INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-4, Oct-Dec-2012 Coden : IJABPT Copyrights@2012

ISSN : 0976-4550

Received: 25th August-2012 *Revised: 04th Sept-2012* *Accepted: 10th Sept-2012*

Research article

A STUDY ON NITROGEN FIXING AND PHOSPHATE SOLUBILIZING MICROORGANISMS ON GROWTH AND PHYSIOLOGY OF PLUMBAGO ZEYLANICA.L

D.Haribabu Rao[1]., T.Vijaya[2]., B.V.Ramana naidu[3]., P.Subramanyam[4]., D.Jayasimha Rayalu[5*]

[1, 2]Department of Botany, Sri Venkateswara University, Tirupathi, Andhra Pradesh.

[3]Department of Botany, S.S.B.N Degree and P.G.College, Anantapur, Andhra Pradesh

[4]Department of Botany, Layola Degree college, Pulivendula, Kadapa

[5]Department of Biotechnology, JNTUA, Anantapur.

ABSTRACT : Organisms that are commonly used as biofertilizers component are nitrogen fixers (N-fixer), potassium solubilizer (K-solubilizer) and phosphorus solubilizer (P- solubilizer), or with the combination of molds or fungi. Most of the bacteria included in biofertilizer have close relationship with plant roots. In this work we have selected plumbago zeylanica.L plant to study the effect of Azotobacter on the growth of roots, stem, and leaves. Also biochemical characterization was done to identify the effect of Azotobacter in Plumbago. The maximum shoot length was recorded in T_4 plants (43.51) on 90th days of plant growth after transplanting the plants. There was a significant increase at 5 % level in the root length from 30th days to 90th days in all the treatments. The maximum number of leaves were found in T_4 treatment followed by T_3 and T_2. Minimum numbers of leaves were found in T_1 (1083). On 60th day and 90th day also the total chlorophyll content was maximum in T_4 treated plants followed by T_3, T_2 plants. The amount of reducing sugars (µg/g) in shoots of T_4, T_3 and T_2 plants on 30th, 60th and 90th days were significantly high when compared to T_1 plants. The content of protein in roots of T2, T_3 and T_4 plants on 30th, 60th and 90th days were significantly high when compared to protein content of T_1 plants.

Key words: Plumbago zeylanica, Azatobacter, Biofertilizer, nitrogen fixing, phosphate solubilizing

INTRODUCTION

Nitrogen and phosphorous are known to be essential nutrients for plant growth and development as production of chemical fertilizers is a highly energy consuming process which uses large amounts of fossil energy. Large quantities of chemical fertilizers are used to replenish soil N and P, resulting in high costs, pollutes ground water, increases risk of chemical spills and severe environmental contamination (Dai J et al 2004). They are cost effective, ecofriendly and renewable source of plant nutrients to supplement chemical fertilizers in sustainable agricultural system. Various sources of biofertilizers include nitrogen fixers, phytostimulators, phosphate solubilizing bacteria, plant growth promoting rhizobacteria (Shekh, B.A. 2006). Application of biofertilizers became of great necessity to get a yield of high quality and to avoid the environmental pollution (Shevananda 2008 and El-Kholy, M.A et al 2005). Biological nitrogen fixation is one way of converting elemental nitrogen into plant usable form (Gothwal et al., 2007). Nitrogen-fixing bacteria (NFB) that function transform inert atmospheric N2 to organic compounds (Bakulin et al., 2007). Nitrogen fixer or Nitropgen fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix atmospheric nitrogen. They are grouped into free-living bacteria (Azotobacter and Azospirillium) and the blue green algae and symbionts such as Rhizobium, Frankia and Azolla (Gupta, 2004). Nitrogen fixation and P.solubilization (Bakulin M.K et al 2006) production of antibiotic (Zaidi A, and Mohammad S 2006) and increased rood dry weight are the principal mechanism for the PGPR. A number of different bacteria that promote plant growth, including Azotobacter sp., Azospirillum sp., Pseudomones sp., Bacillus sp. Acetobacter sp (Shekh, B.A. 2006). Plant growth promoting bacteria are important because of their effects on soil conditions, nutrient availability, growth and yields. Plumbago zeylanica Linn. belongs to the family Plumbaginaceae. P. zeylanica is a branched evergreen shrub reaching up to 2 m. It can be seen throughout the tropical and subtropical countries of the world. Four species of the genus Plumbago have been reported.

