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Assessment of microbial inoculants on enzyme activity and chlorophyll content of *Guizotia abyssinica (L.f) Cass* plants genotypes

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Abstract
This experiment was carried out to study the efficiency of bio inoculants on *Guizotia abyssinica (L.f) Cass* plants genotypes on hydrolytic enzyme activty, Chlorophyll content. Four hydrolytic enzyme activities were activated in the roots from three varieties of Niger plantlets by all three (*Pseudomonas aeruginosa, Azotobacter chroococcum, Glomus leptonica*) bioinoculants. Celluase and Protease activity 0.0090, 0.0080 and 0.0088, 0.0081 µmoles/ml/min respectively in RCR-18 and DNS-4 varieties were found maximum from *Pseudomonas aeruginosa*, and *Azotobacter chroococcum* inoculums and peroxidase activity, *Pseudomonas aeruginosa* showed maximum enzyme activity 289.6 Units/ml. Similar trend of results was observed with Chlorophyll content since the highest records appeared with application of *Pseudomonas aeruginosa* in *Guizotia abyssinica (L.f) Cass* var RCR-18, recorded highest chlorophyll content with 2.36 gm /tissue. This study shows significant difference of Enzyme activity and growth enhancement with treatment of bio-inoculants.

Keywords: *Guizotia abyssinica (L.f) Cass,* Chlorophyll content, PGPR (Plant Growth Promoting Rhizobacteria), Enzyme activity, Bio-inoculants

1. Introduction
Abundant microorganisms are present in the rhizosphere zone, and rhizoplane. Very important and significant interactions were reported among plant, soil, and microorganisms [4]. These interactions may be beneficial, harmful or neutral, and that can significantly influence plant growth and development [1, 2, 26]. The microorganisms colonizing plant roots include bacteria, algae, fungi, protozoa and actinomycetes. There Enhancement influence in plant growth with the development by application of these microbial inoculants is well documented [9, 18, 20]. Verma *et al.* (2001) [46] have proved that the microbial activities of cellulase and pectinase enzymes showed the potential for inter- and intracellular colonization to the plant roots several. N_2-fixing bacterial strains isolated from Rhizosphere of rice roots were able to produce hydrolytic enzymes. However, these hydrolytic enzymes are sensitive to some abiotic factors, in particular chemical fertilizers [51]. There are still certain aspects which need further investigations for obtaining maximum benefits in terms of improved plant growth from this naturally occurring microbial population particularly under stress conditions. The presence of ROS (Relative Oxidative Stress) can cause cellular damage through oxidation of lipids and proteins, chlorophyll bleaching, damage to nucleic acids, ultimately leading to cell death [5, 12]. Plants develop self defense mechanisms by producing antioxidant enzymes like superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase [7]. The enzymes like peroxidases have also been reported to play an important role in lignifications and suberization [21]. Plants also develop certain other self defense mechanisms to protect themselves from pathogen attack. These include accumulation of secondary metabolites and synthesis of defense proteins [13, 10]. Induction of hydrolytic enzymes and the construction of defense barriers. These strategies help the plant to maintain its growth under stress environment by mitigating the negative impact of stress on plant growth and development.
Metabolic changes involved in the defense mechanism of plants are correlated with changes in activity of key enzymes in primary and secondary metabolism.

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria they found in the rhizosphere, at root surfaces and in association with roots. PGPR synthesize hydrolytic enzymes, such as chitinases, glucanases, proteases, and lipases, which can lyse pathogenic fungal cells. Of different microbial populations present in the rhizosphere, bacteria are the most abundant microorganisms, cause a pronounced effect on plant growth and enzyme activation. Zahir *et al.,* (2004) [50]. Exploring the mechanisms of growth promotion by PGPR and mycorrhizae could be very useful for enhancing plant growth by using these microbial populations together, particularly under stressful environments. Although a number of studies have shown that combined application of PGPR and fungi could be a meaningful approach for sustainable agriculture [35]. Niger oil has a fatty acid composition typical for seed oils of the Compositae plant family (e.g. safflower and sunflower) with linoleic acid being the dominant fatty acid. The linoleic acid content of Niger oil was approximately 55% in seed grown in India [34]. The Niger oil is used for cooking, lighting, anointing, painting and cleaning of machinery [11, 37, 36]. Niger oil also is a substitute for sesame oil for pharmaceutical purposes and can be used for soap-making. Niger seed oil has been identified as a potential biodiesel crop because of the presence of 50–60% the oil called biocrude, which can be converted into biodiesel by chemical or lipase mediated esterification [19]. Study on mycorrhizal and PGPR association with Niger is nearly meager and lesser known, [14] unexploited oil yielding plants of the tropics and subtropics grown under dry-land agriculture. Therefore this present work is to evaluate activities of several hydrolytic enzymes in the roots of Niger plants treated with microbial inoculants

