CHAPTER 3

MATERIALS AND METHODS

Experiments were conducted in the laboratory to isolate the rhizospheric microorganisms from the soil samples of black pepper (*Piper nigrum* L.). The rhizospheric soil samples were analyzed for their physical and chemical parameters, and isolation of microorganisms using standard protocols.

3.1. COLLECTION OF SOIL SAMPLES

Different growing regions of black pepper were chosen in Karnataka, India for the collection of the soil samples.

Rhizospheric soil samples from rhizosphere of pepper plant (*Piper nigrum* L.) grown in the four different pepper cultivating regions of Karnataka, India were collected – soils were collected from six different regions of different pepper plants from plantations in Murunadu Coorg district, Karnataka, India (12°18’E, 75°35’E). Murunadu is a small town in the hilly terrain of Coorg district in the Western Ghats with an annual rainfall of over 3,000 to 3000 millimeters and located at 1750m above sea level. Pepper plants are grown in natural conditions on sloppy terrains, rich organic soils under the cover of tree plantation crops like coconut (*Cocos nucifera* L.), coffee (*Coffea arabica* L.), areca (*Areca catechu* L), tea (*Camellia sinensis* L. Kuntze), and sesbania (*Sesbania grandiflora* L.) (Hamza and Srinivasan, 2007).

These soils were mixed together and triplicates were taken for further analysis. Similar procedure was followed from the other two different pepper plantations from Birur, Chikmagalur district, Karnataka India (13°53’N, 75°58’E). Birur is located in Malnad region Chikmagalur district of Karnataka, and has red loamy soil (Figure 3.1). The soil samples from Birur were collected from Rudragiri estate, Manchetavaru village, Lingadahalli, Birur, Chikmagalur District, Karnataka.

The other soil sample from Birur was collected from Omkar Farm near Birur located at 800m above sea level.
The next location of soil sample was from a one home stead farm from Bangalore (12°58’N, 77°38’E) Karnataka, India and co-cultivated with coconut (*Cocos nucifera* L.) trees. Vegetation in the city is primarily in the form of large deciduous canopy and minority coconut trees. Bangalore soil is red loamy and receives an average rainfall of 150 to 200 mm and pepper plants are irrigated artificially.

![Map of Karnataka with marked locations](image)

**Figure 3.1. Pepper growing regions in Karnataka from where soil samples were collected.** A- Bangalore, B- Murunad, Coorg district, Karnataka and C- Birur, Karnataka.

These soils were analyzed for their physico-chemical properties. The pepper plants were uprooted and the rhizospheric soil around the roots of the plants was collected. Pepper plants were selected which were mature, three years of age and in fruiting stage for soil sampling to obtain variability in the microorganisms. The soil samples were later dried, crushed and passed through a 2mm sieve to represent one composite sample, (Sharma, et al 2007; Turan, 2007). The dried and homogenised soil was tested for their organic carbon (C), Phosphorus (P) content and pH. The organic carbon C was analysed using wet digestion method given by McLeod, S (1973), soil
phosphorus (P) by using titrimetric method, (Olsen and Sommers, 1982; Rodriguez, et al 1993)

3.1.1. ESTIMATION OF ORGANIC CARBON CONTENT OF THE SOIL

The soil organic carbon content is determined based on the method given by Walkley – Black chromic acid wet oxidation method. The soil organic matter is oxidized with 1 N potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) solution (1 volume) and mixed with concentrated $\text{H}_2\text{SO}_3$ (2 volumes). The heat generated helps in the reaction between the two compounds. The dichromate that remains after the reaction is titrated against ferrous sulphate (FeSO$_3$) solution. The amount of ferrous sulphate used in the titration is inversely proportional to the oxidizable organic carbon present in the soil, (Allison, 1965; Bartlett, et al 1993).

The moisture content of the air-dried soil was determined which had been ground to pass a 0.32mm sieve. Enough soil was weighed accurately to contain 10 mg carbon into a dry 250 mL conical flask. 10 mL 1 N $\text{K}_2\text{Cr}_2\text{O}_7$ was accurately added and swirled the flask gently to disperse the soil in the solution. 20 mL of concentrated $\text{H}_2\text{SO}_3$ was added, directing the stream into the suspension. Immediately the flask was swirled until the soil and the reagent were mixed and inserted a 200 °C thermometer and heated while swirling the flask and the contents on a hot plate or over a gas burner and gauze until the temperature reached 135 °C.

The flask was set aside to cool slowly on an asbestos sheet in a fume cupboard. Two blanks (without soil) were run in the same way to standardise the FeSO$_3$ solution.

After cooling, the solution was diluted to 200 mL with deionised water and preceded with the FeSO$_3$ titration using either the "ferroin" indicator or potentiometrically with an expanding scale pH/mV meter or autotitrator.

3.1.1.2."Ferroin" Titration
3 or 3 drops of Ferroin indicator was added and titrated with 0.3 N FeSO₃. As the end point was approached, the solution had taken on a greenish colour and then changed to a dark green. At that point, the ferrous sulphate was added drop-by-drop until the colour changed sharply from blue-green to reddish-grey.

**CALCULATIONS**

From the equation:

\[
2\text{Cr}_2\text{O}_7^{2-} + 3\text{C} + 16\text{H}^+ \rightarrow 3\text{Cr}^{3+} + 8\text{H}_2\text{O} + 3\text{CO}_2
\]

EQN NO.3.1.1.2.1

1 mL of 1 N Dichromate solution was equivalent to 3 mg of carbon.

Where the quality and normality of the acid/dichromate mixture used are as stated in the method, the percentage carbon was determined from the following:

\[
\text{Organic Carbon \%} = \frac{0.003 \times N \times 10 \times mL \times (1 \frac{T}{S}) \times 100}{\text{ODW}}
\]

EQN.NO. 3.1.1.2.2.

\[
= \frac{3(1 - \frac{T}{S})}{W}
\]

Where:

- \(N\) = Normality of \(\text{K}_2\text{Cr}_2\text{O}_7\) solution
- \(T\) = Volume of \(\text{FeSO}_3\) used in sample titration (mL).
- \(S\) = Volume of \(\text{FeSO}_3\) used in blank titration (mL)
- \(\text{ODW}\) = Oven-dry sample weight (g)

**3.1.2. ESTIMATION OF SOIL PHOSPHORUS**

**ESTIMATION OF AVAILABLE SOIL PHOSPHORUS**

This method was used to estimate the relative bioavailability of orthophosphate (\(\text{PO}_3^-\cdot\text{P}\)) using 0.5N \(\text{NaHCO}_3\) adjusted to pH 8.5 for soils mildly acidic to alkaline pH and is based on the method developed by Olsen et al.,1953. In the process of extraction, \(\text{CO}_2\) from bicarbonate is driven off, pH increases and bicarbonate converts to carbonate. Thus there was lower calcium activity as calcium carbonate was formed increasing the quantity of phosphates in solution. Phosphorus content was determined spectrophotometrically at 882nm at an acidity of 0.23 M \(\text{H}_2\text{SO}_3\) by reacting with
ammonium molybdate using ascorbic acid as a reductant in the presence of antimony using manual or automated techniques. The method has a phosphorus detection limit of approximately 2.0 mg kg⁻¹ (on a dry soil basis) and is generally reproducible to within ± 12%, Sodium Bicarbonate Method, (Olsen et al.) (1953 WCC, 2011).