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They are Plumbago indica, Plumbago rosea, Plumbago capensis, and P. zeylanica (Arunachalam et al., 2010). P.zeylanica is known by different names in different parts of the world. Such names include white leadwort or Ceylon leadwort (in English), bleiwurz or zahnkraut (in German), chitrak or chitramol (in India), ensain or enkin (in Arabia), sanza (in Swahili), and inabiri among the Yoruba (in South- West Nigeria). The whole plant, roots, powder of the root, leaves and stem-bark are widely used as medicinal herbs throughout Asia and Africa. In traditional herbal medicine, the root part of P. zeylanica is used for treatment of different ailments such as parasitic diseases, scabies and ulcers (Olagunju et al., 2006), piles, diarrhoea, skin diseases and leprosy (Uma et al., 1999), fever or malaria, rheumatism, intestinal parasites, anemia due to 'stagnant blood', internal and external trauma,toxic swelling and furunculous scabies (Olagunju et al.,2006; Jeyachandran et al., 2009; Jiangsu New Medical College, 1979; Dai et al., 2004).

MATERIALS AND METHODS

Selection of plant

Plumbago zeylanica L. is an important medicinal plant. This plant was brought under cultivation in many Asian countries. There is a need to develop efficient, low cost cultivation methods for Plumbago zeylanica which are suitable to various climatic conditions to obtain higher yield, hence Plumbago zeylanica is selected in the present study.

Collection of seeds

The seeds of Plumbago zeylanica L. were collected from S.V. University, Campus area Tirupathi, Andhra Pradesh. The seeds of uniform size were separated and surface sterilized with 0.05 % sodium hypo chloride, after thorough washing with tap water before sowing.

Collection of biofertlizers inoculums

Culture of Bacillus megatherium and Azotobacter chrocooccum was obtained from Regional Biofertilizers Development Centre, Bangalore Division, India.

Preparation of mediums

Pikovskaia's broth and Ashby medium were used for Bacillus megatherium and Azotobacter respectively.

Experimental design

The pot culture experiment was carried out under green house conditions to know the response of Plumbago zeylanica L plant to Azotobacter chrocooccum and PS bacterial (Bacillus megatherium) inoculation. The treatment details of the pot experiment were generalized as follows:

T1 : Control (No inoculation)

T2 : Inoculation with Azotobacter chrocooccum (nitrogen fixing bacteria)
T3 : Inoculation with Bacillus megatherium (PSB)
T4 : Inoculation with Azotobacter chrocooccum and Bacillus megatherium

Cultivation of plants

Plumbago zeylanica.L plants were grown in plastic pots contained sterilized mixture of soil and sand (1/1, w/w). Six experimental replications were maintained for each treatment and the pots were arranged in a completely randomized design. The plants were grown under natural photoperiods (23.5/18 ºC day/night, 4000 – 6000 Lux light intensity) for three months during which only deionized water was applied. Inoculum of 20ml of Bacillus megatherium culture and/or 20ml of A. chrocooccum was mixed with soil as per treatments and filled the pots.

Physiological parameters

The growth parameters were measured on Shoot length, Root length, Number of leaves, Leaf area, Fresh shoot biomass, Fresh root biomass, Dry shoot biomass, and Dry root biomass every 30^{th}, 60^{th} and 90^{th} day of the plant growth in all the treatments.

Biochemical Parameters

Estimation of chlorophyll content (Arnon (1949), starch (Mc Cready et al., (1950) carbohydrates (Highkin and Frankel (1962) and total proteins (Lowry et al., (1951) every 30^{th} day.

Statistical analysis:-

The data was statistically analyzed of the by two way analysis of variance. **SPSS version 11.55** was used. The values were mentioned corresponding to each table in the results.

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RESULTS AND DISCUSSION

Effect of Azotobacter and PSB on growth and yield of Plumbago zeylanic L.Shoot length (cms): (Table: 1, fig: 1)
It was observed that all the inoculated plants exhibited increased shoot length compared to un-inoculated plants. The maximum shoot length was recorded in T_4 plants (43.51) on 90th days of plant growth after transplanting the plants. There was a significant difference at 5 % level among all the treatments and also among the days..

Root length (cms): (Table :2, fig:- 2) :-

The plants grown under T_4 treatment exhibited maximum root length (31.64) and the root length was found minimum (13.15) in T_1 treatment on 90th days of plant growth. There was a significant increase at 5 % level in the root length from 30th days to 90th days in all the treatments.

Number of leaves: (Table: 3, fig: 3):-

The numbers of leaves were found increased in all the treatments from 30th days to 90th days when compared to control. The maximum number of leaves were found in T_4 treatment followed by T_3 , and T_2. Minimum numbers of leaves were found in T_1 (1083).