Methodology

Experiment was conducted in the Green house of Department of botany, Karnatak University, Dharwad-580003 India, using Completely Randomized Design (CRD) with 4 replicates. Seeds of three *Guizotia abyssinica (L.f) Cass* genotypes (RCR-18, NO-71, DNS-4) were surface-sterilized with a bleach solution (10%, v: v), and germinated under sterile conditions on wet filter paper at 28°C for three days they were divided into four sets: first set was soaked in water (control), Isolated Bacterial suspension (*Pseudomonas areoginosa P.a, Azotobacter chroococcum A.z*) was shaken in nutrient broth for 72 hours. The bacterial cells were harvested by centrifugation and washed with 0.85% sterilized phosphate buffer solution (PBS). Approximately 108 cfu ml^{-1} of live washed bacteria cells were directly inoculated into the plants in each pot**.** Mycorrhizal *(Glomus leptonicum)* inoculation was carried out by mixing with the growing substrate 10% of a sand: soil (9:1, v: v) based inoculums enriched in fungal propagules and containing chopped mycorrhizal *Allium porrum* L. roots. At potting, plants corresponding to all treatments received an aliquot of a filtrate (B20 mm) of both AM fungal inocula in order to provide the microbial populations accompanying the mycorrhizal inoculation. The suspension obtained was used to inoculate the corresponding Niger plants by injecting 8 ml per plant close to the root system. Control plants were supplied with 8 ml of sterile water.

Extraction of enzyme

At 60 days after seed germination (7 days after 100Mm NaCl treatment), the plant samples were harvested and rinsed with distilled water. The plant sample (1g) was homogenized in 3 ml of 50 mM sodium phosphate buffer (pH 7.2) and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C.The reaction mixture was incubated at 37 °C for seven days, and then 1 ml of the supernatant was assayed for total (sugar DNS method) and protein (Folin Ciocalteau method described by Lowry *et al*. (1951) [28] content according to standard methods. The enzyme activity is expressed in micrograms of reducing sugar produced per milliliter supernatant per milligram protein**.**

Cellulase assay:- The cellulase activity was determined by the method proposed by Teeri & Koivula, (1995) [45] The culture obtained was centrifuged at 8,000 rpm for 6 min and the supernatants were used as the enzyme source. The 2ml reaction mixture of the enzyme contained 1 ml of 1% CM cellulose, 0.5 ml of phosphate buffer (25 mM, pH 7.0) and 0.5 ml of enzyme extract. The tubes were incubated at 37 ^{0}C for 10 min and added with 1 ml of DNS. The tubes were further boiled for

$$\text{Activity} = \frac{\text{Conc. of glucose liberated X ml of enzyme}}{\text{Mol. Wt. of glucose X incubation time}} \text{ X dilution factor}$$

10 min, cooled and the absorbance was measured at 540 nm against the blank, added with DNS before the addition of enzyme. The concentration of glucose released was estimated by standard glucose curve. The activity was calculated according to the following formula.

Protease Assay:- The protease activity was determined by the method proposed by Keay *et al*., (1970) [23]. 0.5 ml of suitably diluted enzyme was added to 1.0 ml of 1% casein and 0.5 ml of phosphate buffer (25 mM, pH 7.0), whole mixture was incubated at 37 ^{0}C for 10 min. The reaction was terminated by the addition of 3 ml of 10% TCA solution. The solution was allowed to stand for 10 min in cool and was filtered. To the clear filtrate, 5 ml 0.4 M Na_2CO_3 and 0.5 ml of Folin Ciocalteau reagent (FCR) was added, mixed thoroughly and incubated at 37 ^{0}C for 30 min, in dark. The absorbance was measured at 660 nm. A blank was prepared by the same procedure, except that TCA was added at '0' time, i.e., in the control tube, TCA was added before the addition of the enzyme solution. The activity of the enzyme was calculated according to the following formula.

$$\text{Activity} = \frac{\text{Concentration of tyrosine X vol. of enzyme X dilution factor}}{\text{Molecular wt. of tyrosine X incubation time}}$$

One unit of protease activity was defined as the amount of enzyme that produced TCA soluble material equivalent to 1 µg of tyrosine from casein per min under assay conditions [30]

Peroxidase:- The protease activity was determined by the method proposed by Wang and Yang, (2005) [47].3.0 ml of reaction mixture was taken in the cuvette containing 100 mM of potassium phosphate buffer (pH 7.0), 0.05 ml of 20 mM guaicol solution, 0.1 ml of enzyme extract and 0.03 ml of 12.3 mM hydrogen peroxide (H_2O_2). The reaction mixture was mixed well and ongoing reaction was read at every 30 sec at 470 nm up to 3 min. against the blank without enzyme.

