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## RESEARCH ARTICLE

**RANDOMIZED FIELD TRIALS OF AZOSPIRILLUM LIPOFERUM WITH ENHANCED PROPERTIES OF DESICCATION TOLERANCE AND PLANT GROWTH PROMOTING TRAITS ON DROUGHT PRONE PADDY FIELDS OF DHARMAPURI**

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**Abstract:**

Azospirillum, are free living plant growth promoting rhizobacteria. Soil samples were collected from Dharmapuri, drought prone field. They were gram negative bacilli, motile, vibrioid in shape. They formed subsurface pellicle in Azospirillum Isolation broth. Out of 13 isolates, Asp 5, Asp 6, Asp 7, Asp 9 and Asp 13 formed distinct subsurface pellicle formation. Two isolates of Azospirillum lipoferum, Asp 7 and Asp 9 capable of producing high amounts of Indole Acetic Acid (IAA) and Exopolysaccharide (EPS) were further characterized for plant growth promoting (PGP) traits like ammonification, HCN production and their tolerance to desiccation of which Asp 9 showed greater PGP traits. Asp 7 and Asp 9 were taken for pot cultivation. Based on shoot and root length parameters, Asp 9 was again found to be efficient than Asp 7. The best strain Asp 9 was then subjected to field study using random block design (RBD). Different treatments were done to the paddy seeds and their growth was monitored during four stages of growth. Soil moisture and pH were constantly monitored throughout the study. The study revealed good growth, utilization of nutrients and considerable increase in grain yield for the strain supplemented with NPK.

**Key Words:** PGPR, RBD

**Introduction**

Modern agriculture depends on Biotechnology to improve bionoculants and in turn contributes to soil amendments. The increased use of various biological processes in the soil will decisively contribute to make agriculture more productive with less harm to the environment. Bacteria of the genus *Azospirillum* are widely distributed in soil and associated with the roots of forage grasses, cereals and non gramineous plants (Bashan and Holguin, 1997). *Azospirillum* spp are widely distributed soil nitrogen fixing bacteria that play an important role in the promotion of plant growth (Steenhoudt and Vandeleiden, 2000).

*Azospirillum* spp are diazotrophs that fix nitrogen as free-living organisms (Ramos *et al.*, 2002). The bacterium obtains the nutrients necessary to replicate and survive from the plant and the plant benefits from the association with the bacterium because the bacteria produces hormones that increase the volume of the roots bulk thereby leading to improved plant nutrition. The ability of *Azospirillum* to fix the atmospheric nitrogen in to the soil and make it available to the plants is an important trait in sustainable farming for increasing crop yield. Application of *Azospirillum* significantly augmented maize growth parameters. Plant inoculation with *Azospirillum* promoted greater uptake of NO<sub>3</sub>, K<sup>+</sup> and H<sub>2</sub>PO<sub>4</sub> in corn, sorghum and wheat (Zavalin *et al.*, 1998).

The versatile metabolism of *Azospirillum* is well suited to competition and to the harsh conditions which exist in the rhizosphere and in soil. *Azospirillum* fixes nitrogen, reduces nitrate, can form cyst-like structures in unfavorable conditions, produces plant hormones, vitamins and siderophores, and efficiently anchors itself to roots with the help of fibrillar material (Patten and Glick, 1996; Bashan and Holguin, 1997). Besides being a crop rhizosphere bacterium, *Azospirillum* is a natural inhabitant of many non-graminae plants and can sometimes promote their growth (Bashan and Holguin, 1997). Briefly, *Azospirillum* can be found in a wide range of habitats in association with many different types of plants.

Various authors have proposed the following direct promoting mechanisms in addition to biological nitrogen fixation: (a) production of phytohormones such as indole 3-acetic acid (IAA), gibberellic acid (GA3) and ethylene (Bashan *et al.*, 2004), zeatin ( Tien *et al.*, 1979), and abscisic acid (ABA). (b) production of plant growth regulatory substances such as polyamines (Thuler *et al.*, 2003), particularly cadaverine (CAD), which may be correlated with root growth promotion (Niemi *et al.*, 2002) and osmotic stress response in plants (Aziz *et al.*, 1997). (c) phosphate solubilization (Seshadri *et al.*, 2000). (d) siderophore production (Saxena *et al.*, 1986).