Leaf area (cm^2): (Table: 4, fig: 4):-

The leaf area differed significantly in all the treatments at 5 % level. On 30th day, the maximum leaf area was found in T_4 plants (9.01) and the minimum in T_1. On 40th and 60th day, all the treatments (T_2, $T_{3,\text{ and }}T_4$,) performed better compared to control (T_1). The highest leaf area was recorded in T_4 plants. The next best treatment was T_3 followed by T_2. The leaf area found to be least in T_1 plants.

Table: 1 Effect of Azotobacter and PSB on shoot length (cms) of Plumbago zeylanica L.

Treatments	Days after treatment		
	30 days	60 days	90 days
T1	5.76	9.47	13.96
	(0.14)	(0.12)	(0.07)
T2	10.53	17.94	21.51
	(0.11)	(0.04)	(0.12)
T3	12.63	18.45	20.71
	(0.07)	(0.05)	(0.05)
T4	21.59	32.69	43.51
	(0.16)	(0.11)	(0.15)

Values within the brackets indicate standard deviation. Each value represents mean of six replications.
Two – way ANOVA for the effect of Azotobacter and PSB on shoot length of Plumbago zeylanica L.
Significant at 5% level

Source of variation	**F - value**	
	Shoot length	
	Calculated	**Table**
Treatments	24.95	8.94
Days	13.6	19.33

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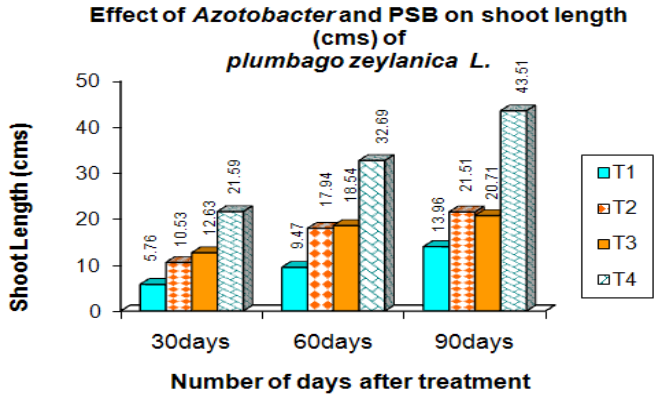

Fig : -1

Table : 2 Effect of Azotobacter and PSB on root length (cms) of Plumbago zeylanica L.

Treatments	Days after treatment		
	30 days	60 days	90 days
T1	4.74	7.52	13.15
	(0.08)	(0.16)	(0.08)
T2	8.19	17.28	21.03
	(0.03)	(0.03)	(0.03)
T3	8.28	16.71	20.43
	(0.04)	(0.03)	(0.11)
T4	12.55	23.40	31.64
	(0.43)	(0.33)	(0.16)

Values within the brackets indicate standard deviation. Each value represents mean of six replications. Two – way ANOVA for the effect of Azotobacter and PSB on root length of Plumbago zeylanica L.

Source of variation	F - value	
	Root length	
	Calculated	Table
Treatments	16.33	8.94
Days	28.75	19.33

Significant at 5% level

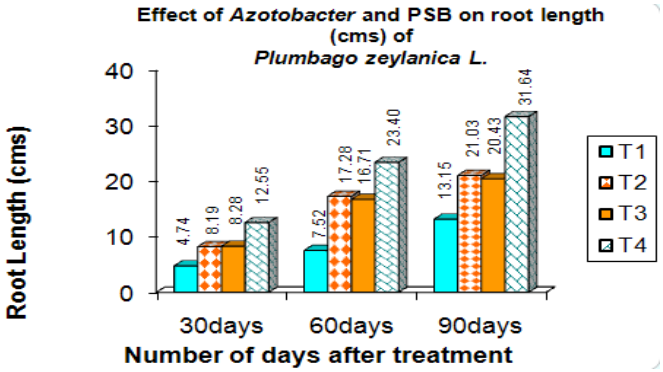

Fig :- 2

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Table : 3;Effect of Azotobacter and PSB on leaf number of Plumbago zeylanica L.

Treatments	Leaves Number		
	Days after treatment		
	30 days	60 days	90 days
T1	4.83	6.50	10.83
	(0.75)	(0.55)	(0.75)
T2	8.67	10.17	18.17
	(0.52)	(0.75)	(0.98)
T3	8.00	8.83	19.67
	(0.89)	(0.75)	(0.52)
T4	11.17	15.67	27.17
	(0.75)	(0.82)	(1.17)

Values within the brackets indicate standard deviation. Each value represents mean of six replications. Two – way ANOVA for the effect of Azotobacter and PSB on leaf number of Plumbago zeylanica L.

