CHAPTER 3:

MATERIALS AND METHODS
This study was conducted in the experimental site of Tezpur University, Napaam, Assam, India for a period of three years (2012-2015). One of the popular upland crop sequences of the region, cereal (wheat) – summer vegetable (okra) - pulse (green gram), was used for the study to investigate the role of inorganic (especially N) fertilizers and organic amendments (FYM, vermicompost and biochar) on the dynamics and enhancement of soil organic carbon (SOC) and its fractions under two water regimes (rainfed and irrigated). The changes in soil physicochemical and biological properties along with crop responses to applied management practices were also studied during the experimental period.

3.1. EXPERIMENTAL SITE

Tezpur University is situated at North Bank Plain Agro-climatic Zone of Assam, India and is geographically located around 26°14´ N and 92°50´ E. The experimental site is presented in Figure 3.1. It falls in the subtropical climatic region having monsoon type of climate with an average temperature of 18 ºC to 36 ºC during the summer months (April to September). Winter extends from the month of October till February with an average temperature ranging from 7 ºC to 22 ºC. The region receives an average total annual rainfall of 1851 mm with peak rainfall during late June to early September. The monthly average temperature (minimum and maximum) and total rainfall during the experimental period are presented in Figure 3.2. The soil of the experimental site is typic inceptisol which is characterized by recent and old alluvium soils having sandy to sandy-loam texture with slight to moderate soil acidity. The basic soil characteristics are presented in Table 3.1.

3.2. EXPERIMENTAL DESIGN AND MATERIALS USED

3.2.1. Experimental layout

Field was initially ploughed with a tractor (3 times) to completely remove the previous vegetation cover. After ploughing, the field was levelled properly and divided into plots of size 2 m × 2 m with a buffering zone of 1 m in between.
Figure 3.1: Experimental site at North Bank Plain Zone of Assam
Figure 3.2: Meteorological parameters during the experimental period (November, 2012 to October, 2015)

3.2.2. Crops
To fulfill the objectives of the present study, one of the popular upland cropping sequence of Assam with - wheat, okra and green gram were used.

3.2.2.1. Varietal description
Wheat variety DBW 39 was used. This is a semi-dwarf variety with a duration of 105-110 days. This variety is derived from ATTLA and HUI and developed by Directorate of Wheat Research, Karnal, Haryana, India.

Okra variety used was OH-397. The average days to first plucking is 40-45 days and approximately 10-12 plucking can be done.

Green gram variety used was Pratap. It was developed by Assam Agricultural University (AAU), Jorhat from the parental material ML 56 and PIMS 1. The average maturity period of the variety is 65-70 days.
Table 3.1: Basic soil physical and chemical properties (Mean ±SE)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Top soil (0-15 cm)</th>
<th>Sub soil (15-30 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>53±0.45</td>
<td>-</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>18±0.32</td>
<td>-</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>27±0.43</td>
<td>-</td>
</tr>
<tr>
<td>Bulk density (Mg m$^{-3}$)</td>
<td>1.11±0.08</td>
<td>1.19±0.05</td>
</tr>
<tr>
<td>Water holding capacity (%)</td>
<td>44.4±1.4</td>
<td>43.1±1.1</td>
</tr>
<tr>
<td>pH</td>
<td>5.69±0.2</td>
<td>5.64±0.2</td>
</tr>
<tr>
<td>Available nitrogen (kg ha$^{-1}$)</td>
<td>225±3.21</td>
<td>176±2.10</td>
</tr>
<tr>
<td>Available phosphorus (kg ha$^{-1}$)</td>
<td>36±0.68</td>
<td>29±0.98</td>
</tr>
<tr>
<td>Available potassium (kg ha$^{-1}$)</td>
<td>230±2.95</td>
<td>161±3.12</td>
</tr>
<tr>
<td>Soil organic carbon (%)</td>
<td>1.29±0.11</td>
<td>0.75±0.05</td>
</tr>
<tr>
<td>Particulate organic carbon</td>
<td>0.64±0.02</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>Microbial biomass carbon (mg kg$^{-1}$)</td>
<td>154±12</td>
<td>134±11</td>
</tr>
<tr>
<td>Humic acid carbon (%)</td>
<td>0.33±0.02</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>Fulvic acid carbon (%)</td>
<td>0.41±0.01</td>
<td>0.33±0.01</td>
</tr>
</tbody>
</table>

3.2.2.2. Seed sowing and harvesting

**Wheat:** Wheat seeds were sown during third week of November (15$^{th}$ to 20$^{th}$) in each year throughout the experimental period and was harvested in first week of April. Seeds were sown at the rate of 100-120 kg ha$^{-1}$ with a row to row distance of 20 cm.