The activity of the enzyme was calculated using the following formula:

$$\text{Activity} = \frac{\text{Optical Density}}{6.39 \times \Delta t \times \text{Volume of the reaction mixture}} \times 10\ \mu m/ml/min$$

Where, r is the area of cuvette (1 cm), Δt, is the time in min. required to increase the absorbance by 0.1, 6.39 extinction coefficient of guaicol dehydrogenation. The specific activity of the enzyme was calculated by dividing the activity with the corresponding total protein concentration of the enzyme extract.

Endoglucanase Assay

A modification of the reducing sugar method described by Khan (1980) [24]. was used for the assay of 1, 4-Endoglucanase activity as follows: Carboxymethyl-cellulose (CMC) was used as enzyme substrate. The reaction mixture contained 2.0 ml of 0.1% (w/v) CMC in 0.1M sodium acetate buffer (pH 5.0) and 2.0 ml of cell-free culture supernatant. The mixture was incubated at 40°C in water bath with shaking for 30 minutes. The released reducing sugar was assayed by Miller's method (1959) [32]. and expressed in glucose equivalent. A unit of activity was defined as amount of enzyme required to liberate 1µmol of glucose per minute under the assay conditions.

Chlorophyll Estimation

One gram fresh leaf sample taken from each replication was analyzed for total chlorophyll content by following the Arnon, (2001) [6]. method.

Chlorophyll content was calculated by following standard formula,

V mg of chlorophyll 'a'/g of =12.7 (A663) – 2.69 (A645) × Fresh leaves 1000 × W

V mg of chlorophyll 'b'/g of = 22.9 (A645) – 4.68 (A663) ×Fresh leaves 1000 × W

V mg of total chlorophyll/g = 20.2 (A645) +8.02 (A663) × of fresh leaves 1000 × W

Where,

A = absorbance at specific wave lengths. V = final volume of chlorophyll extract in 80% acetone

W = fresh weight of tissue extracted

Results and Discussion

Plants was evident in all the harvests, in mycorrhizal as well as in PGPR roots. In mycorrhizal roots, the enzyme activity gradually increased and attained maximum on the 90 days and then declined. A significant increase in enzyme activity was noticed in all roots of *Guizotia abyssinica (L.f) Cass* from 60 days after inoculation. Four hydrolytic enzyme activities were activated in the roots from three varieties of Niger plantlets by all three bioinoculants as shown in (Table. 1.) In roots, a higher and statistically different enzymatic activity from the control was recorded with the three bioinoculants, *Pseudomonas areoginosa,* and *Azotobacter chroococcum* two inoculates showed maximum in Celluase and Protease activit4y 0.0090, 0.0080 and 0.0088, 0.0081 µmoles/ml/min respectively in RCR-18 and DNS-4 varieties as shown in (Table-1 and Fig-1,2). While in Peroxidase activity *Pseudomonas areoginosa* showed maximum enzyme activity 289.6 followed by *Glomus leptonicum* with 279.5 Units/ml. in DNS-4 genotype. *Azotobacter chroococcum* reported (Fig-3, 4) maximum Glucanase activity surpassing the control with 1.94 µmoles/ml/min in No- 71. Among three bioinoculants *Pseudomonas areoginosa,* and *Azotobacter chroococcum* induced maximum enzyme activity in all three Niger varieties.

