endophytic bacteria isolated from the crop plant

Out of the 6 bacterial endophytes introduced into the crop plants, three bacterial endophytes, C2, C5 and C8 were seen to inoculate and grow in all the three field conditions. Bacterial isolate C11 did not grow in any of the field conditions Four out of the six bacterial endophytes were able to inoculate into the plant and grow in the temperature conditions ranging in between 21-28°C. Three bacterial isolates were retrieved from plants grown under 30-33°C.Five bacterial endophytes were isolated from the crops grown in temperature conditions ranging in between 24-32°C. (Fig 3). The temperature can affect the growth of the bacterial isolates. It is one if the important abiotic factors that can affect the growth of microorganisms. The temperature can affect the metabolic activities of the bacterial cells. The optimum temperature required for a bacterial strain to grow usually indicates the temperature needed for the bacterial cell to perform all its activities [21].

From the above observation, most of the bacterial isolates were able to grow at a temperature range between 28°C-31°C. In a study conducted by [22-23], the temperature for the optimum growth of *Bacillus* species were seen to fall between 28°C-31°C. In a study conducted by [24], the optimum temperature for the growth of *Kocuria rocea* was observed as 28°C. The bacterial isolates were isolated from the plants that were grown at temperature range of 21-33°C. *Brevibacterium casei* is usually seen to grow at 30°C [25-26], but in the above studies, the strains were isolated from the samples grown at a temperature range of 21-32°C. It was isolated from field 1(28°C) as well as field 2 (31°C).

Effect of pH on the inoculation and growth of endophytes inside the crop plant

The pH range varied from 7.0-8.0. The pH in the soils collected from control was the least, ranging from 7.0-7.21 and the pH in the soils treated with chemical fertilizers was the maximum. The pH ranging from 7.8- 8.0. the endophytic isolates were seen to grow in the observed pH range. Maximum growth was noted at a pH of 7.4 from the samples collected from the plants frown in field 3 in soil treated with manure. The addition of chemical fertilizers to the soil can change its pH and soil salinity [26], this in turn can affect the growth of the plant and the microorganisms.

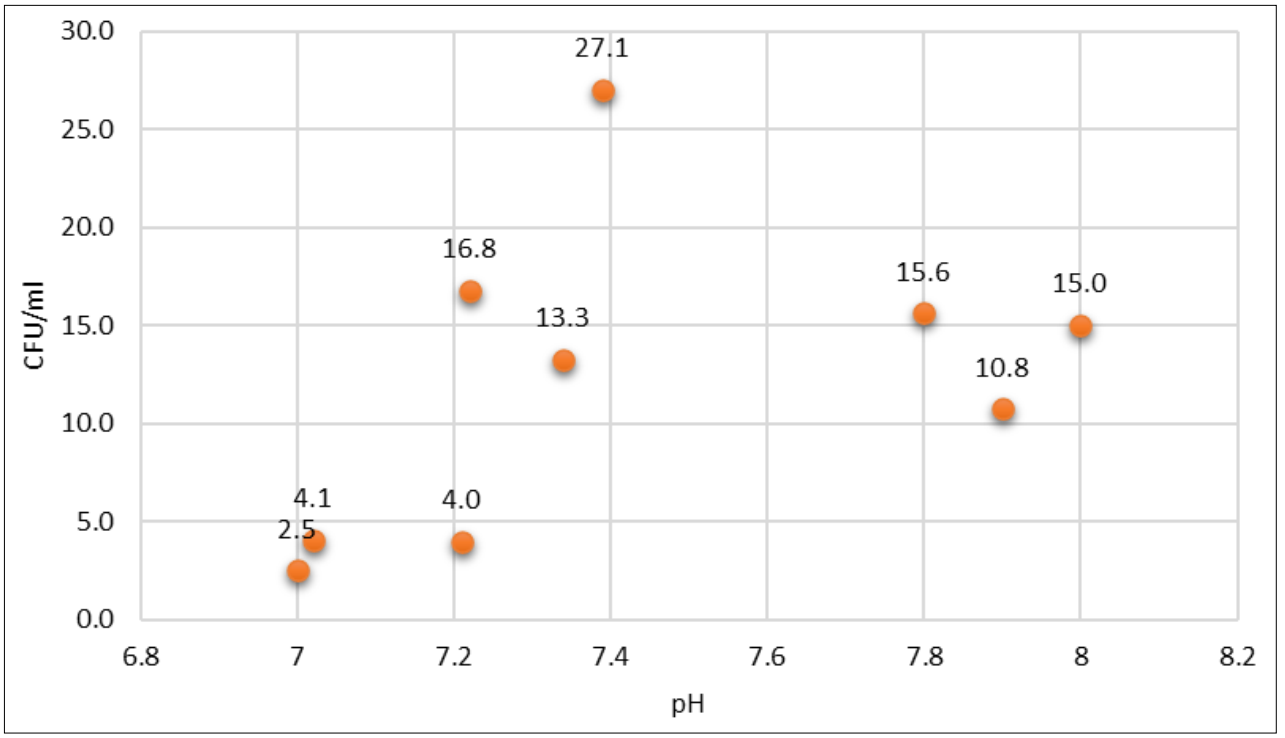

Fig 4 Effect of pH on the growth of endophytes inside the crop plant

The endophytes can live in a wide pH range from 4.0 to 8.0. However, the optimum range is the neutral pH. From the above observation, maximum rate of growth was observed when the pH was 7.4, the rate of growth decreased as the pH came down to 8. A similar result was observed by [27] who in their study to identify the effect of pH on the endophytic growth, noted that the maximum number of colonies were observed when the bacterial endophytes were grown in neutral pH. The least growth was seen when the pH was 4.0.

CONCLUSION

The addition of chemical fertilizers not only affects the soil micro flora but also triggers the balance in the microbial ecosystem present inside the plant which in turn leads to the reduced resistance of the plants towards biotic and abiotic stresses and plant pathogens. The endophytes can be used as an alternative to the chemicals that are used to increase the productivity of the plants. However, these endophytes can be sensitive to certain environmental factors like temperature and soil composition thus affecting its successful growth inside the plant. In the above study it was observed that the endophytes, even though thrives best in their optimum temperature and pH, can withstand the changes in these parameters to some extend thus helping the plants with growth promotion and resistance towards stress. This can be further exploited in agriculture to produce more healthy and resistant varieties of plants reducing the use of chemicals.

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