**Okra:** Seeds of Var. OH-397 were sown in mid-April and harvested in the last week of July each year. Okra seeds were sown at a distance of 45 cm and 30 cm to maintain a row to row and plant to plant distance respectively.
Green gram: Seeds were sown in mid-August in each year with a row to row and plant to plant distance of 30 cm and 10 cm respectively maintaining a seed rate of 18-20 kg ha\(^{-1}\) and was harvested in the last week of October.

3.2.3. Fertilizer
3.2.3.1. Application rate and time
Fertilizers (both organics and inorganics) were applied as per the package of practice recommended by Assam Agricultural University, Jorhat and approved by the Government of Assam, India for this region.

After preparation of the seed bed, inorganic fertilizers were applied as basal doses (except for wheat) one day before seed sowing. The organic amendments were applied 10 days before seed sowing and mixed thoroughly (ploughed) with soil.

Wheat: The recommended doses of NPK is 80:42:34 and 40:20:20 kg ha\(^{-1}\) for irrigated and rainfed condition respectively. Half of N and whole quantity of P and K were applied as basal dose one day before seed sowing whereas the remaining N (50%) was applied at crown root initiation stage (21 days after sowing) just before first irrigation.

Okra: NPK @ 50:50:50 kg ha\(^{-1}\) (100%) was applied as basal dose one day before seed sowing.

Green gram: Fertilizers were applied @ 15:35:10 kg N:P:K ha\(^{-1}\) (100%) as basal dose one day before seed sowing.

3.2.3.2. Sources of fertilizers
Inorganic fertilizers
Commercially available mineral fertilizers are used as sources of inorganic fertilizers as follows. The nutrient content of the applied fertilizers are presented in Table 3.2.

- Urea [CO(NH\(_2\)] was used as a source of inorganic nitrogen.
- Single super phosphate [Ca(H\(_2\)PO\(_4\))\(_2\).H\(_2\)O] was used as phosphorus source.
- Muriate of potash [KCl] was used as potassium source.
**Organic amendments**

The organic amendments used for our experiment was collected locally (FYM and biochar) and prepared near the experimental site (vermicompost). Before application to the field, the physicochemical characterization of applied organic amendments was done using standard methods (as described in section 3.4.1.) and are presented in Table 3.3.

**Farmyard manure:** Cow dung mixed with straw and garden wastes (3:1) was used as FYM for the experiment.

**Vermicompost:** Vermicompost was prepared from crop residue and garden waste in the vermicompost unit of Tezpur University. The feed materials (straw and garden wastes) were mixed with fresh cow dung at a ratio of 2:1 and then after 15 days *Eisenia fetida* was introduced. Vermicomposting was then continuously monitored for optimum moisture content and water was sprinkled after every 6-7 days. The vermicompost was ready after 65 days and sieved through 2 mm sieve before application to the field.

**Biochar:** Commercially available biochar prepared from mixed hard wood was used in the experiment. Before application to the field, biochar was ground and sieved with 2 mm sieve.

**3.2.4. Treatments**

**3.2.4.1. For objective 1**

To fulfil the objective 1, field experiments were conducted for two consecutive years (November, 2012 - October, 2014). Twenty four plots were prepared to accommodate 6 fertilizer treatments with 4 replications and were laid in a completely randomized block design. The recommended inorganic N fertilizer doses for each crop under irrigated condition were modulated keeping P and K dose and other intercultural operations uniform. The following treatment combinations were.