Source of variation	F- value	
	Calculated	Table
Treatments	11.01	8.94
Days	25.45	19.33

significant at 5% level

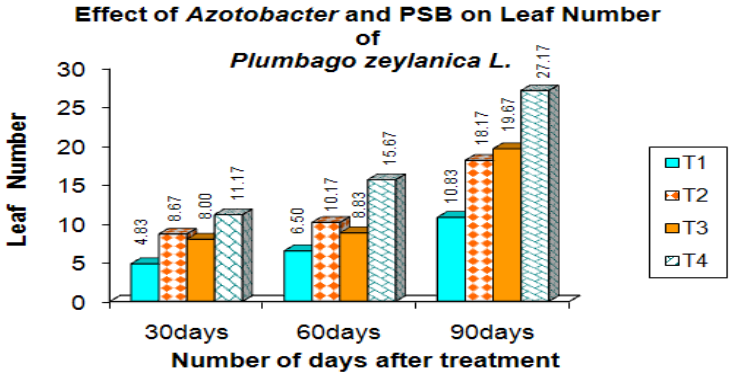

Fig: - 3

Table: 4 : Effect of Azotobacter and PSB on leaf area (cm^2) of Plumbago zeylanica L.

Treatments	Leaf Area (cm2)		
	Days after treatment		
	30 days	60 days	90 days
T1	2.73	4.19	6.10
	(0.97)	(1.99)	(3.74)
T2	6.23	9.44	14.64
	(3.67)	(5.15)	(7.99)
T3	6.47	9.68	14.82
	(3.84)	(5.23)	(8.39)
T4	9.01	16.16	26.09
	(5.22)	(10.59)	(14.27)

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Values within the brackets indicate standard deviation. Each value represents mean of eight replications. Two – way ANOVA for the effect of Azotobacter and PSB on leaf area (cm^2) of Plumbago zeylanica L.

Source of variation	F- value	
	Calculated	Table
Treatments	9.947	8.94
Days	10.69	19.33

Significant at 5% level

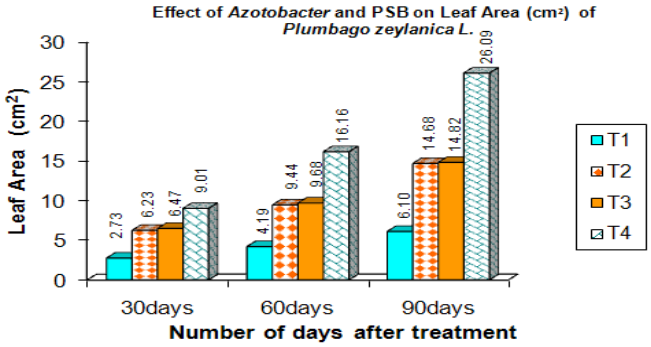

Fig : - 4

BIOCHEMICAL STUDIES

Chlorophyll estimation (mg/g): (Table: 5 , fig:5 a, fig:5 b and fig:5 c) :-

On 30th day, the total chlorophyll content was maximum in T_4 and minimum in T_1 plants. On 60th day and 90th day also the total chlorophyll content was maximum in T_4 treated plants followed by T_3, T_2 plants. Minimum amount of chlorophyll was found in T_1 plants on these days. Varied difference in chlorophyll- content was observed among the treatments on 30th, 60th and 90th day samples. On 30th, 60th and 90th day, more amount of chlorophyll-a was found in T_4 plants followed by T_3 and T_2 plants. The control plants exhibited low levels of chlorophyll-a in all the days. The chlorophyll-b was also observed maximum in T_4 plants and minimum in T_1 plants on 30th, 60th and 90th days' sample. There was a significant difference in chlorophyll-a, chlorophyll-b and total chlorophyll content among the treatments and different days of a given treatment at 5 % level.

Table: 5: Effect of Azotobacter and PSB on chlorophyll content of Plumbago zeylanica. L

Treatments	Chlorophyll 'a' (mg/g)			Chlorophyll 'b' (mg/g)			Total Chlorophyll (mg/g)		
	Days after treatment								
	30 days	60 days	90 days	30 days	60 days	90days	30 days	60 days	90Days
T1	0.70	0.92	1.34	1.04	1.22	1.26	1.73	2.13	2.60
	(0.01)	(0.01)	(0.01)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)	(0.03)
T2	0.78	1.02	1.36	1.28	1.40	1.68	2.06	2.41	3.04
	(0.01)	(0.02)	(0.01)	(0.01)	(0.01)	(0.02)	(0.02)	(0.03)	(0.03)
T3	0.99	1.11	1.42	1.13	1.35	1.44	2.14	2.46	2.86
	(0.04)	(0.02)	(0.02)	(0.01)	(0.01)	(0.03)	(0.09)	(0.02)	(0.04)
T4	1.00	1.46	1.91	1.33	1.54	1.80	2.33	3.00	3.71
	(0.02)	(0.01)	(0.02)	(0.02)	(0.01)	(0.02)	(0.03)	(0.02)	(0.04)