The results also revealed that, the enzyme activity of *Guizotia abyssinica (L.f) Cass* plants had rich chlorophyll content was influenced by enzyme activity of bioinoculants. Plants inoculated with triple inocula showed a significantly increased plant height compared to uninoculated plants. The total chlorophyll content of as influenced by inoculants treatments are presented in (Table -2). The chlorophyll content of the leaves of *Guizotia abyssinica (L.f) Cass* increased upto 90 days after inoculation and then gradually decreased with increasing age of the plant. The plant inoculated with three bioinoculants recorded significant total chlorophyll content ranging from 1.80 to 2.30 mg g-1 of plant shown in (Table -2). In *Guizotia abyssinica (L.f) Cass* var RCR-18, *Pseudomonas areoginosa* recorded highest chlorophyll content with 2.36 gm /tissue followed by *Azotobacter chroococcum*, 2.27 gm/tissue, in NO-71 variety 2.13 gm /tissue was maximum chlorophyll content treated with *Azotobacter chroococcum* and in DNS-4 genotype there was no significant changes in chlorophyll recorded *Pseudomonas areoginosa* showed more production 2.13 gm /tissue. Overall out of three genotype of *Guizotia abyssinica (L.f) Cass* RCR-18 recorded highest total chlorophyll content with respect treatment of *Pseudomonas areoginosa* shown in (Fig -5 & 6).

Mycorrhizae and PGPR play an important role in improving plant growth through various mechanisms. In addition to fungal and bacteria population, also represent a significant portion of soil rhizosphere microflora and influence plant growth [25] The symbiotic association generated by fungi with plant roots (mycorrhizae) increases the root surface area, and therefore enables the plant to absorb water and nutrients more efficiently from large soil volume. Several attempts to test this hypothesis by means of both genetic and molecular approaches have been undertaken recently [33]. This experiment was carried out to examine the possibility of production of cell wall hydrolyzing enzymes during the process of intracellular establishment Production of endoglucanase and exoglucanase enzymes has been reported [15] in the roots of lettuce and onion during the penetration of the host by the VAM (vesicular– arbuscular mycorrhizal) fungus *Glomus mosseae*. The intracellular establishment of VAM symbiosis between fungus and plant roots requires penetration of the host cell by the fungus. Despite these mechanisms, PGPR may also enhance plant growth and development by the virtue of their key enzymes (ACC-deaminase, chitinase) and also by the production of substances such as exopolysaccharides, rhizobitoxine, etc. that help plants to withstand stress conditions [17, 39]. The production of enzymes related to pathogenesis (PR-proteins) by strains of rhizobacteria is considered the largest property of the antagonistic strains [38]. Among these enzymes can be highlighted chitinases, lipoxygenases, peroxidases, and glucanases. Increased activity of peroxidase has been correlated with resistance in many plant species, including rice, wheat and sunflower [49]. The action of lipoxygenase products contributes to the defense reactions involving the inhibition of growth of the pathogen and induction of phytoalexins [27]

Our findings are concordant with previous studies showing that PGPR inoculations may stimulate the yield and growth of different plant species under saline conditions [8], Jha and Subramanian (2013) [22] had found that inoculation of plant

material with PGPR can protect paddy plants against salt stress through an increase in plant growth parameters and the regulation of ion concentration and antioxidant enzymes. The mechanisms that promote plant growth include: nitrogen fixation, phosphorus solubilization, production of siderophores, plant growth regulators and organic acids as well as protection by enzymes like ACC-deaminase, chitinase and glucanase cellulose [17, 20]. Peroxidase is an important enzyme involved in morphogenesis and auxin oxidation. This enzyme is sensitive to environmental fluctuation and is considered as the measure of plants resistance to abiotic stress. Increased activities of chitinase and peroxidase after exogenously applied fungal polysaccharides were demonstrated by Slováková *et al*. (1993) [42] in cucumber and bean leaves, Plant growth promoting rhizobacteria has long been known to promote plant growth under abiotic stress conditions. PGPR inoculations elevated the chlorophyll reading values compared with the control. These findings are similar to those of Yildirim *et al*. (2008) [48] who found that PGPR increased the chlorophyll reading values of radish leaves under salt stress. Similarly increase in chlorophyll and soluble protein content was also recorded in shola species [41] with inoculation of *Azospirillum*+ *Phosphobacterium*. The positive influence of bioinoculants, FYM and IF on the chlorophyll content was in conformity with the findings of Sunil (2005) [44], who observed that, higher chlorophyll content in mulberry leaves was due to addition of organic manures, biofertilizers and inorganic fertilizer Sandhya *et al*. (2010) [40] reported that *Pseudomonas* inoculated maize plants showed increased antioxidant enzymes activity on exposure to drought stress. Our study confirms the relationship among the antioxidative enzymes and the rhizobacteria. Peroxidase and catalase activities increased by *Pseudomonas* treatments. These results could also be worth for future practical protection of different plants against pathogen attacks with induction of numerous histological and biochemical defensive responses.

