CHAPTER 3
MATERIALS AND METHODS

The present investigation was carried out at Post graduate Department of Botany P.V.P. College, Pravaranagar (Loni). Dist. Ahmednagar (M.S.) during 2006-2013.

3.1 Climatic conditions:-

Geographically the campus of P.V.P. College Loni (Pravaranagar) is situated between 19 degree 47 and 19 degree 57 North latitude and between 74 ° 32 and 70 ° 47 longitudes. The altitude varies from 495 m to 569 m above the mean sea level. This tract is in the eastern side of western ghat and falls under rain shadow area.

Climatically, the area falls in semi arid tropics with average annual rain fall 55 cm, which is erratic, and unevenly distributed in 15 to 45 rainy days in different months. Out of the total annual rainfall, above 80% receives from south west monsoon from June to September.

The annual mean maximum temperature is 37.9 degree Celsius with a range between 33°c to 43°c and annual mean temperature is 17.2°c with a range between 3 ° Celsius to 23°c. The mean relative humidity ranges between 35 to 59 percent respectively. Agro climatically, the area falls in scarcity zone of Maharashtra.

3.2 Collection of soil samples:-

Soil samples of ten different wheat fields were collected from different sites in Pravaranagar and Shrirampur area. Soil samples were air dried, cleaned and sieved to fine particles.

3.3 Isolation of *Azospirillum spp.*:-

1 g soil samples were inoculated in sterile Nfb medium and incubated for 8 days at 25°c ± 2. *Azospirillum spp.* were identified with microscopic observations on pigment basis and purified by transfer and retransfer on the same medium.
3.4 Gram staining: - (Dube and Maheshwari 2005).

Bacterial smears were prepared on glass slides. Smears were fixed to slides by gently heating them on spirit lamp. The smears were rinsed with crystal violet solution for 1 min then rinsed with water and then flooded with 1 gram iodine mordant for 1 min. Again smears were rinsed in running tap water for 2 sec and films were blotted to dry. The smears were then flooded with 95% ethanol for 30 sec. Again they were rinsed with water and counter stained with safranin (0.25%) for 10 sec. The films were dried with blotter paper and observed under 100 X magnification.

3.4.1 Shape:-

Selected strains were grown on Nfb semi liquid medium for two days and one drop of each isolate was placed on glass slide, stained with carbol fuschin and observed under 40 x magnifications (Beijerinck, 1901; Tchan and New 1984).

3.4.2 Size: (Dube and Maheshwari 2005).

The measurements of isolated cells were done with the help of stage micrometer. One drop of five days old culture was placed on glass slide and stained with carbol fuschin and observed under 40 x magnifications.

3.4.3 Motility:-

A drop of sample was placed in the center of cover slip. Cover slip was inverted over the wall of well of cavity slide in such a way that the drop was not in contact with side wall of well. Vaseline was applied on the edge of cover slip to avoid drying. The slide was observed under 100 x magnifications.

3.4.4 Staining of capsules: - (Dube and Maheshwari 2005).

A loopful *Azospirillum spp.* broth was mixed with small drop of India ink. This bacterial suspension was spread over glass slide and smear was air dried. Smear was fixed to slide by gently heating on spirit lamp. The smear was stained with crystal violet for 1 min. rinsed with water and cover slip was placed. The smear was pressed with a filter paper until the ink was sepiya colour beneath the cover of glass. The slide was observed under 100 x magnifications.
3.4.5. Staining of cyst:- (Dube and Maheshwari 2005).

*Azospirillum spp.* culture broth of 8 days was immersed in a stains reagent. A drop of bacterial suspension was spread on a clean glass slide and covered with cover slip. The slide was observed under 100 x magnifications.

3.4.6. Staining of Poly-β- hydroxybutyrate (PHB) granules: (Dube and Maheshwari 2005).

A drop of 8 days old *Azospirillum spp.* culture broth was placed on slide. Bacterial smear was prepared and fixed to slide by gently heating on spirit lamp. The slide was immersed in 0.31% Sudan black B solution for 5 to 15 min. Then the slide was drained, dried and immersed in xylene. Again slide was kept out, dried and counter stained with 0.5% aqueous safranin. The slide was rinsed in water to remove excess stain. The slide was observed under 100x magnification microscope.

3.5  Biochemical activity:-

3.5.1. Detection of catalase activity:- (Dube and Maheshwari 2005)

A drop of 8 days old culture broth of *Azospirillum spp.* was placed on glass slide. Few drops of H2O2 (30%) diluted to 1:10 were added over the culture. After 20 seconds the bubbles were observed positive activity of catalase was recorded.

3.5.2. Thermal death point:- (Dube and Maheshwari 2005).

Test tubes of uniform thickness were selected. In each test tube 10ml Nfb medium was added and sterilized in an autoclave at 15 lb pressure. The sterile culture medium tubes were inoculated with 1 ml four days old culture of each isolate. Inoculated tubes were subjected to different temperature viz, 10, 20,30,40,50 and 60$^\circ$C for 10 min. in hot water bath. After heat treatment the culture tubes were incubated for 5 days at room temperature (25 ± 2$^\circ$C). The observations regarding presence and absence of growth were recorded.

