3

Materials and Methods

The study was conducted in a greenhouse located at Khetri, Kamrup (M) district of Assam, India, for two years (2018 - 2019). Two pulse crops viz. *Vigna radiata* L. (mung bean) and *Lathyrus sativus* L. (grass pea) were cultivated to study the effects of drought (at two different crop growth stages) and applied soil amendments (biochar and FYM) on plant growth, grain quality, soil biological properties, and mineralization of soil nitrogen and phosphorus.

3.1. Experimental location

Khetri is situated at the Lower Brahmaputra Valley Agro-climatic zone of Assam, India, and is geographically located around 26º06'50.9" N 92º03'27.2" E. The exact location of the greenhouse is presented in figure 3.1 and the region falls in a subtropical climatic zone with a monsoon type of climate. The cultivation period of *Vigna radiata* and *Lathyrus sativus* extends from November to February. The recorded air temperature during the cropping period was 9ºC - 34ºC with an average temperature of 20.8ºC. Relative humidity was 33%-98% with an average of 74.4%. The soil of the experimental site is typic inceptisol, characterized by recent and old alluvium soils having sandy to sandy-loam texture with the pH of 4.5.

3.2. Experimental design and materials used

3.2.1. Experimental Layout

Fifty-four plots of $1m^2$ each were prepared to accommodate all the treatment combinations with three replications of each treatment in a factorial randomized block design (Factorial RBD). A buffer zone of 0.3m was kept between the blocks to keep the treatments isolated. A distance of 10cm was maintained between the seeds along with a

distance of 30cm was kept between the rows as per the recommended package of practice.

Seeds were sown in 10th of November, 2017, 2018, and harvested on physiological maturity after 105 DAS. Water withdrawal was done for 15 days to simulate the drought conditions during vegetative (40-55 DAS) and reproductive stages (65-80 DAS) of crop growth.

3.2.2. Crops

The study was conducted with two test crops viz. *Vigna radiata* and *Lathyrus sativus* because of their contrasting nature. *Vigna radiata* being the popular grain legume cultivated globally, whereas *Lathyrus sativus* is considered as an orphan legume.

3.2.3. Soil amendments

3.2.3.1. Application rate and time

Selection of soil amendments were made based on locally available materials and conventional techniques. Selected soil amendments were applied following the package of practices recommended by the Assam agricultural university, Jorhat[1], and approved by the government of Assam, India, for this region. Soil amendments (biochar or FYM) were applied at the rate of 1 ton ha^{-1} before one week of seed sowing.

Table. 3.1: Table showing pH and bulk density of the basic soil and after the application of soil amendments.

Soil	pH	Bulk density
Control	4.5	1.19
Biochar amended	4.9	1.10
FYM amended	4.8	1.14

3.2.3.2. Sources of the soil amendments

FYM for the study was prepared by collecting cow dung from a local cow shed and mixed with straw residues at a ratio of 3:1. Biochar was produced conventionally by collecting mixed wood residues from a nearby sawmill. Following procedure was used for pyrolysis:

Procedure:

A sand bed over a bed of Bricks was prepared on which a metallic kiln could be placed firmly. A fire was ignited on the sand bed over a metallic basal plate. After the fire was properly ignited, the kiln was put over the plate so that air flow is not restricted. The feed material was fed into the kiln up to the 1/3 mark and fumes were allowed to disappear. When the flames started to rise above the kiln, the air inlet was sealed till the fire settled down. The kiln was covered from above once the fire settled down and sealed with clay so that no air exchange could occur. The kiln was allowed to cool down properly as the temperature inside the kiln remains high even after the outer kiln cools down. The biochar thus prepared was extracted and the process may be repeated if required.