Conclusion

The present study suggests that bio-inoculations could alleviate the deleterious effects of growth conditions in soil by activating enzyme activity. Our study confirms, *Pseudomonas* sp. treatment has increased maximum enzyme activation on the three genotypes of Guizotia *abyssinica (L.f) Cass*. Exploration for existence of valuable enzymes as well as understanding the appropriate physiological role of many hydrolytic enzymes in plants is still an open area of investigation. In addition, enzyme producing bioinoculants were successfully used in combination with other biocontrol agents, leading to a synergistic inhibitory effect against pathogen and enhances the Plant Growth promotion.

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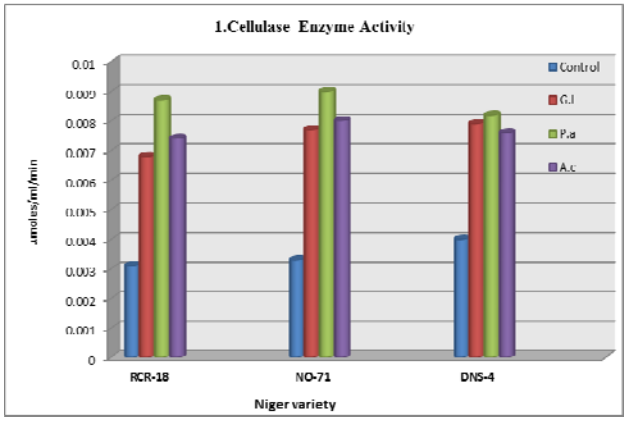
Table 1: Showing the pot experimental data on 3 varieties of Niger with respect Enzyme Activities in plant roots for 90 days.

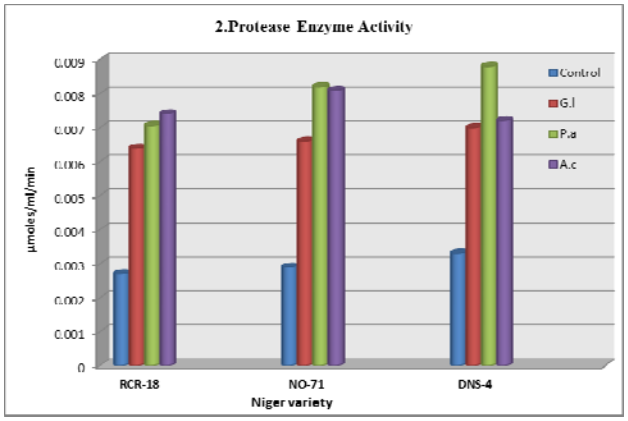
Sl no	Treatments	Enzyme activity (µmoles/ml/min)											
		Cellulase µmol/ml/min			Protease µmoles/ml/min			Peroxidase Units/ml.			Glucanase µmol/min		
		RCR-18	NO-71	DNS-4	RCR-18	NO-71	DNS-4	RCR-18	NO-71	DNS-4	RCR-18	NO-71	DNS-4
1	Control	0.0031	0.0033	0.0040	0.0027	0.0029	0.0033	141.62	153.60	149.07	0.33	0.23	0.47
2	*Glomus leptonicum*	0.0068	0.0077	0.0079	0.0064	0.066	0.0070	276.9	279.5	264.9	0.74	0.86	0.98
3	*Pseudomonas areoginosa*	0.0087	0.0090	0.0082	0.00706	0.0082	0.0088	278.9	272.8	289.6	0.98	1.76	1.12
4	*Azotobacter chroococcum*	0.0074	0.0080	0.0076	0.00741	0.081	0.0072	260.5	263.3	274.3	0.84	1.94	1.15

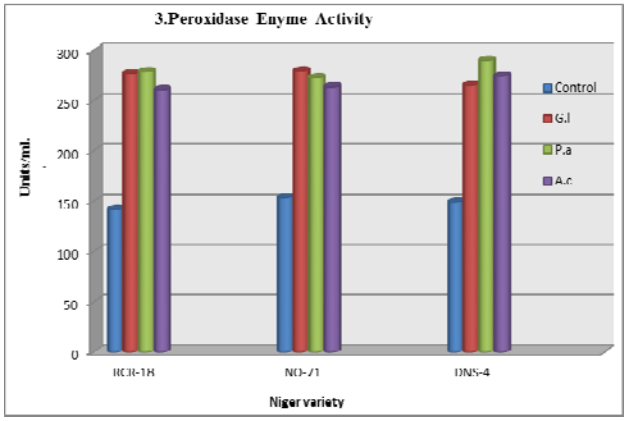
The specific activity was expressed in µmol of the reaction product per minute per mg protein

Table: Showing the pot experimental data on 3 varieties of Niger with respect Chlorophyll content in plant leaves and for 90 days.