Today, the most common explanation for the effect of rhizobacteria on plants is based on the production of phytohormones that alter plant metabolism and morphology, leading to improved mineral and water absorption. The Additive Hypothesis of Bashan and Levanony (1990) proposes that multiple mechanisms operate simultaneously or in succession to promote plant growth.

## Material and Methods

### I. Collection of soil samples

Soil samples were collected from the rhizosphere region of five different paddy fields of Dharmapuri, in the regions of Karimangalam, Toppur, Makkanur, Pennagaram. Samples were collected in polythene bags from the selected sites at a depth of 10-15 cm. The samples were then immediately transported to lab for further processing.

### II. Isolation

*Azospirillum* was isolated from each sample by serial dilution and spread plate method. *Azospirillum* isolation agar was used as selective medium for *Azospirillum*.

### III. Subsurface pellicle formation

- The loopful of culture was taken and inoculated on to semi solid nitrogen free basal medium.
- The medium was incubated at 32°C for 48 hours and checked for sub surface pellicle formation.

### IV. Morphological and Biochemical characterization

Gram staining, motility and biochemical tests such as indole, methyl red, voges-proskauer, citrate, urease, nitrate, catalase, oxidase, utilization of different carbon sources, starch hydrolysis, gelatin hydrolysis, phosphate solubilization were performed.

### V. Characterization for plant growth promoting traits

#### a) Production of ammonia (Cappuccino and Sherman, 1992)

- 10 ml of peptone water was taken in test tube.
- A loopful of culture was inoculated into it.
- The tube was incubated for 48-72 hours at room temperature.
- 0.5 ml of Nessler's reagent was added.

#### b) Production of HCN (Lorck, 1948)

- Nutrient agar was prepared and amended with 4.4g glycine per litre.
- A loopful of culture was inoculated into modified medium.
- A Whatman filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate.
- Plate was sealed with parafilm and incubated at room temperature for 4 days.

### VI. Screening for desiccation tolerance (Kumaran and Elango, 2013)

- Isolates were transferred into sterile 1.5 ml eppendorf micro capillary tubes and the tubes were kept open in a sterile petriplate.
- The petriplate with the tubes were then placed in incubator at 37° C .
- After one week incubation, the dried cells from the tubes were washed with 1 ml of sterile distilled water with vigorous agitation for the complete removal of the bacterial cells.
- Bacterial viability was determined intermittently by plating on yeast extract glucose agar.

### VII. Bacterization

**a) Mass cultivation of *Azospirillum***

*Azospirillum* was transferred to a flask containing 1000 ml of sterile *Azospirillum* isolation broth. The flask was incubated in a rotary shaker at 32°C for 72-80 hours.

**b) Procurement of host seeds**

The seeds of paddy (ADT 36) were bought from Tamilnadu Horticulture Department, Anna Nagar, Chennai.

**c) Seed dressing**

The seeds were surface sterilized with 0.1% mercuric chloride solution for one minute. Then, it was washed with sterile distilled water several times and then the seeds were aseptically transferred to a flask containing *Azospirillum* and the flask was again incubated in a rotary shaker for 4 hours.

**d) Drying**

After the incubation, the seeds were dried in shade at room temperature.

**VIII. Pot cultivation**

The seeds were divided into 4 experimental designs.

- 1<sup>st</sup> set contains *Azospirillum* ( Asp 7) seed dressed.
- 2<sup>nd</sup> set contain *Azospirillum* ( Asp 9) seed dressed.
- 3<sup>rd</sup> set contain *Azospirillum* (commercially available) biofertilizers seed dressed.
- 4<sup>th</sup> set contain only seed (no bacteria).

They were sowed in 4 pots in duplicates and drought conditions were maintained.

**IX. Study of plant growth parameters**

Assessment of root and shoot length

For assessment of root and shoot length, paddy plants were randomly uprooted and washed with running tap water and the results were tabulated.

**X. Field study****a) Experimental field**

The isolate which showed best root and shoot length was taken for field study. Toppur was selected as field area in Dharmapuri district. The field had 895.56 mm rainfall, temperature of 38° C and 35% humidity during October to January. Its latitude and longitude are 11° 54' N and 78° 6' E.