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Values within the brackets indicate standard deviation. Each value represents mean of six replications.Two – way ANOVA for the effect of Azotobacter and PSB on chlorophyll content of Plumbago zeylanica

Source of variation	F- value					
	Chlorophyll a		Chlorophyll b		Total chlorophyll	
	Calculated	Table	Calculated	Table	calculated	Table
Treatments	11.93	8.94	17.89	8.94	18.02	8.94
Days	38.21	19.33	25.97	19.33	46.75	19.33

Significant at 5% level

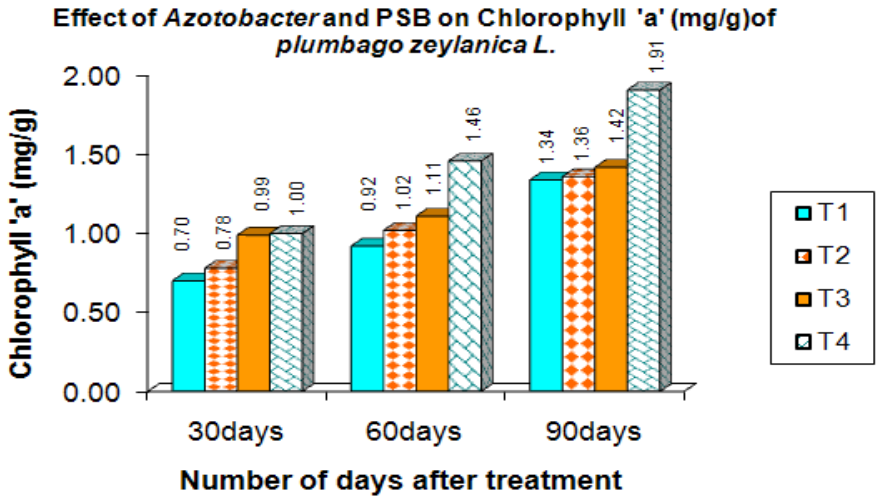

Fig : -5 (a)

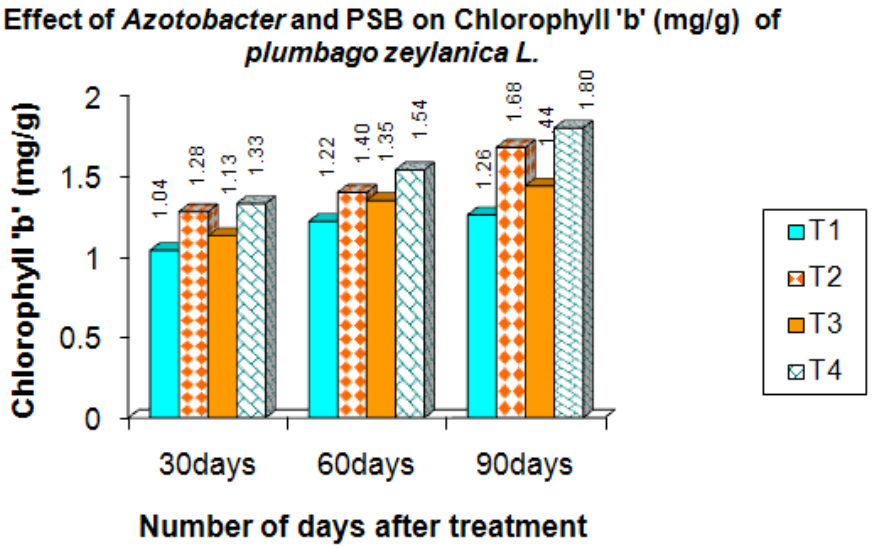

Fig – 5 (b)

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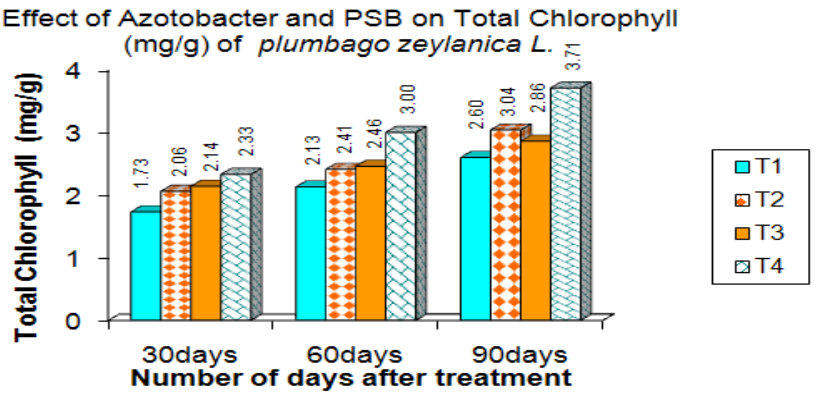