Photographs showing different stages of conventional preparation of biochar

(a) Preparation of sand bed and igniting the fire

(b) Letting the fire ignite properly

(c) Putting the kiln over the fire and filling with feed

(d) Sealing the bottom air vents

(e) Letting the fire settle

(f) Sealing off the top with metal sheet and clay

(g) Allowing the kiln to cool down before extracting the biochar

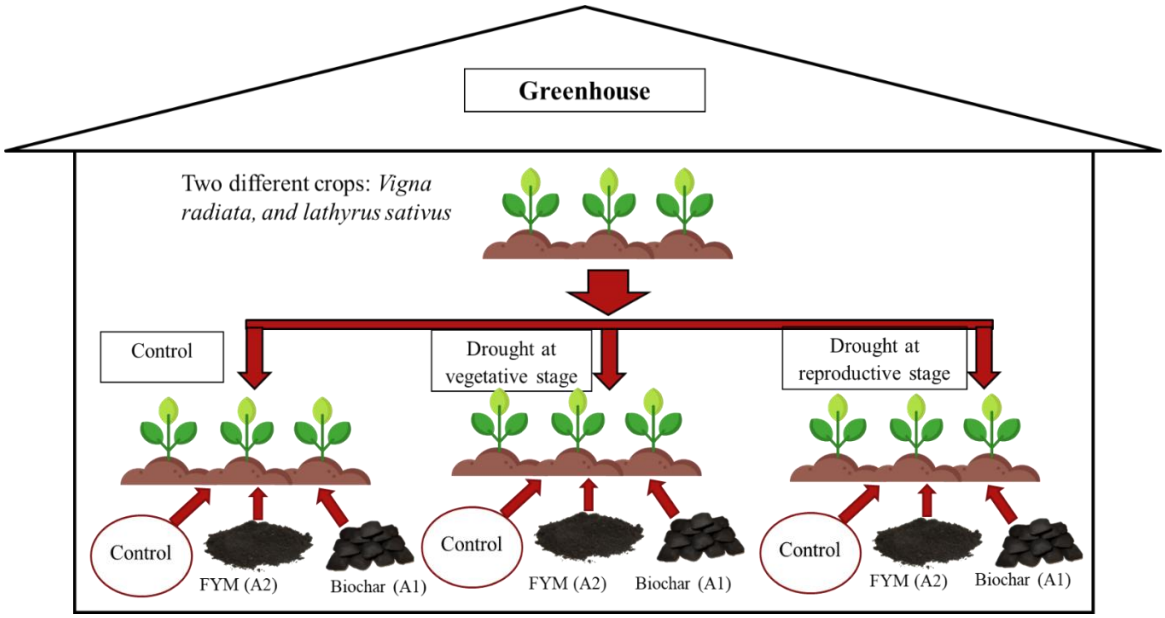


Figure 3.1: Experimental design

Index:

C1= Crop 1 (*Vigna radiata)*

C2= Crop 2(*Lathyrus sativus)*

T0= Control (No stress)

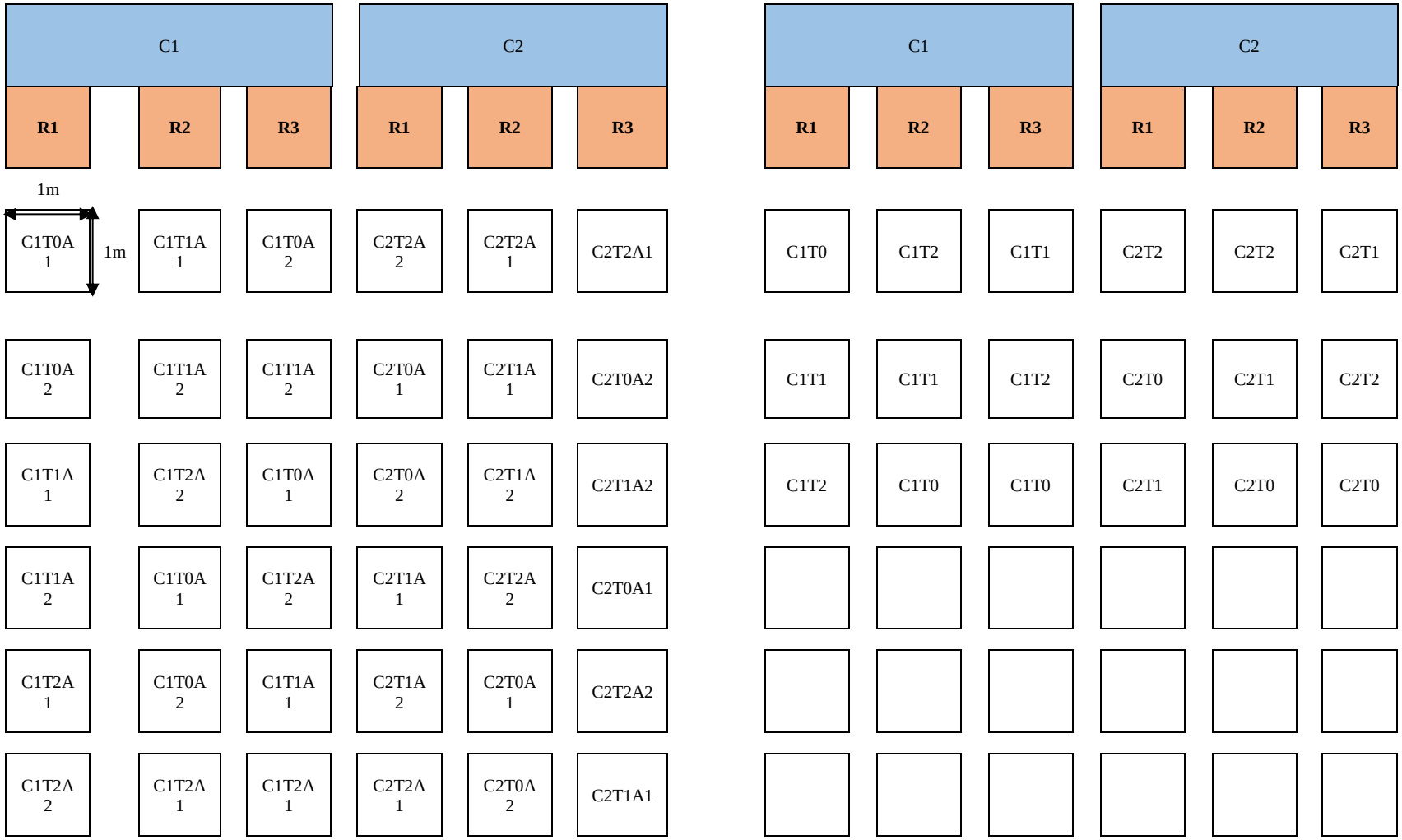
T1= Moisture deficit stress at vegetative stage.