**b) Mass production of the isolate**

*Azospirillum* was transferred to a flask containing 1000 ml of sterile *Azospirillum* isolation broth. The flask was incubated in a rotary shaker at 32°C for 72-80 hours. The OD value of the culture was spectrophotometrically measured and the cell number was adjusted to 10<sup>7</sup> CFU/ml.

### c) Carrier blending

Peat was used as the carrier material. The neutralized and sterilized carrier material was spread on a clean, sterile plastic tray and bacterial cultural ( $10^7$  CFU/ml) was mixed well manually to get a final concentration of  $10^6$  CFU/g of carrier.

### d) Field design

The experimental design was completely randomized block with three replications  $R_1$ ,  $R_2$  and  $R_3$ . The plot consisted of 6 lines of 25m long separated by 20cm. The different treatment combination is as follows.

T<sub>1</sub>. Asp,

T<sub>2</sub>. Azo + NP (75%) K (100%),

T<sub>3</sub>. Asp + NP (75%) K (100%),

T<sub>4</sub>. Asp +Azo,

T<sub>5</sub>.Asp + Azo + NP (75%) K (100%),

T<sub>6</sub>. CA Asp + CA Azo + NP (75%) K (100%),

T<sub>7</sub>. CA Asp + NP (75%) K (100%),

T<sub>8</sub>. CA Azo + NP (75%) K (100%),

T<sub>9</sub>. CA Azo+ CA Asp,

T<sub>10</sub>. NP (75%) K (100%) (Asp-Asp 9, Azo-Azotobacter, CA-Commercially Available)

Randomized block design

| R <sub>1</sub>  |                | R <sub>2</sub> |                 | R <sub>3</sub>  |                |
|-----------------|----------------|----------------|-----------------|-----------------|----------------|
| T <sub>4</sub>  | T <sub>9</sub> | T <sub>2</sub> | T <sub>8</sub>  | T <sub>4</sub>  | T <sub>7</sub> |
| T <sub>1</sub>  | T <sub>6</sub> | T <sub>5</sub> | T <sub>10</sub> | T <sub>1</sub>  | T <sub>9</sub> |
| T <sub>8</sub>  | T <sub>2</sub> | T <sub>3</sub> | T <sub>7</sub>  | T <sub>5</sub>  | T <sub>6</sub> |
| T <sub>10</sub> | T <sub>5</sub> | T <sub>9</sub> | T <sub>4</sub>  | T <sub>3</sub>  | T <sub>2</sub> |
| T <sub>7</sub>  | T <sub>3</sub> | T <sub>6</sub> | T <sub>1</sub>  | T <sub>10</sub> | T <sub>8</sub> |

## XI. Study of plant growth parameters

Assessment of root and shoot length

For assessment of root and shoot length, paddy plants were randomly uprooted during the four stages of growth such as active tillering, panicle initiation, flowering and harvesting.

## XII. Tests for soil parameters

### a) Soil moisture test (Gravimetric method)

Procedure

- 100g of soil sample was taken in a tray and kept in the oven.
- The same was kept at 105°C till it attains a constant weight for 24-36 hours.
- Then, the sample was brought to room temperature.
- The weight of the tray was taken. The loss in weight is equal to moisture contained in 100g soil sample.

Calculation

$$\text{Moisture (\%)} = \frac{\text{Loss in weight}}{\text{weight of tray} - \text{weight of soil}} \times 100$$

### b) Soil pH (Mc Keague, 1978)

Procedure

- 10g of soil sample was taken and dissolved in distilled water in a beaker.
- The soil suspension was stirred well and allowed to settle.
- The electrodes were immersed carefully into the soil suspension.
- The solution was stirred well before taking the reading.
- The functional switch was adjusted to the particular pH range and the pH was recorded.

## Results

*Azospirillum* spp are commonly used free living plant growth promoting rhizobacteria capable of affecting the yield of numerous plant spp of agronomic importance. This study aims at conducting randomized field trials of the bacterium in drought prone condition. The isolates formed subsurface in *Azospirillum* isolation broth. Dobereiner and Day (1976) reported micro-aerophilic growth in semisolid agar stagnant conditions were helpful for the inoculation of the organism. Since *Spirillum lipoferum* grows in a typical pellicle 1 to 4mm below the surface, this method was particularly useful for studying the substrates and growth conditions for nitrogen fixation.