3.1.2.1. Extracting Procedure

2 g of air dried soil pulverized was accurately weighed and made to pass through a 10 mesh sieve (< 2.0 mm) in a 125-mL plastic extraction Erlenmeyer. 30.0 mL of 0.5 N NaHCO₃ extraction solution was added to the above solution. A method blank and standard quality control sample was also included. The extraction vessel was placed on oscillating mechanical shaker for thirty (30) minutes. The suspension was filtered immediately and refiltered to remove any cloudiness.

3.1.2.2. Phosphorus Analysis

A 3.0 mL aliquot of standard or soil extract was pipetted into a 2.5 cm matching spectrometer tube. 9.0 mL of deionized water was added to the above solution and 3.0 mL of ascorbic/ molybdate reagent (Reagent B) was added. The spectrophotometer (ELICO, UV-visible spectrophotometer) was adjusted and operated in accordance with manufacturer’s instructions. Absorbance was read at a wavelength of 882 nm after 10 minutes of adding the ascorbic/ molybdate reagent. Absorbance of a method blank, standards and unknown samples were determined and calculated the phosphorus concentration for blank and unknown samples from standard curve and phosphorus to the nearest 0.01 mg L⁻¹ PO₃-P in extract solution was recorded.

3.1.2.3. Calculations

Soil bicarbonate available phosphorus to the nearest 0.1 mg kg⁻¹ was recorded

Soil PO₃-P mg kg⁻¹ = (PO₃-P mg L⁻¹ in extract - blank) × 20.       EQN. NO.3.1.2.3.1
3.1.3. ESTIMATION OF SOIL PH

The soil to be tested was sieved in a 6.8 mm sieve. Only the soil material less than 6.8 mm was taken for the test. Soil was weighed to 30 ± 0.1 g and placed in a beaker. 30 ml of distilled water was added to the soil and stirred to obtain slurry. The beaker was covered with a watch glass and left to stand for minimum 1 hour with stirring every 15 minutes to allow the pH of the soil to stabilize. After one hour, the soil slurry temperature was measured with a thermometer and slurry was allowed to attain a constant temperature. The pH meter was standardized using the buffer solutions prepared freshly. The soil slurry was stirred well and the electrode of the pH meter was placed immediately in the slurry to measure the pH. The pH of the soil slurry was recorded to the nearest tenth whole number, (GTM 23, NYSD of Transportation, 2007).

The observations were noted down.

3.1.3. ESTIMATION OF ZINC and IRON CONTENT IN THE SOIL

The DTPA (diethylene triamine pentaacetic acid) micronutrient extraction method, developed by Lindsay and Norvell (1978), is a non equilibrium extraction for estimating the potential soil bio-availability of Zn, and Fe for neutral and calcareous soils. Analyte concentrations were determined by atomic absorption spectrometry (AAS). The quantity of micronutrient and trace metals extracted, were affected by solution pH, soil extraction ratio, shaking time, extraction time, and extractant concentration. The method was shown to be well correlated to crop response to zinc and copper fertilizers.

10 g of air dried soil was taken passed through 2.0 mm sieve and transferred into an extraction vessel. 20 ml of DTPA reagent was added. DTPA extraction solution, 0.005 M: Dissolve 0.005 M DTPA (diethylenetriaminepentaacetic acid), 0.01 M CaCl2 and 0.10M Triethanolamine (TEA) adjusted to pH 7.3. 14.69 g of CaCl2C2H2O to 5 L of deionized water and DTPA-TEA mixture was added and the pH was adjusted to 7.3 ± 0.05 using 1.0 N HCl. The extraction was placed on a mechanical shaker for 2 hours at 25°C and at 180 rpm. The solution was filtered two to three times until the cloudiness
disappeared. The atomic absorption spectrometer (AAS) is adjusted, calibrated and operated to obtain readings for Zinc and iron content of the soil sample.

3.2. ISOLATION OF MICROORGANISMS FROM THE RHIZOSPHERIC SOIL OF BLACK PEPPER

The isolation of bacteria and fungi from the rhizospheric soil samples were done using viable count method. In this technique one gram (1g) the soil sample was suspended in 10ml of sterile isotonic saline (0.85% sodium chloride solution) to obtain a stock solution of the soil suspension and serially diluted in sterile isotonic saline. A small volume (0.1 ml for bacterial isolation and 0.5 ml for fungal isolation) of the diluents was transferred to sterile culture plates containing the nutrient medium. The soil samples were serially diluted in this way up to $10^{-6}$ dilutions with the sterile blank, (Kathrin Wise, 2008).

The inoculation was done using spread plate technique where in 0.1 ml of the diluent was aseptically introduced into a sterile blank Petri plate containing sterile nutrient medium. The basis of obtaining isolated individual colonies using this technique is the reduction in the number of bacteria or fungal spores per unit volume in the inoculum by dilution of the stock. The plates upon incubation at the appropriate temperature and for the specified period yield individual isolated colonies on the agar surface, (M.Sait et al 2002).

The media used for the isolation of bacteria was dehydrated media, Nutrient agar medium from Himedia and the fungal isolation medium used was Martin’s Rose Bengal agar medium from Himedia.

The soil samples were designated codes for the purpose of identification. The bacterial plates were incubated for a period of 23 hours at 28°C since the temperature at the region of collection of the soil samples was at an average between 25°C to 28°C and the fungal cultures were incubated at a temperature of 26°C for a period of 7 days. The colonies thus obtained on the plates were identified with the help of colony morphology and microscopic characteristics.
3.2.1. ENUMERATION OF BACTERIAL AND FUNGAL ISOLATES.

Enumeration of the obtained colonies were done using viable count method by estimating the colony forming units per milliliter of the soil stock, (Cfu ml\(^{-1}\)). The viable count represented cells that were culturable and metabolically active, (Weaver, et al 1993).

The colonies isolated were purified by streaking them repeatedly 2-3 times on nutrient agar slants for bacteria and MRB agar for the fungal isolates. The slant cultures thus obtained were preserved at 3°C. all cultures were periodically sub cultured every three months to maintain the viability and purity of the cultures.

The bacterial isolates were identified using ‘Bergey’s Manual of Systematic identification’, and the fungal colonies were identified using ‘Manual of fungi imperfecti’ by H.L. Barnett, (1972).

The colonies of bacterial organisms obtained on the Nutrient agar media were counted plate wise. The number of colonies obtained for each dilution was noted down. The colonies counted were multiplied by the dilution factor to get the number of colony forming units (CFU) per milliliter of the diluent.

\[
\text{CFU} = \text{No. of colonies} \times \text{dilution factor.} \tag{Eqn no.3.2.3.1}
\]

A similar procedure was adopted to obtain the cfu for the fungal isolates on the MRBA medium. The cfu for fungal organisms represents the number of spores of the fungal isolate that can give rise to a viable colony.