Fig : - 5 (c)

Carbohydrate content of Root: (Table:6 , fig:6a, fig:6b, fig:6c) :-

The amount of reducing sugars (µg/g) in shoots of T_4, T_3 and T_2 plants on 30th, 60th and 90th days were significantly high when compared to T_1 plants. Maximum amount of reducing sugars were recorded in the shoot samples of T_4 plants on 30th, 60th and 90th day. In T_1 plants the amount of reducing sugars was found minimum.

There was a significant increase in the amount of non-reducing sugars (µg/g) in T_4, T_3 and T_2 plants over T_1 (control) plants on 30, 60 and 90 days. The amount of non-reducing sugars being maximum in T_4 plants on 30th, 60th and 90th day were followed by T_4, T_3, T_2 and T_1. In T_1 plants the amount was 276.96, 462.99 and 634.22 respectively.

The amount of starch content (mg/g) in shoots of T_4, T_3 and T_2 plants on 30, 60 and 90 days was significantly high when compared to starch content of shoots in T_1 plants. Maximum amount of starch content was recorded in T_4 plants and minimum in T_1 plants. The amount of starch in T_4 plants on 30th, 60th and 90th day was 7.42, 12.64 and 15.75. In T_1 plants it was 4.40, 5.78 and 6.27 respectively. There was a significant difference in the levels of reducing sugars, non reducing sugars and starch level content of shoot between the treatments and different days of a given treatment at 5 % level.

Table: 6 : Effect of Azotobacter and PSB on carbohydrate content in root of Plumbago zeylanica.L

Treatments	Reducing sugars (µg/g)			Non-reducing sugars (µg/g)			Starch (mg/g)		
	Days after treatment								
	30 days	60 days	90days	30 days	60 days	90 days	30 days	60 days	90 days
T_1	316.96	498.74	693.09	276.96	462.99	634.22	4.40	5.78	6.27
	(0.68)	(0.33)	(0.45)	(0.24)	(0.41)	(0.40)	(0.09))	(0.09)	(0.13)
T_2	488.98	776.12	883.99	412.17	636.95	824.02	6.37	8.51	12.52
	(0.61)	(0.48)	(0.18)	(0.33)	(0.08)	(0.21)	(0.14)	(0.07)	(0.17)
T_3	471.84	764.14	895.89	398.95	614.87	803.23	5.85	8.37	13.17
	(0.40)	(0.27)	(0.46)	(0.19)	(0.29)	(0.29)	(0.10)	(0.12)	(0.12)
T_4	554.27	882.09	1076.40	587.03	791.92	933.03	7.42	12.64	15.75
	(0.47)	(0.18)	(0.40)	(0.42)	(0.28)	(0.40)	(0.14)	(0.07)	(0.08)

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Values within the brackets indicate standard deviation.Each value represents mean of six replications. Two – way ANOVA for the effect of Azotobacter and PSB on

Carbohydrate content in root of Plumbago zeylanica**Source of variation**	**F- value**					
	Reducing sugars		**Non- reducing sugars**		**Starch**	
	Calculated	**Table**	**Calculated**	**Table**	**calculated**	**Table**
Treatments	28.06	8.94	158.7	8.94	8.762	8.94
Days	100.10	19.33	468.2	19.33	14.62	19.33

Significant at 5% level

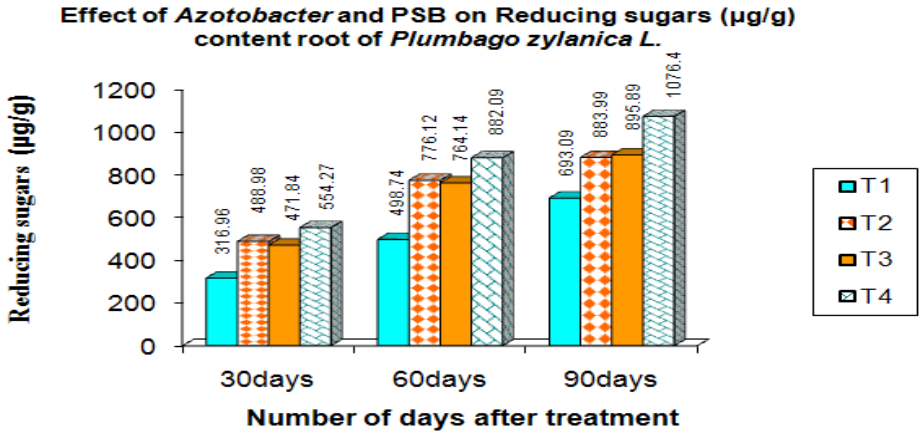

Fig :- 6(a)