T2= Moisture deficit stress at reproductive stage.

A1= Amendment 1 (Biochar)

A2= Amendment 2 (Farmyard manure)

Figure 3.2: Field layout of the study



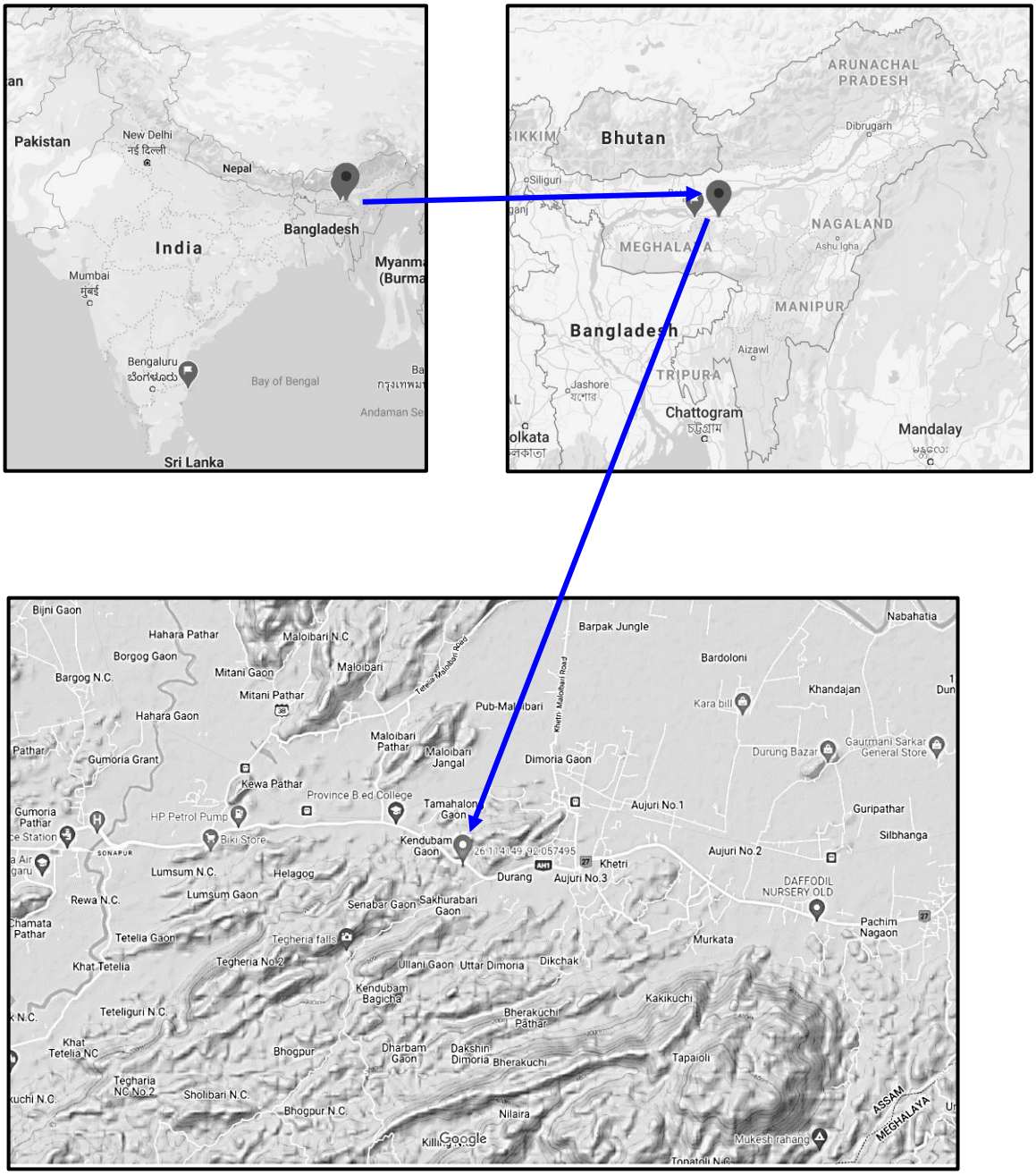


Figure 3.3: Experimental site at Lower Brahmaputra valley Agro-climatic zone Assam (Source: Google Maps)