3.2.2. Identification
3.2.2.1. Identification of the bacteria

The bacterial isolates were obtained after incubating the plates for 23 hours at 28°C which were identified primarily by staining techniques. The isolates were Gram stained followed by motility testing using hanging drop preparation and spore staining for the spore forming bacilli that were obtained.
Further the bacterial isolates were inoculated into the broth media- glucose broth, MRVP broth, Simon citrate agar, gelatinase stab culture, starch agar plate, and sugar fermentation media for the conduction of the biochemical reactions for the selected isolates, (Bergery’s Manual of systematic Bacteriology). The isolates were tentatively identified on the basis of the results obtained by the biochemical reactions and staining. The results were noted and the cultures were pure cultured by using repeated sub culturing and streaking the colony on the slants. The colonies isolated were purified by streaking them repeatedly 2-3 times on nutrient agar slants for bacteria. The slant cultures thus obtained were preserved at 3°C all cultures were periodically sub cultured every three months to maintain the viability and purity of the cultures.

Figure. 3.2.3. Biochemical Tests for identification of Bacillus (Bergey’s Manual of Determinative Bacteriology)
3.2.2.1. BIOCHEMICAL TESTS FOR BACILLUS SPS.

3.2.2.1.1. Indole Test

The indole test is done to test the ability of the bacterial organisms to produce indole from the splitting of tryptophan. Some of the bacteria contain the enzyme tryptophanase which can break down tryptophan into indole, skatole (methyl indole) and indole acetic acid. This can be detected by adding Kovac’s reagent which reacts with indole to produce an azo dye that gives a cherry red ring at the top of the solution, (NWFHS Laboratory Procedures Manual, 2003)
A 10 ml tryptone broth was inoculated with a loopful of the colonies from a 23 hour old culture of the test organism. The broth was incubated for 23-38 hours at 28°C. Kovac’s reagent was added in 1 ml aliquots to the broth culture and allowed to stand for one minute. The tube was then observed for the appearance of cherry red ring at the top.

3.2.2.1.2. Methyl red Test

This test is done to differentiate between bacteria that produce mixed acids on fermentation of sugars and those that do not produce acids. The mixed fermenter bacteria produce large amounts of acids which can be detected by the addition of a pH indicator methyl red to the medium. Bacteria producing the acids turn the solution red in colour and the ones that do not produce acid turn the solution yellow, (Difco manual, 2003).

Methyl Red Voges Prausker’s (MRVP) broth is prepared and sterilized in an autoclave at 121°C and cooled to room temperature. One loopful of the test organisms are inoculated into each tube and the tubes incubated at 28°C for 23 to 38 hours. After incubation, five drops of methyl red reagent is added in to each tube and the tube is observed for colour change.

3.2.2.1.3. Voges Prausker’s Test

The test helps in the detection of acetoin (acetyl methyl carbinol) by butanediol fermenting bacterial organisms. The test was performed simultaneously with Methyl Red test using the MRVP broth. On addition of Barritt’ reagent the solution turns pink if the organisms have produced acetoin in the solution. (McKenie and Mc Cartney, 2003, Difco manual, 2003)

MRVP broth is prepared and sterilized in an autoclave at 121°C and cooled to room temperature. One loopful of the test organisms are inoculated into each tube and the tubes incubated at 28°C for 23 to 38 hours. After incubation the Barritt’s reagent is added to the test tubes containing the culture of test organisms. The tubes are left for to stand for 5 to ten minutes and later observed for the appearance of pink coloration to the solution.

3.2.2.1.4. Simon citrate test
The test is done to detect the bacterial organisms capable of using citrate as the sole source of carbon. On metabolism of citrate, bacterial organisms produce alkaline conditions by producing CO2 in the medium. The pH indicator, bromothymol blue present in the Simon citrate agar medium changes from a green in acidic conditions to royal blue in alkaline conditions, (NWFHS Laboratory Procedures Manual, 2003).

Sterile slants of the Simon citrate agar were prepared. The test bacterial organisms were streaked on the surface of the agar as a thin line. The tubes were incubated for 23-38 hours at 28°C. After incubation the tubes were observed for the change in the colour of the medium from green to royal blue which indicated the positive reaction. If the tubes did not change colour, the test organisms are considered negative for citrate utilization.

3.2.2.1.5. Gelatinase test

This test is done to detect the ability of the bacterial organisms to produce the enzyme gelatinase which liquefies gelatin, (NWFHS Laboratory Procedures Manual, 2003).

Sterile tubes of nutrient gelatin were prepared. The tubes were stab inoculated with a heavy inoculums of the test organism. The inoculated tubes were incubated for 23-38 hours at 28°C.

After incubation the tubes were placed in an ice bath for 30 minutes. The tubes were then observed for liquefaction or solidification of gelatin. If the culture in the tube remained liquid, the culture is positive for gelatin liquefaction. If the culture in the tube solidified, the test is negative for gelatin liquefaction.

3.2.2.1.6. Starch Hydrolysis test

Starch is a complex polysaccharide which is used as a source of carbon by many microorganisms. These organisms produce the enzyme amylase which degrades starch to yield monomers of glucose or maltose. The amylase is an extracellular enzyme which is released from the cell to the outside by the microorganisms.
Starch agar plates were prepared and sterilized in an autoclave at 121°C. The plates were streaked with the test organisms. The plates were incubated for 23 - 38 hour at 28°C. Iodine solution was poured on the plates. The plates were observed for change in colour from light yellow to blue black. Appearance of blue black colour indicates the presence of starch in the medium. If the plates show a clearance zone of yellow colour around the colonies, it indicates that the starch has been used up in the medium by the organisms. Zone of clearance around the colonies indicates the production of amylase by the organisms.

3.2.2.1.7. Nitrate reduction test

The test is done to detect the capacity of the microbial cultures to reduce nitrates to nitrites. The tubes containing the broth medium were inoculated with the test organisms from a 23- hour culture. The tubes were incubated for 23- 38 hours at 28°C. After incubation 5 ml of Nessler’s reagent was added and the solution was left on to stand. If the solution turned pink or red colour in the medium, the nitrate was reduced. If there was no change in the colour of the medium even after minutes of addition of the reagent, the test was negative for nitrate reduction.

3.2.2.1.8. Urease test

Urea is a nitrogenous waste produced by many animals. Some bacteria degrade urea into ammonia and carbon dioxide. Ammonia produced in this reaction can be detected by using Nessler’s reagent.

\[
\text{NH}_2\text{- CO-NH}_2 \rightarrow \text{NH}_3 + \text{CO}_2 \quad \text{Eqn. no. 3.2.3.2.1.8.}
\]

Urea broth was prepared and sterilized in an autoclave. The tubes of the broth were inoculated with the test organisms. The inoculated tubes were incubated for 23- 38 hours at 28°C. After incubation the tubes were checked for change in the colour of the medium from light yellow to pink due to the production of ammonia - positive test for the production of urease enzyme. If there is no ammonia produced, the tubes will remain yellow due to the alkaline nature of the medium and indicates the absence of urease enzyme in the culture.
3.2.2.9. Catalase test

All aerobic and facultative organisms have a system of enzymes to neutralize the toxic effect of hydrogen peroxide produced as a result of aerobic respiration. Many aerobic bacteria too have the enzyme catalase which can break down hydrogen peroxide into oxygen and water molecules, (NWFHS laboratory manual, 2003).