The presence of ammonifiers in soil enhances nitrogen fixation. Two isolates of *Azospirillum lipoferum*, Asp 7 and Asp 9 were subjected to test for their ability to produce ammonia. Both the isolates were found to produce ammonia efficiently. Production of hydrogen cyanide (HCN) by bacteria makes it a biological control agent. The

isolates were tested for their ability to produce HCN. Both the isolates, Asp 7 and Asp 9 were capable of producing HCN.

Chart 1 shows the desiccation tolerance rate of isolates, Asp 7 and Asp 9. Both the isolates showed good number of colonies on day 1. Asp 7 had a 25% decrease in colony number on day 3 whereas Asp 9 had only a marginal decrease on day 5 and negligible growth on day 7 for Asp 7. But a sizeable number of colonies was observed on day 5 and day 7 for Asp 9 which states a better desiccation tolerance. The exopolysaccharide (EPS) of Asp 9 could be the major factor for its survival for a week.

Chart 2 and 3 shows the assessment of shoot and root length in pot experiment. Based on the shoot and root length parameters Asp 9 was selected as a better strain for field studies. Four stages namely active tillering, panicle initiation, flowering and harvesting were considered for the study. The increasing order of shoot length was, active tillering stage -  $T_{10} > T_6 > T_5 > T_9 > T_7$ ; panicle initiation and harvesting stage -  $T_{10} > T_6 > T_5 > T_9 > T_3$ ; flowering stage -  $T_{10} > T_6 > T_5 > T_9 > T_7$ . The increasing order of root length was, active tillering stage -  $T_{10} > T_6 > T_5 > T_9 > T_3$ ; panicle initiation stage -  $T_{10} > T_5 > T_6 > T_3 > T_9$ ; flowering stage -  $T_{10} > T_6 > T_9 > T_5 > T_3$ ; harvesting stage -  $T_{10} > T_6 > T_5 > T_9 > T_3$ .

Moisture was found to be 13% at the time of sowing. Though the moisture content declined during the stages of crop development, the isolate contributed to the growth as it was viable even in drought conditions. At the time of sowing, the pH of the soil was found to be 6.8. During the four stages of the crop, the pH considerably favoured the growth of the plant. The pH was neutral during flowering and harvesting stages. The study needs to be further extended on grain yield, quality parameters, strain improvement strategies and their molecular characterization needs further insights.

## Discussion

Drought is one of the major environmental stresses that limit the growth of plants and the production of crops (Shinozaki *et al.*, 2003). Desiccation affects microbial population structure (Ilyas *et al.*, 2008). Exposure to extreme environmental conditions as imposed during dry season alters soil microbial activity (Castro-Sowinski *et al.*, 2007). The introduction of beneficial bacteria can normalize and, in some cases, improve plant performance in stressful environments and thereby preserve or enhance yield (Bensalim *et al.*, 1998).

Four stages namely active tillering, panicle initiation, flowering and harvesting were considered for the study. Plant height was significantly increased with advancements in plant growth. The increasing order of shoot length for active tillering stage was  $T_{10} > T_6 > T_5 > T_9 > T_7$ .  $T_5$  (Asp + Azo + NP (75%) K (100%)) shared the third position which was next only to  $T_6$  (CA Asp + CA Azo + NP (75%) K (100%)). The increasing order of shoot length for panicle initiation stage was  $T_{10} > T_6 > T_5 > T_9 > T_3$ . At this stage,  $T_3$  though held last showed a better performance in shoot length when compared to active tillering. A similar data was obtained in harvesting stage as well. The increasing order of shoot length for flowering stage was similar to active tillering stage where  $T_{10}$  and  $T_6$  topped the list. Kavitha Mary Jackson and Ilamurugu (2013) in their field experiment obtained maximum plant height of 111 cm in  $T_9$  - NPK (100%) at harvesting stage followed by 94 cm in  $T_6$ - AM Fungi (Colonized root bits) + (Sand based inoculum) + Azophos + NP (75%) K (100%).