3.3. Sampling

3.3.1. Soil sampling

Soil samples were collected from the top 15 cm of soil depth at the completion of the stress phase at either stage of crop growth and at harvest. Moreover, three samples of the soils from different areas of the greenhouse where randomly collected (before addition of soil amendments) to study the basic soil parameters. Three sub-samples of soil collected from each replication were mixed thoroughly to form a composite sample. The field-

moist composite sample was divided into two sub-samples. One set of sub-sample was stored in zipper bag at 4°C to stabilize the soil microbiological activity disturbed during soil sampling and handling until analyzed for biochemical and microbial parameters to fulfil the objective two. Care was taken to ensure minimal air inclusion in the zipper bags to avoid disturbance in the microbial diversity. The second sub-sample was air dried at laboratory conditions, sieved through a 2 mm sieve and stored in plastic zipper bags until soil physico-chemical parameters was analyzed.

3.3.2. Plant Sampling

On completion of the stress at either stage of crop growth, the plant samples were collected to analyze the biochemical parameters. At the same time, the non-destructive measurement was made for plant physiological parameters. Leaf water potential was measured using a plant water status console (PMS instrument Co. USA). The leaf photosynthesis rate was determined using a portable photosynthesis system (Model: Li-6400, Make: Li-Cor Instruments, USA).

3.4. Analyses

3.4.1. Objective 1

3.4.1.1. Ammoniacal Nitrogen

Soil ammoniacal N was measured according to Wang and Øien (1986)[2] by indophenol method where extraction for 10g soil was made for 1 hr with 2M KCl. 10ml of the extract was added with 10 ml of the NaOH-tartrate solution and mixed well. Now, 7ml of the alkaline phenol solution along with 5ml sodium hypochlorite reagent was added to it for colour development. The colour intensity was read at 635nm after 30 minutes using a spectrophotometer.

3.4.1.2 Nitrate Nitrogen

Nitrate N was estimated following Narayana and Sunil (2009)[3]**.** Soil sample (1g) was extracted with 5 ml of 0.5% sodium carbonate and filtered using Whatman filter paper No. 41 repeatedly. 25ml of the aliquots were mixed with 1ml of sulfanilic acid (0.5%) and 1 ml of HCl solution (2 mol L^{-1}) and agitated for 5 minutes to complete the diazotization reaction. An azo dye was created with 2 ml of 2 mol L^{-1} sodium hydroxide solution and 1 ml of 0.5% methyl anthranilate. The mixture was diluted to 10ml with water and the absorbance of the red colored dye was measured at 493 nm against the reagent blank.

3.4.1.3. Soil Organic Nitrogen

Soil organic N was measured following the method of Bremner (1960)[4]. Soil sample were extracted with 2 mol L^{-1} KCl (soil to solution ratio: 1:5) for 60 minutes. After carefully discarding the supernatant, the soil was once again extracted with 2 mol L^{-1} KCl (soil-to-solution ratio 1:2.5, w:v) for 20 min. After collection of the supernatant, the pellet soil was shaken for 20 minutes while being cleaned with distilled water, dried at 60°C, and then subjected to the Kjeldahl method for determining its N content.

3.4.1.3. Soil Microbial Biomass Nitrogen

The microbial biomass N of soil was estimated using the method of Joergensen and Brookes (2005)[5]. Soil samples (25g each) kept at 4°C was taken in beakers. One set of the samples were then put in 250 ml conical flasks with 100 ml of 0.5 M K_2SO_4 (extractant to soil ratio: 4:1) and shaken for 30 min. The duplicate sample sets were used for the fumigation treatment in a dessicator containing a beaker with ethanol-free $CHCl_3$, incubated for 24 hours at 25°C in dark, and extracted following the same procedure. The extractions were then analysed for total N following Kjeldahl method using chromium (III) potassium sulfate dodecahydrate, Zn powder and $CuSO_4$ as reducing agents.

1. Calculation of extractable total N

$$\text{N } (\mu g/g \text{ soil}) = (S - B) \times M \times N \times (VK + SW)\, A \times DM$$

S= HCl consumed by sample extract (µl)
B = HCl consumed by blank extract (µl)
M = molarity of HCl
N = molecular mass of nitrogen (14)
VK = volume of K_2SO_4 extractant (ml)
SW = total amount of water in the soil sample (ml)
A = sample aliquot (ml)
DM= total mass of dry soil sample (g)

2. Calculation of microbial biomass N

Biomass N = *E*N/*k*EN

*E*N= (total N extracted from fumigated soils) − (total N extracted non-fumigated soils)

*k*EN= 0.54 (Brookes et al., 1985; Joergensen and Mueller, 1996)[6,7]

3.1.4.4. Soil Total Nitrogen

Total soil N in the air dried samples were measured using CHN analyser (Model: 2400 SERIES 2, Make: PERKIN ELMER, USA).