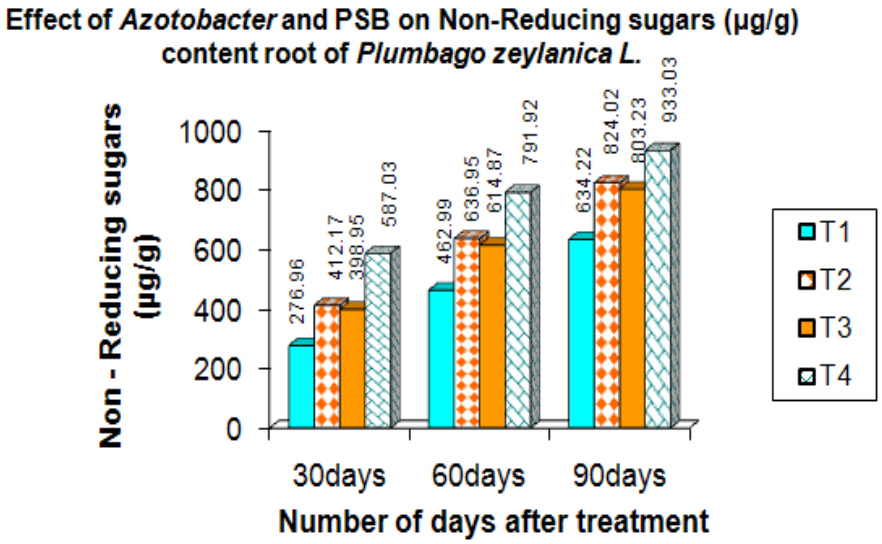

Fig :- 6(b)

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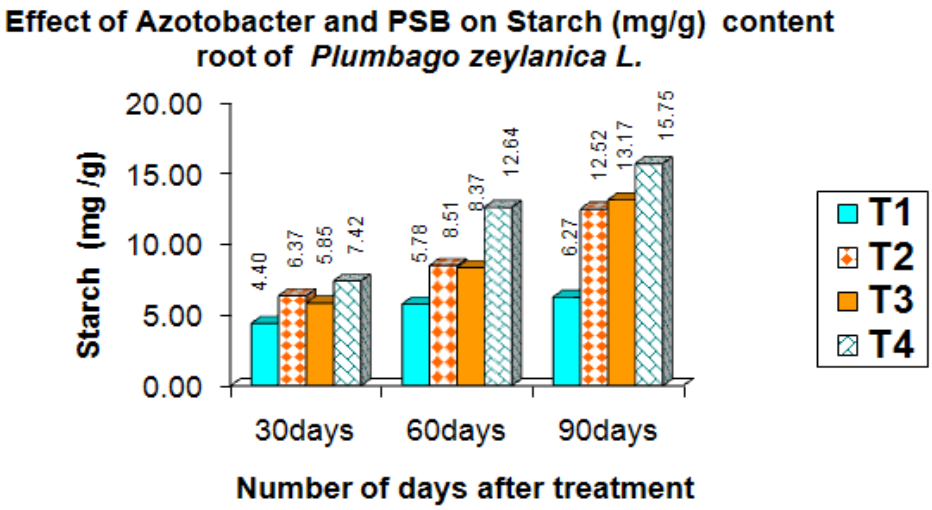

Fig :- 6(c)

Protein estimation (mg/g): (Table: 7, fig : 7)

The content of protein in roots of T2, T_3 and T_4 plants on 30th, 60th and 90th days were significantly high when compared to protein content of T_1 plants. Maximum shoot protein content was recorded in T_4 plants and minimum in T_1 plants. The protein content in T_2 plants was less than T_3, T_4, plants. There was significant difference in the levels of protein content of root between the treatments and different days of a given treatment at 5 % level.

Table : 7 Effect of Azotobacter and PSB on Protein (mg/g) content root of Plumbago zeylanica.L

Treatments	Protein content (mg/g)		
	Days after treatment		
	30 days	60 days	90 days
T_1	0.87 (0.01)	1.21 (0.01)	1.47 (0.02)
T_2	1.70 (0.01)	1.87 (0.02)	2.06 (0.01)
T_3	1.63 (0.03)	1.92 (0.02)	2.07 (0.01)
T_4	1.93 (0.01)	2.19 (0.02)	2.96 (0.02)

Values within the brackets indicate standard deviation. Each value represents mean of six replications. Two – way ANOVA for the effect of Azotobacter and PSB on Protein content root of Plumbago zeylanica.