Sl no	Treatments	Chlorophyll/gm tissue								
		RCR-18			NO-71			DNS-4		
		Chl-a /gm tissue	Chl-b /gm tissue	Total Chl-/gm tissue	Chl-a /gm tissue	Chl-b /gm tissue	Total Chl /gm tissue	Chl-a /gm tissue	Chl-b /gm tissue	Total Chl /gm tissue
1	Control	1.26	0.35	1.65	1.21	0.46	1.69	1.44	0.50	1.95
2	*Glomus leptonica*	1.50	0.61	2.12	1.28	0.52	1.80	1.49	0.65	2.15
3	*Pseudomonas aeruginosa*	1.64	0.74	2.36	1.43	0.66	2.10	1.50	0.65	2.16
4	*Azotobacter chroococcum*	1.57	0.69	2.27	1.36	0.64	2..13	1.52	0.63	2.15







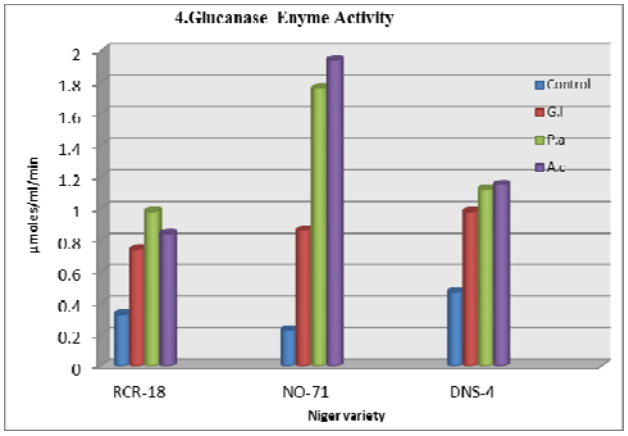
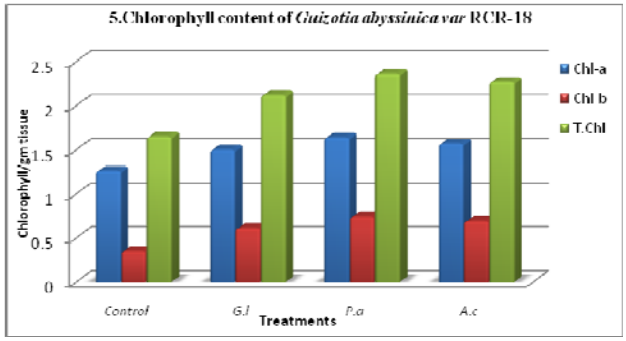
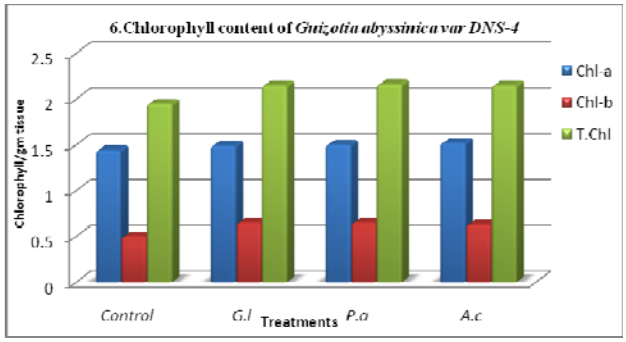


Fig 1-4: Showing the pot experimental data on 3 varieties of Niger with respect Enzyme Activities in plant roots for 90 days.





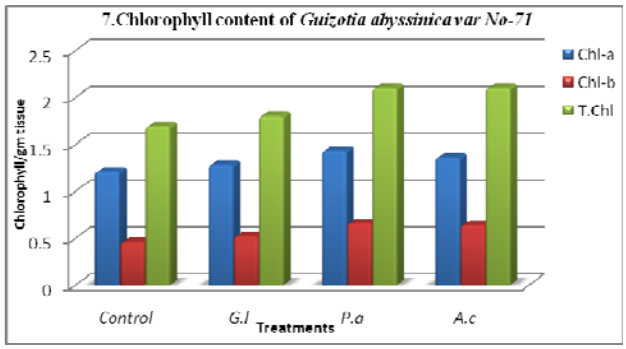


Fig 5-7: Showing the pot experimental data on 3 varieties of Niger with respect Chlorophyll content in plant leaves and for 90 days.

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