3.1.4.5. Soil Oxidizable Carbon

Oxidizable soil carbon content was estimated following the protocol of Walkley and Black (1934)[8]. In a conical flask, 1g of soil (0.2mm) was treated with 20 ml of concentrated H_2SO_4 and 10 ml of 1N $K_2Cr_20_7$. Orthophosphoric acid and 200 ml of distilled water were added gradually on it. After adding 1 ml of diphenylamine indicator the solution mixture was titrated back using 0.5 N ferrous ammonium sulphate solution until the appearance of green color.

The calculation of oxidizable organic carbon was done as follows:

$$\%\ Oxidizable\ organic\ C = \frac{Vk\left(1 - \frac{V_s}{V_b}\right)}{W} \times S_k \times 0.3$$

Where,

V_k = Volume of $K_2Cr_2O_7$ (ml)

V_s = Volume of ferrous ammonium sulphate in samples (ml)

V_b = Volume of ferrous ammonium sulphate in blank (ml)

S_k = Strength of $K_2Cr_2O_7$ (N)

W = Weight of the soil (g)

3.1.4.6. Phosphorus Fractionation

Soil P was fractionated using the method of Zhang (2009)[9]. The soluble and loosely bound P was extracted with 1M NH_4Cl by shaking for 30 min and the supernatant was diluted with deionized water into a 50 ml volumetric flask (extract A).

Al-P in the residue was extracted with 0.5 M NH_4F (pH 8.2) for an hour and the supernatant was collected (extract B). The soil residues were subsequently washed with saturated NaCl solution, combined and stored for further analyses.

Fe-P in the residue were extracted with 1 M NaOH by shaking for 17 hours and collecting the supernatant into a 100 mL volumetric flask (extract C). Saturated NaCl washings of the residues were combined with extract C and brought to volume for further analyses.

Reductant soluble P was extracted with $Na_3C_6H_5O_7.2H_2O$ (0.3 M) and 5 mL of $NaHCO_3$ (1 M) by heating at 85°C for 15 minutes. After adding 1g of sodium dithionate ($Na_2S_2O_4$), the mixture was quickly agitated and heated for 15 minutes. The supernatant was collected in a 100 ml volumetric flask (extract D) and the residue was washed twice

with saturated NaCl. The washings were combined with extract D and oxidized by exposing to air before further analyses.

The Ca-P in the soil residue was extracted with H_2SO_4 (0.25 M) by centrifugation and the supernatant was collected in a 100-mL volumetric flask (extract E). Saturated NaCl washings of the residue were collected and made up to volume.

An aliquot containing 2 to 40 g P was transferred to separate 50 ml volumetric flasks from extracts A, B, C, D, and E. Flasks containing extracts C and E were filled with five to ten ml of deionized water, five drops of p-nitrophenol indicator, and the pH was then adjusted with either 2 M HCl or 2 M NaOH until the indicator showed a bare change in color. Flasks containing extracts C and E changed color from yellow to colorless, and colorless to yellow, respectively. The extract B was added in volumetric flask with 15 ml of 0.8 M H_3BO_3. The phospho-molybdate method was used to calculate the concentrations of phosphorus in the various solutions (Murphy and Riley, 1962)[10].

3.4.2. Objective 2

3.4.2.1. Soil Aryl Sulphatase activity

Arylsulphatase activity was calculated following Tabatabai & Bremner (1970)[11]. Field moist soil (1g) was extracted with 4ml of acetate buffer (500mM, pH 5.8) for two hours. 1ml distilled water was added to the samples and the reaction was terminated by adding 1 ml of 500 mM $CaCl_2$ and 4 ml of 500 mM NaOH. Samples were then shaken in a rotary shaker and intensity of p-nitrophenol was measured at 400nm after centrifugation. Sample controls were measured similarly with substrate instead of distilled water. A standard curve was generated using a range of p-nitrophenol concentrations in distilled water between 0 and 50 ug ml^{-1}.

3.4.2.2. Soil *ß*-Glucosidase Activity

Soil *ß*-Glucosidase Activity was measured following the protocol of Tabatabai (1983)[12]. 1g of field moist soil was extracted using 4 ml tris buffer (pH-12) and 1 ml substrate (25 mM). Soils were then vortexed and incubated at 20°C for 2 hours. After adding 1 ml of distilled water to the samples, the reaction was stopped by adding 1 ml of 500 mM NaOH and incubated at 20°C for 30 minutes. 1.5 ml aliquots were centrifuged and the color intensity of extracted samples were measured at 400nm in a spectrophotometer (Model: Biospectrometer kinetic, Make: Eppendorf, USA). Sample controls were analyzed similarly with 1ml of substrate instead of distilled water.