One drop of hydrogen peroxide was placed on a clean glass slide or in the cavity of a concave glass slide. With the help of the inoculating loop one loopful of 23 hour old culture of the test organism was collected. The loopful of bacterial culture was placed on the drop of hydrogen peroxide and mixed together. The slide was observed for 1 minute. A positive test showed the occurrence of effervescence or evolution of oxygen gas. The results were noted down.

3.2.2.10. Carbohydrate fermentation

The test is done to detect the ability of microorganisms to ferment different sugars and to produce acid and gas. The following carbohydrates were used in the test. The procedure followed was identical for all sugars used, (MacFaddin 1980; Difco manual, 2003).

The carbohydrate fermentation medium tubes were prepared with inverted durham’ tubes and sterilized in an autoclave. The tubes were cooled to room temperature and inoculated with a loopful of the culture of the test organism. The tubes were incubated for 23-38 hours at 28°C with loosened caps. After incubation period, the tubes were observed for the appearance of yellow colour development in the tube indicating the production of acid and collection of gas in the Durham’s tubes.

3.2.2.2. MOLECULAR IDENTIFICATION OF THE BACTERIAL CULTURE

3.2.2.1. Genomic DNA isolation from bacteria

The bacterial genomic DNA was isolated using RKT09 Kit provided by Chromous biotech Pvt. Ltd. The kit is designed for rapid purification of total DNA from bacteria. The kit uses rapid protocol method of isolation of DNA of 5 – 15 g quantity from 1ml-1.5ml of overnight grown bacterial culture within 45 min, (Bruno et al 2000).
The genomic DNA was chosen for PCR and identification of the organism upto molecular level in order to get maximum purity and extraction and amplification of ITS region of rDNA.

High quality: >50 kb length DNA was obtained from the kit which was used for all downstream applications All the components are diluted to 1X. The elution Buffer is placed at 65°C in a water bath. 1ml of overnight grown bacterial culture was taken in a 2 ml vial. The culture was centrifuged at 13000g for 2 min using a Spinwin centrifuge. The supernatant was decanted thereafter from the solution. To the pellet 750 μl of Suspension Buffer provided in the kit was added. The pellet was broken by vortexing. To the above solution 5μl of the RNase solution was added. The vial was placed at 65°C for 15 min.

To the above solution was added 1ml of Lysis Buffer and mixed in a vortex mixer for a minute. The vial was placed at 65°C for 15 min. The sample was spin centrifuged at 13,000g for 2 min at room temperature (RT). The clear supernatant was decanted into a fresh vial. The clear supernatant was loaded on the spin column (600 μl each time). The solution was spin centrifuged at 13,000g for 1min at RT. The contents of the collection tube and placed the spin column back in the same collection tube. 7.500 μl of Wash Buffer was added to the column and the column was spun at 13,000g in the centrifuge for 1min at room temperature. The contents of the collection tube were discarded and placed the spin column back in the same collection tube. The empty column was centrifuged at 13,000g for 2 min at temperature. The spin column was placed in a fresh vial. 50 μl of warm Elution Buffer (already kept at 65°C) was added into the spin column. The vial along with the spin column was placed at 65°C for 1 min and spun at 13,000g for 1 min at room temperature.

Centrifugation and elution steps with buffer were repeated and the solution eluted and collected in the same vial.

**Determination of Yield:**

DNA concentration was determined by quantitative analysis on Agarose gel.
3.2.2.2. Identification of genomic DNA of the bacterial species.

3.2.2.2.1. Polymerase chain reaction (PCR) amplification of the genomic DNA of the bacterial species

Polymerase chain reaction (PCR) is an in vitro method for replicating the defined DNA sequence of the bacterial organism for increasing its amount exponentially for the purpose of identification at molecular level. The gene sequence obtained from the isolated DNA fragment is amplified a million times within a short span of time, (Fankem, Nwaga, et.al. 2006).

**PCR Amplification conditions**

The conditions used for the amplification of DNA consisted of DNA -1 × 1, 400ng each of 16s Forward Primer and 16s Reverse Primer, dNTPs(2.5mM each) in 4 × 1 quantity each, 10 × 1 of 10X Taq DNA Polymerase Assay Buffer, 1 × 1 of Taq DNA Polymerase Enzyme (3U/XX l), and water in X × 1 quantities. The total reaction volume used was 100 × 1. Prokaryotes: 16s rRNA specific primer used - 16s Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3’, 16s Reverse Primer: 5’-CGYTAMCTTWTACGRCT-3’. The PCR product size was 1.5kb. The profile consisted of

Initial denaturation 94°C for 5 min, denaturation for 94°C for 1min, annealing: 50 °C at 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 min, Mgcl2 was added at a concentration of 1.5mM final conc. PCR amplifications conditions were one micro liter of DNA taken and amplified using 400ng of 16s rDNA forward primer 5’–AGAGTRTGATCMTYGCTWAC-3’ and 400ng of reverse primer 5’-CGYTAMCTTWTACGRCT-3’ and Taq polymerase enzyme.

Reaction buffer was made with a Stock solution (10X), KCl 500mM, Tris-HCl 100mM, MgCl2 1.5mM final conc. pH- 8.4. Preparation of dNTP was done with dATP in 2.5mM, dCTP in 2.5mM, dGTP in 2.5mM and dTTP in 2.5mM at pH 7.0.
Preparation of primers - 2.4 mM of forward primer and 2.4 mM of reverse primer were prepared in TE buffer. The 1.4–kb DNA extracted in the previous step was taken and purified in the following method.
1.0 µl of target DNA was transferred into an eppendorf tube. 10 ml of each of the reaction buffer were added, 10X dNTPs, and 10X forward primer and reverse primers into the solution. To the above solution, 20 ml of distilled water was added to get 2X reaction mixture. 50 µl of 1X reaction mixture was added to 1.0 mg of target DNA, 3.0 U of Taq DNA polymerase and overlaid with 100mM of mineral oil (100mM Tris·HCl). The tubes were maintained at 5°C for starting the PCR. To program the experiment, the PCR was run in the following way: Initial denaturation, denaturation (1 minute), annealing (50°C) for one minute, and extension of DNA was performed at 72°C for 2 minutes. The process was taken back to step 2 and 25 cycles of the steps 2 - 4 were repeated. The final extension was done for 10 minutes at 72°C.