3.5.3 C1 Cellulase activity:- (Sadashivam and Manikam 2004).

0.5 ml 8 days old culture broth was taken in test tube. A dry Whatman No. 1 filter paper disc of 7 mm diameter and 32 mg weight was inoculated in culture broth and
mixture was incubated for 50 min at 50°C in water bath. After incubation 0.5 ml DNSA reagent was added. Then the mixture was again heated in boiling water bath for 5 min. After heating the tubes were taken out and 1 ml potassium sodium tartarate solution was added. Then tubes were allowed to cool to room temperature and final volume of mixture was made to 5 ml with distilled water and absorbance was recorded at 540 nm. Standard graph of glucose 50 µg to 1000 µg/ml was plotted and conc. of glucose in culture broth was estimated with the help of standard graph.

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C_1 \text{ Cellulose activity} = \frac{\text{Amount of glucose liberate (µm/ml/min.)}}{\text{Molecular weight of glucose}} \times \text{Incubation time}
\]

**3.5.4 Cx Cellulase activity (Endo- β- 1, 4 glucanase activity):** (Mukadam 1982 and Sujata et al; 2006)

This activity was tested by Ostwald’s viscometer. 5 ml. 0.5% carboxymethyl cellulose (CMC) solution 0.2 ml citrate phosphate buffers (pH 5.8) and 3 ml culture broth were taken in 100 ml beaker. This 10 ml enzyme substrate mixture was poured in a clean viscometer and viscosity was measured at 0, 5, 10, 15, 20 and 30 min. at 5 min time interval. During the incubation period viscometer was kept at 30°C constant temp.

**3.5.5 Pectinase (Poly 1, 4 α D galacturonide glycanohydrolase) activity:** (Papdiwal 1982 and Sujata et al; 2006).

5 ml 2% pectin substrate, 3ml culture broth of 8 days old and 2ml distilled water were taken in 100 ml beaker and pH was adjusted to 5.0. The mixture was poured in Ostwald’s viscometer and viscosity was measured at 0, 5, 10, 15, 20, 30 min of 5 min time interval. During incubation period the viscometer was kept at 25°C constant temperature.

**3.6 Biocontrol activity:**

**3.6.1 Isolation of seed borne fungi:** (Tabassam et al; 2003).

Wheat varieties like HD2189, 496, LOK-1, Trimbak and 2123 were selected for isolation of seed borne fungi. For isolation of seed borne fungi seeds were treated with 0.1% mercuric chloride solution for 3 min and serially washed with 3 times with sterile distilled water. After washing seeds were blotted with sterile blotter. Ten seeds of each
variety in triplicates were placed on PDA plates and were incubated for 7 days. For external seed borne fungi 10 seeds of each variety without surface sterilization in triplicates were placed at equidistant on PDA medium in each plate and plates were incubated for 7 days at 25°C. After 7 days fungal forms were isolated and identified with standard literature.

3.6.2 Streak method: - (Nirmala and Singh; 2002)

The isolates were tested against pathogens like Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Penicillium chrysogenum. Eight days old culture plates of these isolates were used as inoculums. These cultures were inoculated against each Azospirillum spp. on solidified PDA plates which were underside marked into two equal halves. Azospirillum inoculated by streak method however fungal inoculums were inoculated with disc of 6 mm. Inoculated plates were incubated at 25 ± 2°C in an incubator. The colony diameter of fungal growth was measured after 8 days. Biocontrol efficiency was estimated by calculating percent decrease in colony diameter using following formula.

Percent decrease in the colony diameter = \( \frac{B - A}{A} \times 100 \)

Where,

B = Colony diameter away from Azospirillum spp.
A = Colony facing towards Azospirillum spp.

3.7 Estimation of growth regulators (Tien et al; 1979)

Growth regulators such as auxin (IAA, NAA), cytokinin, kinetin, gibberellic acid were estimated by HPLC.

3.8. Estimation of chlorophyll a, b and total chlorophyll: - (Arnon; 1949).

The leaves were washed to remove the dust particles, blotted to dry partially and cut down in small pieces. 0.2gm leaf pieces were weighed and homogenized in the mortar and pestle with 10 ml 80% cold acetone. The homogenate was centrifuged at 5000 rpm
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for 5 minutes and the supernatant was transferred to 25 ml volumetric flask. The residue was re- extracted with cold 80% acetone. The supernatant was mixed and the final volume of 25 ml was made cold with 80% acetone. Chlrophyll ‘a’, chlrophyll ‘b’, and total chlrophyll was estimated by measuring the absorbances at 645 and 663nm. Chlrophyll content was expressed in mg per g of fresh weight of leaves by using the formula.

Mg. chlrophyll ‘a’/gm tissue = 12.7 (A663) - 2.69 (A645) x ……V……... 

1000 x w

Mg. Chlrophyll ‘b’/gm tissue = 22.9 (A645) - 4.68 (A663) x ……V……... 