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Source of variation	F- value	
	Root Protein	
	Calculated	Table
Treatments	23.74	8.94
Days	12.53	19.33

Significant at 5% level

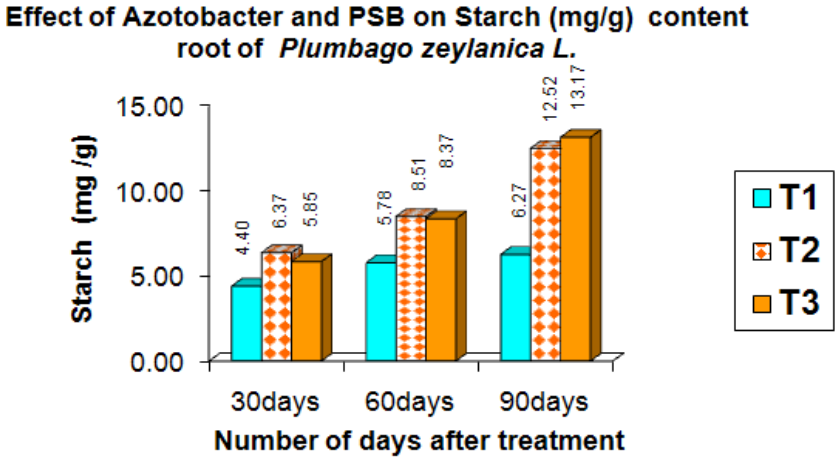

Fig :- 7

Lipid estimation (mg/g): (Table: 8, Fig :8)

30th, 60th, and 90th day, the highest amount of lipid content was recorded in the roots of T4 plants followed by T3, T2 and control T1 plants . There were significant differences in the root lipid content among the treatments and different days of a given treatment at 5 % level.

Table: 8 : Effect of Azotobacter and PSB on Lipid (mg/g) content root of Plumbago zeylanica

Treatments	Lipid content (mg/g)		
	Days after treatment		
	30days	60days	90days
T_1	4.10	6.45	10.33
	(0.01)	(0.02)	(0.02)
T_2	7.56	10.69	14.18
	(0.01)	(0.01)	(0.01)
T_3	6.89	9.75	13.28
	(0.02)	(0.01)	(0.01)
T_4	9.67	12.35	18.79
	(0.01)	(0.01)	(0.02)

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Values within the brackets indicate standard deviation. Each value represents mean of six replications. Two – way ANOVA for the effect of Azotobacter and PSB on Lipid content root of Plumbago zeylanica

Source of variation	F- value	
	Root Lipid	
	Calculated	Table
Treatments	33.95	8.94
Days	77.34	19.33

Significant at 5% level

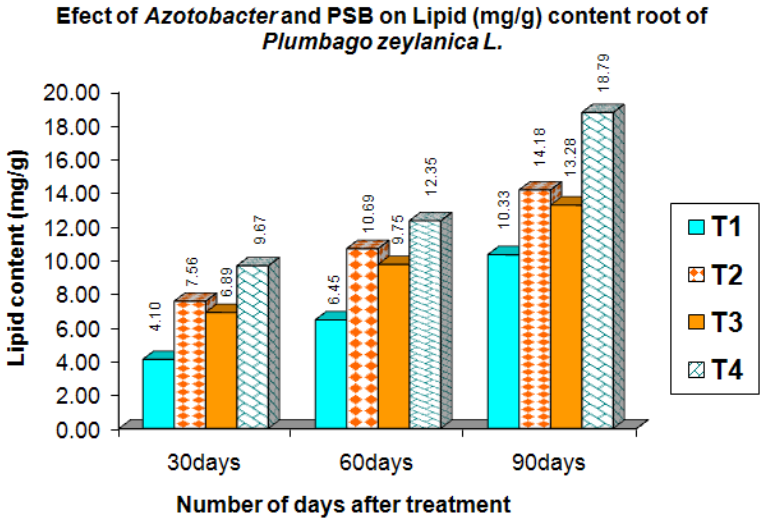

Fig: - 8

CONCLUSION

Azotobacter has ability to fix molecular nitrogen and therefore increase the soil fertility and stimulate plant growth, Azotobacter are widely used in agriculture particularly in nitrogen biofertilizers such as azotobacterin. We concluded that the Azotobacter has significant effect on the growth of root, shoot and leaves of Plumbago zeylanica. Also there was a change in carbohydrate and protein content in Plumbago due the effect of Azotobacter. There was a significant difference in chlorophyll-a, chlorophyll-b and total chlorophyll content among the treatments and different days of a given treatment at 5 % level.

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