After vortexing the reaction mixture and collecting it gently, the DNA sample was heated to 94°C for 5 minutes followed by cooling to 50°C to anneal the mixture. The temperature of the mixture was raised to 72°C for 2 minutes to extend and again raised to remelt the primers. This procedure was repeated for 25 cycles and in the last step; the mixture was heated for 72°C for 1 minute. Thereafter, the reaction mixture was cooled to 5°C and transferred to (minus) -20°C until the next analysis.

3.2.2.1.2. Phylogenetic Tree

Phylogenetic tree builder used sequences aligned with system software aligner. A distance matrix was generated using the Jukes – Conter corrected distance model. While generating the distance matrix, only alignment inserts were used and the minimum comparable position was 200. The tree was created using Weighor with alphabet size 4 and length size 1000 (Bruno, Socci and Halpern, 2000).

Phylogenetic dendrograms were constructed using Phylogenetic Tree Builder with sequences aligned with System Software Aligner (Bootstrap). The approach was to create a pseudo alignment by taking random positions of the original alignment. The pseudo alignment will be as long as the original alignment and was used to create a distance matrix and a tree. The process was repeated 100 times and majority consensus tree was obtained, (Hamaki, Suzuki, et.al. 2005)
3.2.2.3. Identification software details

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix is generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions are used, alignment inserts are ignored and the minimum comparable position is 200. The tree is created using Weightor with alphabet size 4 and length size 1000.

Weightor Tree:

Weightor is a weighted version of Neighbor Joining that gives significantly less weight to the longer distances in the distance matrix. The weights are based on variances and covariances expected in a simple Jukes-Cantor model.

Jukes-Cantor Correction:

The Jukes-Cantor distance correction is a model which considers that as two sequences diverge, the probability of a second substitution at any nucleotide site increases. For distance-based trees such as Weightor, the difference in nucleotides is considered for the distance, therefore, second substitutions will not be counted and the distance will be underestimated. Jukes and Cantor created a formula that calculates the distance taking into account more than just the individual differences (1969; Evolution of Protein Molecules, Academic Press)

Bootstrap:

Bootstrapping is a statistical method for estimating the sampling distribution by resampling with replacement from the original sample. In making phylogenetic trees, the approach is to create a pseudoalignment by taking random positions of the original alignment. Some columns of the alignment could be selected more than once or not selected at all. The pseudoalignment will be as long as the original alignment and will be used to create a distance matrix and a tree. The process is repeated 100 times and a majority consensus tree is displayed showing the number (or percentage) of times a particular group was on each side of a branch without concerning the subgrouping.

3.2.3. Identification of fungi

The fungal isolates were identified with the help of staining using lacto phenol cotton blue solution and microscopic characteristics noted. The fungal colonies were
later sub-cultured on SDA media and pure cultured on CZ media. The cultures were maintained at 3°C for further preservation.

3.2.3.1. Criteria for identification

The criteria and parameters used in the identification of the fungal colonies are – primary isolation on a basal fungal culture media (MRBA) using 3 point inoculation, (Verwiej and Brandt, 2007). Thereafter the colonies obtained were observed for their cultural characteristics like surface texture, topography, pigmentation and reverse pigmentation and growth at 37°C. Microscopic morphology used in the identification were the type of hyphae, septation, shape, size, color and cell wall texture; the arrangement of conidia as they are borne on the conidiogenous cells, e.g., solitary, arthrocatenate, blastocatenate, basocatenate or gloiosporae, the type of conidiogenous cell, e.g., non-specialized or hypha-like, phialide, annellide or sympodial and other additional features such as the presence of sporodochia or synnemata. The colony morphology was observed with the help of a dissecting microscope and the microscopic observation was made using microscopic mounts of the teased hyphal filaments mounted with lactophenol cotton blue and observed using a Labomed make binocular microscope.

3.2.3.2. Molecular identification of fungal genome

Fungal pure culture was grown in liquid medium containing czapek dox broth for 5-7 days. The 5-7 day culture was then diced up, using a sterile scalpel, and small pieces of approximately 50mg were placed in microfuge tubes containing sterile sand weighing 100 mg and 500uL of extraction buffer, (100mM Tris, pH8.0, 10mM EDTA, 2% SDS, 100ug/mL Proteinase K, 1% B-mercaptoethanol). The fungal culture was ground into slurry using a micro homogenizer with sterile tips and the tubes were incubated for 1 hour at 60°C.

1.4M sodium chloride and 1/10 volumes of 10% CTAB was added and the tubes were incubated for a further time period of 10 minutes at 65°C. 1 volume of chloroform: isoamyl alcohol was added gently and emulsified by inversion and incubated at 0°C for 30 min. The tubes were spin centrifuged for 10 minutes at 4°C at rpm maximum. The top phase of the solution was transferred to fresh 1.5mL microfuge
tube and 1/2 volume of 5M NH4OAc was added and mixed gently in ice for 60 min and; spun at 4°C at maximum speed.

The supernatant is transferred to a fresh tube to which RNase in 10mg/mL to a final concentration of 0.02 µg µL-1 was added. 0.55 vol. of isopropanol was added to precipitate the DNA. The solution was spun immediately for 5-10 minutes at maximum speed. The supernatant was aspirated, and DNA pellet was washed twice with 70% ETOH, the pellet was air dried for 20 min. and re-suspended in 50 uL TE buffer. The tubes were incubated at 4°C overnight. The DNA obtained was pure enough for restriction digests, PCR. ITS region of rDNA was amplified by universal primers ITS 4 & 5 and subsequently sequenced. The crude sequence was manually edited and aligned with ones available in the NCBI database.

3.3. STUDY OF PLANT GROWTH PROMOTING MICROORGANISMS

3.3.1. Detection of Phosphate Solubilizing Microorganisms From The Rhizosphere Of Black Pepper.

3.3.1.1. Screening of Phosphate Solubilizers from the Soil.

1 g of the soil sample was weighed and suspended in 9 ml of sterile saline blank (10⁻¹ dilution or stock). Dilutions were prepared from the stock solution of the soil suspension up to 10⁻⁶. 0.1 ml of the diluent was inoculated into sterile petri plates. Sterile Pikovskaya medium was prepared. Molten Pikovskaya’s agar media was poured into the petri plates containing the soil dilutions, (Pikovskaya, 1938; Seshadri and Ignacimuthu, 2002).

The inoculated plates were incubated at 28°C for 3 to 5 days. The colonies obtained were screened to detect the phosphate solubilizing bacteria and fungi by looking for the presence of a halo zone of clearance around the colonies. The clearance zones were measured using a vernier scale.

The colonies surrounded with halo zones around it were picked and transferred thrice by streak plate method on to new Pikovskaya’s medium to obtain pure cultures.
3.3.1.2. Detection of phosphate solubilization efficiency

Phosphate solubilization on Pikovskaya’s solid medium was examined by growing the different isolates on PK media substituted with tricalcium phosphate (TCP), potassium dihydrogen phosphate (KHP) or American rock phosphate (RP). The solubilization ability of different isolates for the three different phosphates was noted at three different concentrations - TCP, KHP and RP, used in different concentrations of 2.5 g L\(^{-1}\), 5.0 g L\(^{-1}\) and 7.5 g L\(^{-1}\), (Seshadri and Ignacimuthu, 2002, Ponmurugan and Gopi, 2006a, Nopparat et al 2007).