1000 x w

Mg total chlrophyll/gm tissue = 20.2(A645) + 8.02 (A663) x ……V……... 

1000 x w

Where,
A = absorbance at specific wavelength
B = Volume of chlrophyll
C = Fresh weight of tissue

3.9 Nitrogen content of straw and spike:-(Jackson;1971).

The nitrogen content in the dried straw and spike husk was estimated by Micro-Kjeldahl’s method. The dried straw and spike husk were powdered in a grinder. 0.5 gm powder was taken in a digestion flask along with 1gm of catalyst mixture, 5 ml conc. H2SO4 and 5 ml of H2O2. The digestion flask was heated on a heating plate till the solution become clear. Discolored solution was diluted up to 50 ml with distilled water and used as acid extract for estimation of nitrogen content.10 ml of 4% boric acid solution was pipetted in to 50 ml beaker and 3 drops of mixed indicator was added into beaker. Then this beaker was kept under the condenser and the tip of condenser was dipped in the boric acid solution, then 10 ml digest was poured in the distillation chamber followed by 5 ml of 50% NaOH. This was titrated against standard 0.02N Hcl till the colour changed green to pink and persisted for 30 sec. Nitrogen was estimated from 1 ml of 0.02N Hcl required and it was expressed as percent nitrogen.
3.10 Phosphorus content of straw and spike:-(Jackson;1971)

The phosphorus content in the dried straw and spike husk was estimated by Vanado-molybdate method. The digested samples used for estimation of nitrogen content were used for estimation of phosphorus content. 10 ml of digest was taken in 50 ml capacity volumetric flask and 10 ml Vanado-molybdate reagent was added. Final volume was made to 50 ml with distilled water. The contents were mixed and allowed to stand for 30 minutes. The colour intensity was measured on spectronic 20 at 470 nm.

3.11 Total nitrogen and phosphorus uptake:-

Total nitrogen and phosphorus uptake of straw and spike was estimated by following formula.

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\frac{\text{Nitrogen/Phosphorus content} \times \text{Dry matter}}{\text{uptake} (\%)} = \frac{\% \text{N/P content of straw} + \% \text{N/P content of spike}/2}{100}
\]

\[
\frac{\text{Dry matter of spike} + \text{Dry matter of spike}/2}{100}
\]

3.12 Mutation: - (Gupta; 1964 Bose and Venkatraman; 1962).

Culture of Azospirillum spp. was mixed for 4 days used for mutation treatment. A 20 ml culture was poured in separate sterilized petriplates in laminar air flow in aseptic condition. The plates were exposed to UV radiation for 20 min. 5ml treated culture broth was taken for respective cultural plates after 5 minutes interval and poured in sterile Nfb semi liquid medium and incubated for 7 days. Mutants were identified on pigment basis and their effect was studied by seed dressing in the pot experiment.

3.13 Fertilizer dose:

As per recommended dose of NPK (40:50:100 kg/ha.) chemical fertilizers like urea and single super phosphate, were worked out as per treatment and plot size.
3.14 Field study:

Field experiment was carried out to study the effect of *Azospirillum sp.* alone and in combination with graded levels of nitrogen on growth and yield of wheat.

3.14.1 Experiment design:

The experimental plot was laid down in Randomized Block Design (RBD) with 8 treatments and 3 replicates. The plot size was 2.5x2.25 sq. feet. Wheat variety Lok-1 was selected for field study. Seeds were sown with a row spacing 22.5 cm at the rate of 51 gm per plot. After sowing light irrigation was given. Subsequent irrigations during crop period were given as per crop requirement. The observations of the plant growth parameters viz, plant height, number of tillers per plant, root length, number of leaves per tiller, leaf area, fresh weight, dry weight of each tiller, spike length and spike weight were recorded at 30, 60 and 90 days.

3.14.2 Harvesting of crop:

Crop was harvested after 120 days after sowing. The grain and straw yield of wheat was recorded.

3.14.3 Total dry matter:

Plant samples were collected from each treatment at 30, 60 and 90 days after sowing. Samples were rolled in brown papers, kept in oven at 60°C for 12 hrs for drying and dry weight was recorded.

3.15 Varietal response: (Dube 2002)

Five local varieties HD2189, Mohan wonder, 496, Trimbak and 2123 were selected for field experiment. Seeds were sterilized with 0.1% HgCl₂ for 5 min and serially washed with sterile distilled water. After washing seeds were dressed with selected formulations of culture broth and dried in shade. Experimental plots were laid down in randomized block design with 2 treatments and 3 replicates and sowing was done at 22.5cm row spacing. The effect of selective formulation was studied with different parameters viz % germination, plant height, number of tillers, root length,
number of leaves, leaf area, chlorophyll content, dry weight, fresh weight, spike length, spike weight, nitrogen and phosphorus uptake.

3.16 Statistical analysis:

The data obtained from field experiments were subjected to statistical analysis by using standard methods (Panse and Sukhatme 1978). The standard error from the treatments and the critical difference at the 5 % level of significance were worked out and used for the comparison between treatments.