- **T1:** Control
- **T2:** NPK @100% of the recommended inorganic NPK
- **T3:** N @ 120% and PK @ 100%
The experimental layout is given in Figure 3.3.

### 3.2.4.2. For objective 2

For objective 2, field experiments were conducted from November, 2013 to October, 2015. Twelve fertilizer treatments (combining organic amendments and inorganic fertilizers) under 2 water regimes (rainfed and irrigated conditions) with 3 replications (72 plots) were laid in a factorial randomized block design. The treatments were laid with water regime as the main plot effect and fertilizer treatment as sub-plot effect. The following fertilizer treatments were used:

- **T1**: Control
- **T2**: NPK @100% of the recommended inorganic NPK
- **T3**: N @ 50% and PK @ 100%
- **T4**: Farmyard manure (FYM) @ 5 t ha\(^{-1}\)
- **T5**: Vermicompost @ 5 t ha\(^{-1}\)
- **T6**: Biochar @ 5 t ha\(^{-1}\)
- **T7**: 100% NPK + 2.5 t ha\(^{-1}\) FYM
- **T8**: 100% NPK + 2.5 t ha\(^{-1}\) vermicompost
- **T9**: 100% NPK + 2.5 t ha\(^{-1}\) biochar
- **T10**: 50% N + 100% PK + 5 t ha\(^{-1}\) FYM
- **T11**: 50% N + 100% PK + 5 t ha\(^{-1}\) vermicompost
- **T12**: 50% N + 100% PK + 5 t ha\(^{-1}\) biochar

The experimental layout is given in Figure 3.4.
Figure 3.3: Experimental layout of objective 1
Figure 3.4. Experimental layout of objective 2
3.2.4.1. For objective 3
To fulfil objective 3, laboratory incubation experiments were conducted to study the soil C-mineralization. Soil samples at harvest of each crops from the experiments conducted under objective 2 were used for this study. Composite soil samples (100 grams) from each treatment were incubated at 27±3 ºC at 60% of water holding capacity for a period of 90 days in sealed polyethylene bottle.

3.2.5. Irrigation
For quick and uniform germination, one pre sowing irrigation (3-4 days before sowing) was applied in all the three tested crops during the experimentation.

Wheat: Two irrigations (flood irrigation) of 60 mm were applied during the crop growth period. The first was applied at crown root initiation stage (22 days after sowing) and the second at heading stage (75 days after sowing) of the crop.

Green gram and okra: Based on the duration of rainless period, irrigation (drip irrigation) was applied at a regular interval of 4-5 days.

3.2.6. Intercultural operations
Wheat: First weeding was done when plants attain 4-5 leaf stage. Another two weeding were also performed at 45-55 and 80-85 days after sowing.

Okra: First weeding at 30-35 days after seed sowing was followed by earthling up to strengthen the collar. Another weeding was performed at 50-55 days after sowing.

Green gram: Single weeding at 20-25 days after sowing was performed.

3.3. SAMPLING
3.3.1. Soil sampling
During the experimental period (November, 2012 to October, 2015), soil sampling was done at harvest of each crop. Iron core of 6 cm diameter and 45 cm long was used to
collect the soil. Soil from the core was separated into 0-15 cm (Top soil) and 15-30 cm (Sub soil) depth layers. Before setting up of the experiment, 5 soil samples were randomly collected from the experimental site to characterise the basic soil parameters which are presented in Table 3.1.

Three sub-samples of soil from different areas of each replication were mixed thoroughly to form a composite sample. The field-moist composite sample was divided into three sub-samples. One sub-sample was oven dried at 105°C for 24 h to determine moisture content and physical soil properties. Second sub-sample was kept at 4°C in plastic bags for few days to stabilize the soil microbiological activity disturbed during soil sampling and handling after which it was analyzed for biochemical parameters. The third sub-sample was air dried at laboratory conditions, sieved through 2 mm sieve and stored in plastic zeeper bags till completion of analysis for chemical parameters.