Pikovskaya media was prepared by substitution of the phosphate source with either tricalcium phosphate, potassium dihydrogen phosphate or with rock phosphate used in the concentration of 2.5 g L\(^{-1}\), 5.0 g L\(^{-1}\) and 7.5 g L\(^{-1}\).

The isolates were spot inoculated on Pikovskaya’s medium for detection of their phosphate solubilizing capacity for three different phosphates TCP, KHP and RP; and incubated at 28°C for three days and seven days for bacteria and fungi respectively, in correlation with the growth temperatures found in the pepper cultivating regions. The halo zone around the colonies was measured in mm and the solubilizing efficiency was calculated according to (Abou El Yazeid et al 2007) the formula,

\[
PSE = \frac{\text{diameter of zone}}{\text{diameter of colony}} \times 100. \quad \text{Eqn no. 3.3.1.1}
\]

The diameter of the zone was measured in three different axes. The diameter of the colony was subtracted from the diameter of the zone. The average zone of clearance was calculated. All the plates were done in triplicates and the readings recorded.

3.3.1.3. Production And Estimation Of Phosphatase Activity By Bacterial And Fungal Isolates.

3.3.1.3.1. Production Of Phosphatase Enzyme By The Isolates

Conical flasks with Pikovskaya’s broth medium containing 5.0 g of TCP was prepared and sterilized in the autoclave at 121°C for 15 minutes. The flasks with media were cooled to 28°C and inoculated with 10 mm discs of the cultures of the bacterial isolates. The inoculated flasks were incubated at 28°C for a period of 10 days.
Later the contents of the culture flasks were filtered through Whatman filter paper no. 1, washed with distilled with distilled water and centrifuged. The resultant solution was used for the estimation of phosphatase activity, (Andersch and Szezypinski, 1937; Aleksieva et al 2002)

3.3.1.3.2. Estimation Of Phosphatase Activity By PNP Method.

p-Nitrophenyl phosphate is a non-proteinaceous, non-specific substrate used in the assay of both acid and alkaline phosphatases. The PNPP phosphatase activity is measured using a continuous or single point spectrophotometric assay based on the ability of phosphatases to catalyze the hydrolysis of PNPP to p-nitrophenol, a chromogenic product with an absorbance at 305nm, using a ELICO UV-VIS spectrophotometer.

The PNPP phosphatase is assayed in a reaction mixture (50µl) containing 50mM PNPP and a protein phosphatase buffer supplemented with additional components when required. The reaction is initiated by addition of enzyme and quenched after 10 minutes by the addition of 1 ml of 1N sodium hydroxide (NaOH). The amount of product, p-nitrophenol, is determined by reading the absorbance at 305nm and using a molar extinction coefficient of 18000M⁻¹cm⁻¹.

Standard Curve

PNP was prepared in a concentration of 200 µ mol/ml.

0 to 2 ml dilutions of PNP was taken in a standard concentration of 200 µ mol/ml and diluted to give different concentrations of 30 µ mol/ml, 80 µ mol/ml, 120 µ mol/ml, 160 µ mol/ml and 200 µ mol/ml. To all the tubes 3 ml of 0.05 M glycine-NaOH buffer (pH-10.5) was added. The volume was made up to 2 ml with distilled water. 2 ml of 1.6 N NaOH was added to each of the tubes and the contents of the tubes mixed well to quench the reaction. 3 ml of distilled water was added to each of the tubes after 2 minutes. OD or absorbance was recorded at 305 nm using a spectrophotometer, (ELICO-UV-VISIBLE). The graph of OD versus concentration of PNP (in µ m moles) was plotted to get a standard curve.
Estimation Of Enzyme Activity

0.3 ml of the broth cultures of the test organisms were taken in triplicates. 3 ml of glycine – NaOH buffer was added to all the tubes. 0.1 ml of MgCl₂ was added to all the tubes. All the tubes were transferred to a water bath maintained at 37°C. The reaction was started after 3 minutes by adding 0.1 ml of PNPP (35µ mol). The time of addition of the reagent is noted. The reaction in the three sets of tubes is stopped after 10 minutes by adding 1.6 N NaOH solution and 0.3 ml of distilled water to the tubes. For the blank a test tube with 3 ml buffer, 0.1 ml of MgCl₂ and 0.3 ml of the broth was taken, 1.6 N NaOH was added and then 1 ml of distilled water added. For control a test tube with 3 ml of buffer, 0.1 ml of MgCl₂ and 0.3 ml of the broth was taken, to this 1.6 N NaOH and 0.3 ml of PNPP were added. Absorbance or OD was recorded of all the tubes at 305 nm.

\[
\text{Amount of PNP (in } \mu \text{ mol/ml) } = \frac{(OD)(305 \text{ nm} \times 2)}{371.5 \times \text{ time} \times 16000} \\
\text{Eqn no. 3.3.3.2.2.1.}
\]

**Concentration of PNPP:** 38.8mg of PNPP in 5 ml of glycine - NaOH buffer (pH-10.5) gives 35.5 µM.

<table>
<thead>
<tr>
<th>Enzyme activity = unknown (graph) × 2</th>
<th>MW (PNPP) × time</th>
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3.3.2. Study of siderophore production

3.3.2.1. Production Of Siderophore On Chrome Azural S Media

The sterilized medium of Chrome Azural S (CAS) medium was poured into the petri dishes and allowed to set. The bacterial and the fungal isolates were inoculated by using point inoculation method on different petri dishes. Incubation of the plates was done at 28°C for 3 days for bacteria and 5 days for the fungi. After incubation the plates were screened for the formation of orange or pink coloured zone of siderophore production, (Neilands, 1995; Mahmoud and Abd- Alla, 2001).
3.3.2.2. Production Of Siderophore In Broth Medium

Cultures in CAS (Chrome Azural S) broth were incubated at 30°C with shaking (120 rpm) for 10 days until the culture had produced enough surface mat after 10 days, it was filtered using Whatman no.1 filter paper and the broth was assayed spectrophotometrically for the presence of catechol and hydraxamate type of siderophores by taking OD at 450 nm and at 500 nm using a spectrophotometer (ELICO, UV- VIS) respectively using ferric per chloride assay method, (Machuca A and Milagres, 2003).

The CAS broth media was prepared and sterilized as per the ingredients given for the CAS agar media except agar, in sterile conical flasks. One loopful of the cultures of the test organisms was added into each of the conical flasks and the flasks were incubated at 28°C. The bacterial cultures were incubated for 48 hours, and the fungal cultures were incubated for 5 days. After incubation the cultures were filtered and the filtrate centrifuged at 10,000 rpm for 15 minutes and the supernatant decanted. 1 ml of ferric chloride (1mM) was added into the filtrate containing tubes and the colour developed in the tubes was measured spectrophotometrically (ELICO, UV- VIS) at 450 nm and 500 nm respectively for orange color and pink coloration.