3.4.2.3. Soil Dehydrogenase Activity

Soil dehydrogenase activity was estimated according to Von Mersi & Schinner (1991)[13]. Field moist soil (1g) was extracted with 1.5 ml of 1M tris buffer (pH 7.0), 2 ml of INT (5 mg ml^{-1}) in 2% v/v N, N-dimethylformamide in foil-wrapped test tubes. After a brief vortexing, the samples were incubated for 24 hours at 20ºC, followed by an addition of 2ml of distilled water. The reaction was stopped by adding 10 ml of 1:1 mixture of N, N-dimethylformamide/ethanol extractant and shaking the mixture for one hour. The aliquots (1.5 ml) were centrifuged five minutes and the absorbance of the supernatants was measured at 464 nm in a spectrophotometer. The controls were analyzed following the aforementioned protocol but the substrate was replaced with distilled water during extraction.

3.4.2.4. Soil FDA Hydrolysis activity

Soil FDA hydrolysis was assessed using the Schnurer and Rosswall (1982)[14]. Field moist soil (3g) was mixed with 50ml of phosphate-buffered saline (PBS) and 250 µl of FDA (2 mg ml^{-1}). Parallel set was run as the control using 250 µl distilled water. The soil suspensions were incubated for 4 hours at 20°C. Following incubation, 250 µl of distilled water was added in the flasks with soil samples, while 250 µl of FDA was added in the controls. The reaction was stopped by extracting 5 ml of the subsamples and 5 ml of acetone was added after vortexing the suspensions. Now, 1.5 ml of aliquots were centrifuged for 5 minutes and the optical density of the supernatant was measured at 490 nm.

3.4.2.5. Phosphomonoestrase Activity

a. Acid phosphomonoesterase

Acid phosphomonoesterase activity was done according to the methods of Speir and Ross (1978)[15]. Moist field soil (1g) was extracted using 4ml of modified universal buffer (pH 4) and 1ml of the substrate (15mM). One control was run using 1ml of sterile distilled water. The soils were quickly vortexed and incubated at 20°C for 2 hours. Then 1 ml of distilled water and 1 ml of substrate were added to samples and controls respectively. The reaction was stopped by adding 1 ml of 500 mM NaOH and the suspensions were agitated on a rotary shaker for 30 minutes. After collection, the aliquots (1.5 ml) were centrifuged and the color intensity was measured at 400nm in a spectrophotometer.

b. Alkaline phosphomonoesterase

Alkaline phosphomonoesterase activity was performed following the protocol of Speir and Ross (1978)[15]. 1g of field moist soil was extracted with 4ml of modified universal buffer (pH 9) and 1ml of substrate (15mM). The soils were vortexed and incubated at 20°C for 2 hours. Following the addition of 1 ml of distilled water, the reaction was stopped by adding 1 ml of 500 mM NaOH and shaken for 30 minutes. Aliquots (1.5 ml) were centrifuged and the color intensity of the extracted samples were measured at 400nm. Sample controls were measured following the same method with 1ml distilled water instead of the substrate.

3.4.2.6. Urease Activity

Urease activity was conducted using a modified version of Kandeler and Gerber (1988)[16], and Naseby and Lynch (1997)[17]. Field moist soil (5g) were extracted using 2.5 ml of urea (80 mM) and 20 ml of borate buffer (75 mM; pH 10) and incubated for 4 hours at 20°C. After 4 hours, 2.5 ml of sterile deionized water was added before extraction with 30 ml of acidified KCl (2M). The suspensions were shaken for 30 minutes on a rotary shaker and the supernatant (1.5 ml) were centrifuged, combined with 1 ml of distilled water, 5 ml of sodium salicylate/NaOH solution, and 2 ml of dichloroisocyanuric acid. The absorbance was measured at 690 nm after it had been exposed to a temperature range of 20°C for one hour. Using a calibration curve of standard ammonium chloride solution (ranging from 0-2.5 ug m^{-1}), the ammonium concentrations were calculated. Controls were extracted with 20 ml of borate buffer with 2.5 ml of distilled water and analyzed in a similar manner.

3.4.2.7. Bacterial, fungal and actinobacterial count

Fungi, bacteria and actinobacteria, defined as the total number of colony-forming units, were enumerated by a classical serial dilution technique (at 10^4 dilution) using Czapek-Dox, nutrient agar media, and glucose-yeast extract respectively.

3.4.2.8. Microbial Biomass Carbon (Fumigation extraction method)

Microbial biomass carbon was measured using the protocol of Voroney (1993)[18]. Three sets of 10g of soil were weighed and the moisture content of the soil was determined with one set. One set was fumigated with chloroform in a dessicator for 24 hours in the dark. Both the fumigated and unfumigated samples were extracted with 25 ml 0.5 M K_2SO_4. The resulting suspension was filtered using Whatman filter paper (No. 1), and 10 ml of the filtrate was taken. 2 ml of 0.2 N $K_2Cr_2O_7$, 10 ml of concentrated H_2SO_4, and 5 ml of orthophosphoric acid were added for digestion followed by heating at 100°C for 30

minutes. 50 ml distilled water was added after cooling and titration was carried out against 0.005 N ferrous ammonium sulphate using 1-2 drops of ferroin indicator to obtain a brick red end point.