3.3.2. Plant sampling
Plant sampling was done at different growth stages of the crop namely, vegetative, flowering and maturity. However, the results presented are the average of the vegetative stage (all sampling till initiation of flowering), flowering stage (flower initiation to maturity) and at harvest. Three plants from each replication were tagged to record the morphological parameters throughout the crop growth period. For measuring the photosynthesis, first fully expanded young leaf of wheat, fourth and fifth fully expanded young leaves from top of okra and fully expanded second and third young leaves from top of green gram were selected.

3.4. ANALYSES
3.4.1. Organic amendments
The organic amendments (FYM, vermicompost and biochar) were air dried and sieved through 2 mm sieve before analysis.

3.4.1.1. Bulk density (BD)
Bulk density was estimated using the method given by Tripathi [1].

Procedure:
1. Weight (W1) and volume (V) of the empty bulk density bottle were taken.
2. The bottle was then filled with the organics upto the rim and repeatedly tapped for about 15-20 times. This tapping was assumed to produce the intensity of packing
3. The final weight (W2) was then recorded after capping the bottle.
4. Bulk density (Mg m\(^{-3}\)) was measured using the following formula
   \[
   \text{Bulk density} = \frac{W2 - W1}{V}
   \]

3.4.1.2. Soil water holding capacity

Water holding capacity of the amendments was estimated following Tripathi [1].

**Apparatus used:**
1. Keen box
2. Flat bottom soaking tray

**Procedure:**
1. The weight (W1) of empty box with a Whatmann No. 42 filter paper at its bottom was recorded.
2. The box was filled (about half of the box) with sieved air dried amendments. The weight of the box (W2) with dry amendments + filter paper was recorded.
3. The box was then kept on a soaking tray and the tray was filled with water till the level was 1 cm above the base of the box. The tray was covered to prevent evaporation and kept undisturbed for 12 hours.
4. A blank test with only filter paper was also run and the weight was recorded (W3).
5. The box is carefully removed from the tray and water from outside was wiped. The weight of the box (W4) was recorded.
6. The box was then oven dried for 24 hours at 110 °C and the weight was recorded (W5).

**Calculation:**

To calculate WHC, the following weights were computed:
1. Total water in wet soil (S1): W4-W1-W3
2. Weight of oven dried soil (S2): W5-W1
   \[
   \text{Water holding capacity(\%)} = \frac{S1}{S2} \times 100
   \]
Table 3.2: Nutrient composition of inorganic fertilizers

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Chemical formula</th>
<th>Nutrient content (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>CO(NH$_2$)$_2$</td>
<td>46</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Single super phosphate</td>
<td>Ca(H$_2$PO$_4$)$_2$.H$_2$O</td>
<td>-</td>
<td>8</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Muriate of potash</td>
<td>KCl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3.3: Physico-chemical characteristics of organic amendments (Mean ± SE)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Farmyard manure</th>
<th>Vermicompost</th>
<th>Biochar</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (1:2.5)</td>
<td>6.7±0.13</td>
<td>6.2±0.12</td>
<td>8.2±0.31</td>
</tr>
<tr>
<td>Total C (%)</td>
<td>20.3±1.23</td>
<td>23.2±0.93</td>
<td>67.3±1.08</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>1.09±0.05</td>
<td>1.30±0.02</td>
<td>1.03±0.04</td>
</tr>
<tr>
<td>C:N</td>
<td>15.61</td>
<td>12.21</td>
<td>65.34</td>
</tr>
<tr>
<td>Total P (%)</td>
<td>0.65±0.01</td>
<td>0.89±0.02</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>Available P (mg kg$^{-1}$)</td>
<td>89.26±1.2</td>
<td>110.28±8.21</td>
<td>98.01±3.21</td>
</tr>
<tr>
<td>Available K (mg kg$^{-1}$)</td>
<td>189.23±9.87</td>
<td>342.17±11.29</td>
<td>289.34±10.21</td>
</tr>
<tr>
<td>Ca</td>
<td>34.62±0.54</td>
<td>41.28±0.32</td>
<td>11.76±0.21</td>
</tr>
<tr>
<td>Micronutrients (mg kg$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>36.97±0.88</td>
<td>46.28±0.21</td>
<td>1.80±0.11</td>
</tr>
<tr>
<td>Mg</td>
<td>10.14±0.12</td>
<td>18.62±0.07</td>
<td>10.91±0.11</td>
</tr>
<tr>
<td>Zn</td>
<td>22.59±0.09</td>
<td>21.02±0.21</td>
<td>0.31±0.08</td>
</tr>
<tr>
<td>Bulk density (Mg m$^{-3}$)</td>
<td>0.88±0.01</td>
<td>1.12±0.01</td>
<td>0.46±0.02</td>
</tr>
<tr>
<td>Water holding capacity (%)</td>
<td>49.21±1.43</td>
<td>55.18±2.08</td>
<td>72.12±2.54</td>
</tr>
</tbody>
</table>
3.4.1.3. pH