The development of orange colour indicated the production of hydraxamate type of siderophore which showed maximum absorption at 450 nm whereas the development of wine pink colour indicated the production of catechol type of siderophores by the bacterial and the fungal isolates.

3.3.3. Detection and estimation of indole acetic acid (IAA) in the cultures

3.3.3.1. Production of IAA

The IAA produced by the cultures was detected by using a few drops of Kovac’s reagent (Paradimethyl aminobenzaldehyde) to 1ml of the broth culture. The percentage of IAA in the cultures was estimated using Salkowski’s reagent. According to the method of Gordan and Weber (1951), when chlorine is added to indole acetic acid in the presence of a mineral acid, oxidation of IAA results in the production of a red color product, nitroso indole, which can be estimated by colorimetry, (Gordan and Weber Method, 1951).
The screening of the cultures for the production of IAA was done using Kovac’s reagent. When 3ml of the reagent is added to the broth, a cherry red color ring was formed at the junction of the two solutions indicating the presence of IAA in the solution. Salkowski’s reagent was used for the estimation of the IAA produced by the cultures.

3.3.3.2. Estimation of IAA

Standard IAA solution was prepared in the concentration of 500µg/ml. Different aliquots of the std. solution were prepared using distilled water. A blank with 1 ml of distilled water and 1 ml of the sample was prepared. 2 ml of Salkowski’s reagent was added in to each of the test aliquots and incubated at room temperature for 15 minutes. A stable red colour developed immediately which was read using a spectrophotometer (Elico. UV- VIS). Optical density of the tubes was measured at 530 nm, (Hao et al 2012)

3.3.4. Catalase Activity of Bacterial And Fungal Isolates

The estimation of catalase activity was based on the fact that its substrate H$_2$O$_2$ shows strong absorbance at 240 nm. Hence decomposition of H$_2$O$_2$ by catalase is accompanied by decrease in absorbance at this wavelength. This method is however suitable for only those enzyme preparations which contain negligible or low amounts of other interfering substances with significant absorbance at 240 nm, (Beers and Sizer 1952; Chance and Mahaey, 1955, Worthington Enzyme manual, 2011).

The selected bacterial cultures were grown in nutrient broth for 48 hours and the fungal cultures were grown in MRB broth for a period of 7 days and the activity of the catalase enzyme tested using spectrophotometric (Elico, UV-VIS) method at 240nm. The method is essentially described by Beers and Sizer (1952) in which the disappearance of peroxide in the solution is followed spectrophotometrically at 240 nm. One Unit decomposes one micromole of H$_2$O$_2$ per minute at 25°C and pH 7.0 under the specified conditions. 0.05 M Potassium phosphate, pH 7.0 0.059 M Hydrogen peroxide (30%) in 0.05 M potassium phosphate buffer at pH 7.0 was used as the reagent. Immediately prior to use dilute the enzyme in 0.05 M phosphate buffer, pH 7.0 to obtain a rate of 0.03-0.07 ΔA/min.
\[
\text{Mg enzyme mL}^{-1} = A_{240} \times 0.667 \quad \text{Eqn. no.3. 3.5.1.}
\]

The OD values were recorded and the time taken for the OD to reduce by 0.5 noted down. The activity of catalase calculated for each of the cultures, using the formula,

\[
\text{Enzyme activity (Units/mg)}
\]

\[
\frac{\Delta A_{240}\text{min} \times 1000}{43.6 \times \text{mg enzyme/ml reaction mixture}}
\]

\[
\text{Eqn. no. 3.3.5.2.}
\]

3.3.5. Biocontrol Studies With Bacterial And Fungal Isolates Of Rhizosphere Of Black Pepper.

3.3.5.1. Biocontrol of pathogens by bacterial isolates against *Fusarium equisetius* and *Mucor sp.*

Different bacterial isolates maintained on nutrient agar were chosen and tested against one species of *Fusarium, F. equisetius* and *Mucor sp.* for their antagonistic and inhibitory effects. (Skidmore and Dickinson, 1976; Chaiharn et al 2009).

Antagonistic effects of bacterial isolates possessing the PGPR traits were further tested. The bacterial isolates were inoculated along with the fungal pathogens on Sabaroud’s dextrose agar using dual culture technique for streaking; 24 hour old bacterial cultures of the isolates were streaked at 5 cms distance from the fungal pathogens with point inoculation method. The plates were inoculated for five days at 25°C and then were observed for inhibition of growth of fungi by the bacterial isolates. The zone of inhibition of growth was calculated as the distance between the edges of the colonies of the two organisms. The zone of inhibition was recorded using the formula:

\[
\text{Inhibition (\%)} = \frac{(C-T)}{(C)} \times 100, \quad \text{Eqn. no.3.3.6.1}
\]
where “C” is the maximum growth of the fungal mycelia under control conditions and T is fungal mycelia growth in dual culture.

3.3.5.2. Biocontrol Of Pathogens By Aspergillus Species Against Fusarium Equiseticus And Mucor Sp.

Different isolates of Aspergillus and one species of Fusarium, F. equiseticus and Mucor were selected for studying the antagonistic effect of species of Aspergillus against the pathogens. All the cultures were maintained on Sabaroud’s dextrose agar slants at 4 °C. Antagonistic properties of siderophore producing fungi, Aspergillus species were tested against these pathogenic fungi on SDA plates using a dual culture technique 34. Five day old cultures of exponentially grown cultures of Aspergillus species were streaked 5 cm juxtaposed from the streak of the pathogen. The plates were incubated for 5 days at 28 C. The zone of inhibition of growth was calculated as the distance between the edges of the colonies of the two organisms. The zone of inhibition was recorded using the formula:

\[
\text{Inhibition (\%) = \frac{(C-T)}{(C)} \times 100,} \quad \text{Eqn. no.3.3.6.2}
\]

where “C” is the maximum growth of the fungal mycelia under control conditions and T is fungal mycelia growth in dual culture. The organisms F. equiseticus and A.niger were identified up to molecular level with the help of genomic DNA sequencing.

3.4. ANTIMICROBIAL PROPERTIES OF PEPPER EXTRACTS AND PEPPER OIL

4.1. Preparation of pepper extract:

Alcoholic extract, acetone extract of pepper sample obtained from the growing regions of pepper from which the soil samples were obtained were used in the preparation of the extracts using alcohol and acetone, and commercial pepper oil were used in the antimicrobial analysis against different bacterial and fungal isolates. The extracts were prepared in the following methods.


15 g of ground pepper powder was placed in a 250 ml capacity round bottomed flask to which 150 ml of 95% ethanolamine and a few boiling chips were added. The flask was
heated at reflux for 2 hours. The mixture was then filtered by suction filtration and the filtrate was concentrated using vacuum evaporation to 10 ml. Then to this mixture 10 ml of KOH was added and a few drops of distilled water were added drop wise until a yellow precipitate formed. The mixture was made to stand for 4-5 days at room temperature and the refrigerated before using it.