Calculation:

1. Soil water content (WS %)

$$WS = \frac{\text{weight of wet soil (g)} - \text{weight of oven dried soil (g)}}{\text{weight of oven dried soil (g)}} \times 100$$

2. Weight of soil sample (oven dry weight equivalent) (MS, g)

$$MS = \frac{\text{weight of wet soil (g)}}{\{100+\text{WS (\%)}\}} \times 100$$

3. Total volume of solution in the extracted soil (VS, ml)

   VS = wet soil weight − oven dry soil weight + extractant volume

4. Determination of extractable C (Ext C in µg ml^{-1})

   a. Volume of $K_2Cr_2O_7$ consumed (Y, ml)

$$Y = \frac{\text{Normality of FAS} \times \text{Titrant volume}}{\text{Normality of } K_2Cr_2O_7} \times 100$$

   b. Volume of $K_2Cr_2O_7$ consumed for oxidizing easily mineralizable C in 10 mL of extractant = 2-Y ml

   c. Amount of extractable C (Ext C in µg ml^{-1})

$$= \frac{600 \times (2 - Y)}{10}$$

5. Total weight of extractable C (µg g^{-1} soil) in fumigated (CF) and non-fumigated (CNF) samples

$$CF\ of\ CNF = Ext\ C \times \frac{VS}{MS}$$

6. Microbial biomass carbon (µg g^{-1} soil or mg kg^{-1} soil)

$$= \frac{CF - CNF}{K}$$

Where, *K*=0.25 and represents the efficiency of extraction of microbial biomass carbon.

3.4.3. Objective 3

3.4.3.1. Root, Shoot and Pod Biomass

Harvested plant materials were dried at 60ºC for 48h and the weight of the dried plant materials were noted.

3.4.3.2. Grain carbohydrates

Following Dubois (1956)[19] and Krishnaveni (1984)[20], total grain carbohydrate was measured using phenol-sulphuric acid digestion. Grain sample (100 mg) was hydrolyzed in a boiling water bath for 3 hours with 5 ml of 2.5N HCl and cooled to room temperature. The samples were neutralized using solid sodium carbonate, diluted to 100ml and centrifuged. 0.1 ml of the sample was then taken in a different test tube with 0.9 ml distilled water. To each tube, 1 ml of phenol solution and 5 ml of concentrated H_2SO_4 was added and thoroughly mixed. The test tubes were shaken, submerged in a water bath for 20 minutes at 25-30ºC and the absorbance was read at 490nm.

3.4.3.3. Grain Crude Protein

Harvested grains were dried at 60ºC for 48h and stored at 24ºC under demoisturized conditions until laboratory analyses. Grains were finely crushed in a mortar pestle and passed through a 0.1 mm sieve prior to analyses. The grain crude protein was estimated in a CHN analyser (Thermo-Fisher, Model: Flash 2000) using the powdered samples and total protein was calculated subsequently by multiplying with the Jones' factor.

3.4.3.4. Grain Phytic Acid Content

Grain phytic acid content was measured using the protocol of Wheeler and Ferrel (1971)[21]. 250mg of finely crushed grains were extracted in 50ml of 3% TCA for 30 minutes. Suspension was centrifuged, and the supernatant was divided into 10 ml portions. $FeCl_3$ solution (4 ml) was added to the aliquots and heated for 45 minutes with 1-2 drops of 3% sodium sulphate in 3% TCA. The content was centrifuged and the supernatant was decanted. The precipitate was thoroughly dispersed in 20 ml of 3% TCA, boiled for 5-10 minutes and centrifuged before being repeatedly rinsed in distilled water. The precipitate was washed in a few ml of water, mixed in 3ml of 1.5 N NaOH and diluted to 30 ml. Now, the solution was heated for 30 minutes and filtered using Whatman No.2 paper. The filtrate was discarded after washing the precipitate in 60–70 ml of hot water. The precipitate thus obtained was removed from the paper using 40 ml hot 3.2 N HNO_3 and the volume was made up to 100ml. A 5 ml aliquot was diluted to 70 ml and 20 ml of 1.5 M KSCN were added to it. The absorbance was measured at 480 nm

within one minute. Each set of samples included a blank sample that was measured as well.

The phytate was calculated as follows:

$$Phytate\ P\ (mg\ 100g^{-1}) = \frac{\mu g\ Fe \times 15}{weight\ of\ the\ sample\ (g)}$$

3.4.3.5. In-vitro Protein Digestibility of the grains

According to Saunders et al. (1973)[22], in vitro protein digestibility was calculated using powdered samples suspended in 0.1 N HCl containing 1.5 mg of pepsin for 3 hours. It was treated with 4 mg of pancreatin in 7.5 ml of 0.2 M phosphate buffer (pH 8.0) containing 0.005 M sodium azide, and the combination was again incubated at 37ºC for 24 hours after being neutralised with 0.5 N NaOH. With the aid of 10 ml of 10% trichloroacetic acid, the process was stopped. After centrifugation, 5 ml of the clear supernatant were collected for analysis using the micro-Kjeldahl technique.

$$\text{Protein digestibility (\%)} = \frac{(\text{T}-\text{B}) \times N \times 14 \times TB}{(X) \times a}$$

Where,

$$X = \frac{250 \times CP\%}{100 \times 6.25}$$

N = Normality of HCl, T =ml of titre, B = ml of blank, a =Number of ml of aliquot, TV = Total volume of the mixture, 14 =Equivalent weight of nitrogen, 250 =Sample weight in mg, CP% = Percent total protein.