Root length was significantly increased with advancements in plant growth till flowering stage. During harvesting, root length decreased as the root nutrients were taken up by plant for maturation. The increasing order of root length for active tillering stage was  $T_{10} > T_6 > T_5 > T_9 > T_3$ .  $T_5$  (Asp + Azo + NP (75%) K (100%)) shared the third position which was next only to  $T_6$  (CA Asp + CA Azo + NP (75%) K (100%)). The increasing order of root length for panicle initiation stage was  $T_{10} > T_5 > T_6 > T_3 > T_9$ . At this stage,  $T_3$  showed a better performance than  $T_9$  when compared to active tillering. The increasing order of root length for flowering stage was  $T_{10} > T_6 > T_9 > T_5 > T_3$  where performance of  $T_3$  decreased. The increasing order of root length of harvesting stage was similar to active tillering stage where  $T_{10}$  and  $T_6$  topped the list. Kavitha Mary Jackson and Ilamurugu (2013) in their field experiment obtained maximum plant root length in  $T_9$  (NPK -100 %) irrespective of crop growth stages. It had 10.9 cm at tillering stage, 15.7 cm at panicle initiation stage, 22.4 cm at flowering stage and 18.3 cm at harvesting stage. Next highest value was recorded by combined inoculation of AM Fungi (Colonized root bits) + AM Fungi (Sand based inoculum) + Azophos ( $T_6$ ) with 10.3 cm at tillering stage, 14.3 cm at panicle initiation stage, 19.6 cm at flowering stage and 15.1 cm at harvesting stage.

Tables

Table I

| TESTS           | Asp 7 | Asp 9 |
|-----------------|-------|-------|
| Gram staining   | -     | -     |
| Motility        | +     | +     |
| Indole          | +     | +     |
| Methyl Red      | +     | +     |
| Voges Proskauer | -     | -     |
| Citrate         | -     | -     |
| Urease          | -     | -     |
| Nitrate         | +     | +     |
| Catalase        | +     | +     |

Table II

| TESTS                    | Asp 7 | Asp 9 |
|--------------------------|-------|-------|
| Oxidase                  | +     | +     |
| Dextrose                 | +     | +     |
| Lactose                  | +     | +     |
| Sucrose                  | -     | -     |
| Mannitol                 | +     | +     |
| Starch hydrolysis        | +     | +     |
| Gelatin hydrolysis       | +     | +     |
| Phosphate solubilization | +     | +     |