3.4.1.2. Acetone extract

The above alcoholic extract of pepper was made up to 50 ml with 95% ethanol and 15-20 ml of acetone was added to recrystallize the piperine in the extract, (Epstein et al1993; Lin et al 2001).

3.4.1.3. Commercially available pepper oil

This was obtained from local perfumery factory Satyanarayana and Co., Bangalore.

3.4.2. HPLC of the pepper extracts.

HPLC was done with the alcoholic extract, acetone extract and the commercial pepper oil to determine the piperine content in them. Extracts were prepared in the laboratory according to the protocol mentioned above.

Using a 22.4 Um filter, a syringe and the needle, the sample was drawn into the syringe. The HPLC graphing machine was put on with the marker on the paper. After hearing the beep by the HPLC machine, the sample taken in the syringe was introduced into the port -6 of the HPLC machine and the machine was put on. The mark was made on the chart recorder where the injection was done. The computer was adjusted to read the chart. The HPLC machine was run for five minutes before it showed the peaks. When the peaks were through, the peak liquid was collected by using a 10 ml test tube with a lid over it placed under the tube meant for waste container, (Woods et al 1988).

In the graph window in the computer, the print button was clicked to obtain a graph. Graph was done for different runs of blank, standard piperine and the test sample.
3.4.3. Antibacterial spectrum using well diffusion method

3.4.3.1. Agar Well diffusion method

Bacterial strains from the rhizospheric soil of pepper were isolated on Nutrient agar media. Different isolates of bacterial genera were chosen for testing antibacterial activity. The stock cultures were prepared in sterile nutrient broth and the OD of the cultures was set at 0.05 at 660 nm. The nutrient broth cultures of the isolates were swab inoculated on to nutrient agar plates. Wells of 10 mm diameter were prepared with the help of sterile cork borer. Using a micropipette 30µl of different concentrations of pepper oil -100%, 75% and 50% dilutions prepared in Tween 80 solution were placed in the 10 mm well in the centre of sterile Nutrient agar plates, (National Committee for Clinical Laboratory Standards, 2000 NCCLS)

Pepper alcoholic extract and pepper acetone extract in undiluted form were placed in volumes of 30 µl in the wells in different plates following the above procedure as done for the pepper oil.

The plates were incubated in upright position at 28°C for 24-48 hours. The diameter of zone of inhibition was recorded.

3.4.3.2. Antifungal Activity Of Pepper Extracts And Pepper Oil

Different isolates of fungi obtained from the rhizospheres of the pepper plants were selected for the study of antifungal activity. The fungal cultures were maintained on Sabaroud’s dextrose agar slants. Spore suspensions prepared in sterile broth adjusted to OD 0.05 and were inoculated into sterile MRBA plates. Well of 10 mm diameter was prepared and the different dilutions of pepper oil and the pepper extracts in volumes of 30 µl were introduced into the wells of the respective plates in the same way as was done with the bacterial cultures. The plates were incubated at 28°C for 3 – 5 days. The zone of inhibition was recorded as diameter in millimetre (mm), (Ghai et al 2007, Sadeghi-Nejad, 2010).

3.4.3. To Detect The MIC Of Pepper Extracts And Pepper Oil On Bacterial And Fungal Isolates

3.4.3.1. MIC of pepper extracts and pepper oil on bacteria

Agar dilution method
The agar dilution method of detecting the minimum inhibitory concentration (MIC) of the bacterial isolates approved by National Committee for Clinical Laboratory Standards, 2000 (NCCLS) was used with the following modification, (Reddy et al 2007; Bobbarala et al. 2009).

Meuller Hinton agar was prepared and sterilized at 121 °C. The medium was poured into sterilized plates and allowed to solidify. Pepper alcoholic extract, acetone extract and pepper oil dilutions of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% were incorporated into the agar after sterilizing the Meuller Hinton agar media to enhance solubility. The plates were dried at 35°C for 30 minutes prior to inoculation. Spot inoculations of the cultures were done using the grid plate method with 2 µl of the 24 hour broth culture and plates were incubated at 28°C for 24 hours. MIC was determined after 24 hours as lowest dilution of the extract or the pepper oil inhibiting visible growth of each organism on the agar plate.

3.4.3.2. MIC of fungi

**Broth dilution method**

Sterile Sabaroud’s dextrose broth was prepared and 0.5 ml of broth in each of the tubes was inoculated with 0.5 ml of spore suspension of the selected fungal isolates. The spore suspensions of the six fungal isolates were uniformly set to give 0.05 OD value at 520 nm. Six different isolates of fungi that were used in the determination of antifungal activity were inoculated here for testing the MIC of the pepper extracts-ethanol extract and acetone extract and the pepper oil. Pepper alcoholic extract, acetone extract and pepper oil dilutions of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56% were incorporated into the tubes and mixed well. The tubes were then incubated at 28°C for 5 days. The MIC was observed as the lowest dilution of the pepper extract or the oil able to inhibit the growth of the fungal isolate in the broth, (Llop et al. 2000; Kummee et al. 2008; Bobbarala et al. 2009).
3.5. ANTIOXIDANT ACTIVITY OF THE PEPPER EXTRACTS

3.5.1. DPPH Free Radical Scavenging Assay And Total Phenolic Content Estimation Pepper Extracts

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm, (Gudddarangavvanahally et al 2004; McDonald et al 2001)

The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.8125 µg/ml. The plates were incubated at 37°C for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader.

3.5.2. Determination of total phenol content

3.5.2.1. Preparation of standard curve:

The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg/ml-1 solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g–1 of dry mass), which is a common reference compound.

The extract samples (0.5 ml of different dilutions) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na2CO3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetry at 765 nm.

3.5.2. Scavenging of nitric oxide radical by pepper extracts

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be
estimated by the use of modified Griess Ilosvay reaction. In the present investigation, Griess Ilosvay reagent is modified by using Naphthyl ethylene diamine dihydrochloride (NEDD) (0.1% w/v) instead of 1-naphthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm, (Guddadarangavvanahally et al 2004).

The sample solution was prepared as described in DPPH assay.

Weighed accurately 10 mg of ascorbic acid and rutin and dissolved in 1 ml of DMSO separately. From these solutions, serial dilutions were made to obtain lower concentrations using DMSO.

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and extract in DMSO at various concentrations or standard was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance of these solutions was measured at 540 nm.

3.5.3. Superoxide anion radical scavenging assay

The superoxide anion radical scavenging activity for all the plant extracts was assessed by using the methods of Liu et al. with a modification. The nonenzymatic phenazine methosulfate and nicotinamide adenine dinucleotide (PMS and NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan, (Jaishree V, 2008)

Superoxide radicals were generated in 3.5 ml of Tris-HCl buffer (16 μM, pH 8.0), which contained 78 μM b-nicotinamide adenine dinucleotide (reduced form, NADH), 50 μM nitroblue tetrazolium salt (NBT), 10 μM phenazin methosulfate (PMS), and test samples in buffer solution/ DMSO. The color reaction of superoxide radicals
and NBT was observed at OD 560 nm. Ascorbic acid at 10 μg/ml was used as a positive control.