3.4.3.6. Protein Fractions in the grains

According to Osborne et al. (1914)[23], protein fractions in the grains were analyzed. Protein fractions were isolated based on their solubility in various solvents as described. 2g of grains (powered) were extracted twice with 30ml of 1M NaCl for 30 min and centrifuged at 3000 rpm. at room temperature using a mechanical shaker. The micro-Kjeldahl method was used to determine the protein content of 10ml of the extract (globulin). The residue was then sequentially extracted using distilled water (albumin), 70% ethanol (prolamin), and 0.2% NaOH (glutelin) and analyzed successively in a similar manner.

3.4.3.7. Grain Mineral Contents

Mineral contents in grass pea grains were estimated using ICP-MS (Thermo-Fisher instruments, Model: iCAP RQ ICP-MS) after microwave digestion of the samples following the method of Petterson et al. (1997)[24], Kingston and Jassie (1988)[25] and later modified by Sah and Miller (1992)[26].

3.4.4. Supporting Data Results for objective 3

3.4.4.1. Photosynthesis

The rate of photosynthesis during the drought imposition phase was determined using a portable photosynthesis system (Model: Li-6400, Make: Li-Cor Instruments, USA) between 10am to 11.30am of the day.

3.4.4.2. Leaf Water Potential

Leaf water potential was measured using a plant water status console (PMS instrument Co., Albany, Oregon) during the drought treatments.

3.4.4.3. Peroxidase Activity

Peroxidase activity was measured according to the protocol of Putter (1974)[27] and Malik (1980)[28]. 0.1 ml of enzyme extract, 0.05 ml of guaiacol solution, 3 ml of buffer solution, and 0.03 ml of hydrogen peroxide solution was added to a cuvette. After thoroughly mixing, the absorbance was read in a spectrophotometer. The time taken to rise in absorbance by 0.05 was noted and the time needed to raise the absorbance by 0.1 minutes was recorded.

3.4.4.4. Superoxide dismutase activity

A modified method reported by Beauchamp and Fridovich (1971)[29] was used for SOD determination. 50 mM potassium buffer (pH 7.8) 13 mM methionine, 2 uM riboflavin, 0.1 mM EDTA, 75 uM NBT, and 50 µl of crude enzyme extract, in duplicate, make up the reaction cocktail. Add double the amount of distilled water to make the volume equal. Control was established that included NBT without enzyme. exposed each tube for 15 minutes to a 400 W bulb. At 560 nm, the absorbance was instantly read. Units/mg of protein are used to express the enzyme activity.

3.4.4.5. Leaf Proline content

Leaf proline content was measured following the protocol of Bates et al. (1973)[30]. Leaf proline content was measured by homogenising 0.5g leaf in 10ml of 3% aqueous sulphosalicylic acid and filtered using Whatman No. 2 filter paper. Glacial acetic acid and acid ninhydrin were added to 2 ml of the filtrate in a test tube and boiled for one

hour in a water bath. The tube was submerged in an ice bath to stop the reaction and 4ml of toluene was added followed by brief vortexing. The absorbance of the toluene layer was recorded at 520 nm. In a similar manner, a series of standards using L-proline were conducted to create a standard curve.

3.4.4.6. Leaf Protein Content

Leaf protein content was measured following Lowry et al. (1951)[31]. 0.1 ml of sample extract was pipetted and the volume was made up to 1 ml with distilled water. Alkaline copper solution was added in 5 ml to all tubes, including the blank. After thoroughly mixing the solution, it is left to stand for 10 minutes. After that, 0.5 ml of Folin-Ciocalteau's reagent was added, thoroughly mixed, and incubated for 30 minutes at room temperature in the dark. The absorbance of blue color was observed at 660 nm. The sample's protein content was determined using a standard graph. The amount of protein is expressed as mg/g sample.

3.5. Statistical Analyses

Analysis of variance (ANOVA) was done for the data sets at $P \leq 0.05$ using Statistical Package for the Social Science (SPSS 16.0) for Windows. Tukey's honestly significant difference (HSD) test was done alongside for the data sets to indicate a significant difference among the data at 5% significance level ($P \leq 0.05$) and Pearson's correlation (2 tailed) was performed using SPSS 16.0 for Windows. The graphical representation of the data was done using Graphpad Prism 8.3.0.

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