Chart I

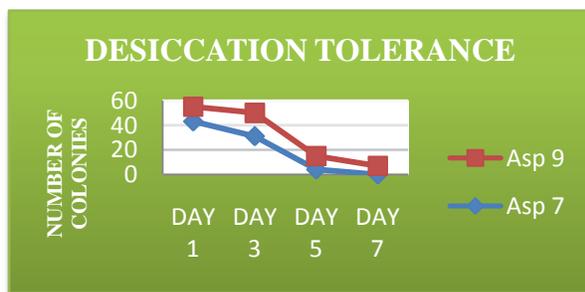


Chart II

Chart III

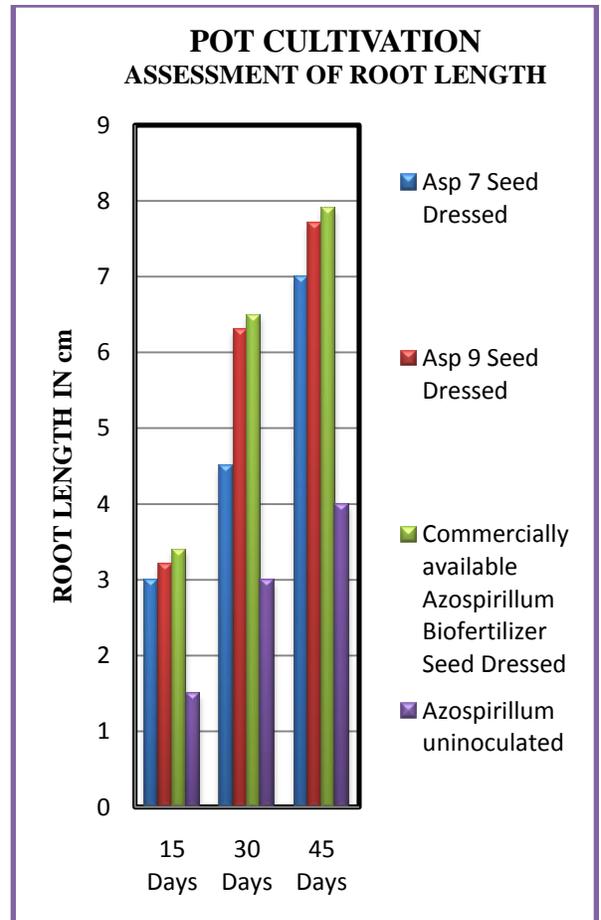
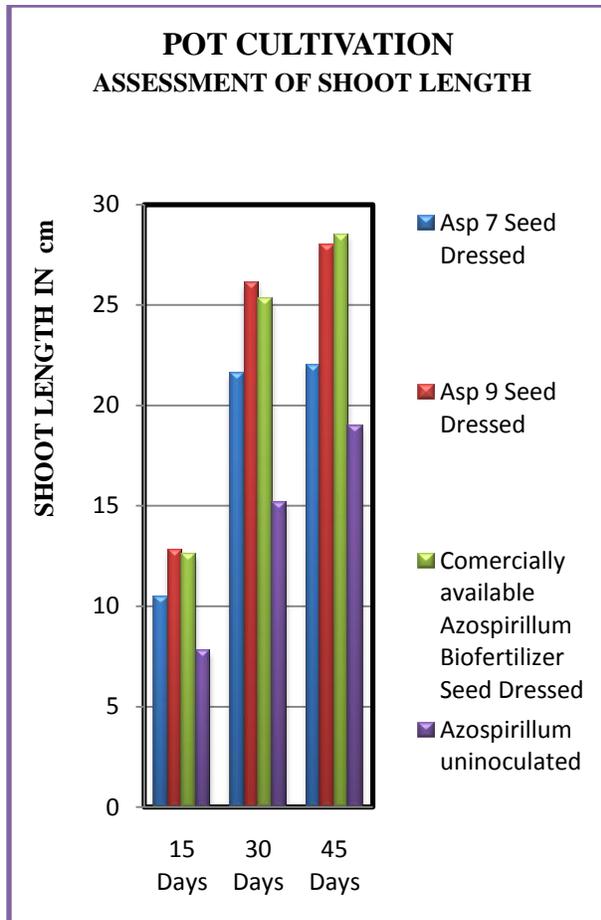


Chart IV

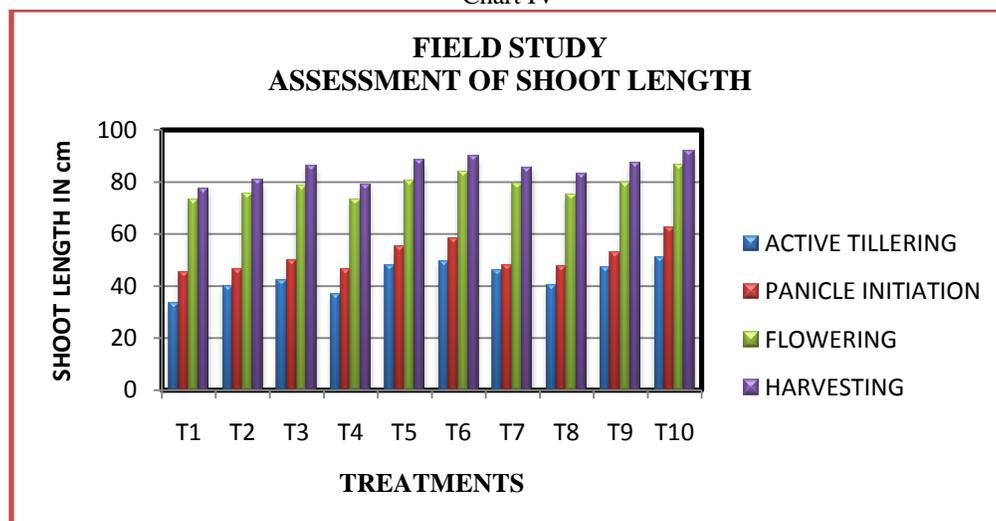


Chart V

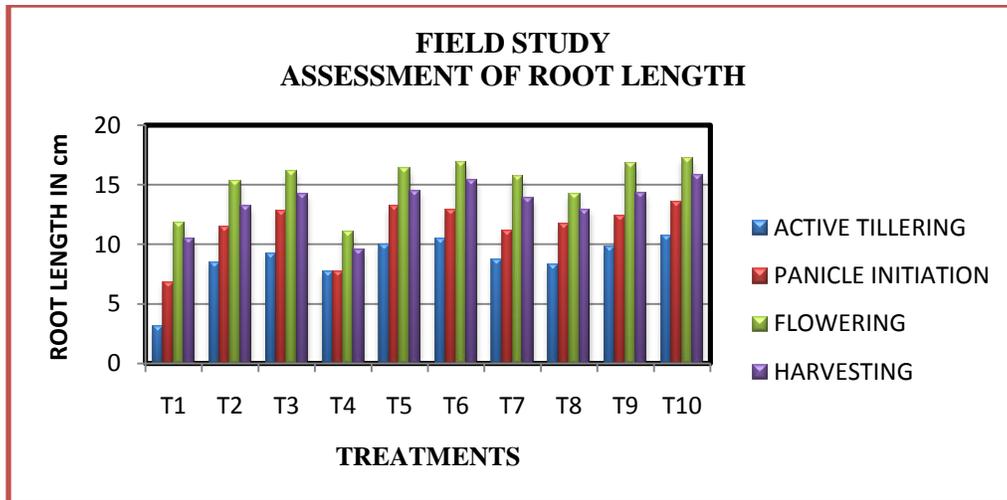


Chart VI

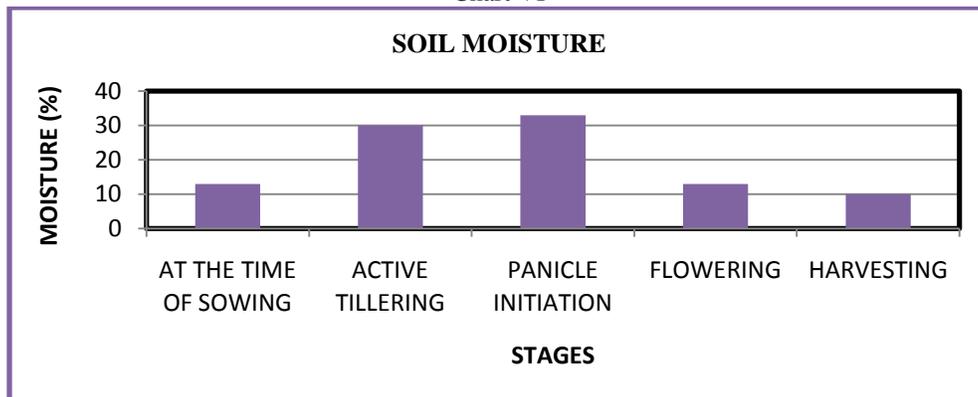


Chart VII

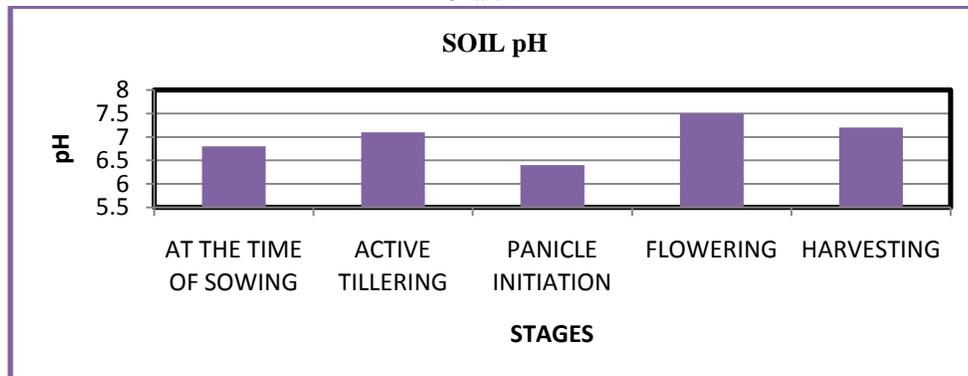


Figure legends

Figure 1  
AMMONIA PRODUCTION

Figure II  
HCN PRODUCTION



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