Procedure:
1. 10 g of sample was taken in a conical flask.
2. Distilled water (50 mL in farmyard manure and vermicompost and 200 mL in biochar) was added to it and placed in shaker for 30 minutes.
3. After 30 minutes pH was measured using pH meter (HI96107, Hanna Instruments, Australia).

3.4.1.4. Total organic carbon and total nitrogen

Total organic carbon and total nitrogen in powdered samples was analyzed using CHN analyzer (Series 2 CHN S/O Analyser 2400, Perkin Elmer, USA).

3.4.1.5. Total P, K and nutrient content

Nutrients in the organic amendments (P, K, Ca, Mg, Fe and Zn) were measured using wet digestion technique as given in Tripathi (2009).

Reagents:

Procedure:
1. 1 g of organics was taken in a 100 mL volumetric flask and 10 mL of di-acid mixture was added to it.
2. The flask was placed on hot plate at low heat (40-50 ºC) in a digestion chamber for 30 minutes.
3. The flask was then heated at high temperature (100 ºC) until the production of red fumes ceased.
4. The content was evaporated till the volume reduced to 2-3 mL and the liquid became colourless.
5. After cooling, 30 mL of double distilled water was added and filtered through Whatmann No. 42 filter paper and the volume was made up to 100 mL.
3.4.1.4.1. Estimation of Ca, Mg, Fe and Zn: The nutrients in the filtered solution were quantified using inductively coupled plasma-atomic emission spectroscopy (Optima 2100 DV, Perkin Elmer, USA).

3.4.1.4.2. Estimation of total P:
Reagents:
1. Vanadomolybdate reagent
2. Phosphorous standard solution: 0.2195 g of analytical grade KH₂PO₄ was dissolved in 200 mL of distilled water and then diluted to 1 L. This solution contained 50 µg P/mL (50 ppm).

Preparation of standard curve:
For preparation of the standard curve, 0, 1, 2, 3, 4 and 5 mL of the 50 µg P/mL standard solution was transferred to 50 mL volumetric flask to get 0, 1, 2, 3, 4, and 5 ppm of P respectively. Following similar procedure the absorbance was recorded and plotted against concentration to get the standard curve.
Once the linear calibration curve was established, the slope of the curve was determined and then the concentration of the unknown solution was calculated by using the equation:

\[ A = mc. \]

Estimation of P
1. 5 mL of the digested sample was taken in a volumetric flask
2. 10 mL of Vanadomolybdate reagent was added and the volume was adjusted to 50 mL by adding deionised water.
3. The absorbance of solution was recorded after 30 minutes at 420 nm. The P concentration was calculated using the standard curve

\[ P(\%) = \frac{\text{Sample conc. (ppm)}}{\text{Wt. of sample (g)}} \times \frac{1}{\text{aliquot (mL)}} \times \frac{100}{\text{final volume (mL)}} \times \frac{1000}{10000} \]

3.4.1.4.3. Estimation of total K: The total K in the solution was directly analyzed by using flame photometer (Systronics BD-MSI).
3.4.2. Soil analysis
3.4.2.1. Soil texture

Soil texture was estimated by International Pipette method as given by Piper [2].

Reagents:
1. Na-hexametaphosphate (5 g L\(^{-1}\))

Procedure:
1. 20 g of soil was weighed and transferred to a shaker cup.
2. 5 mL Na hexametaphosphate was added and stirred for 5 minutes.
3. The contents were then poured into a 500 mL graduated cylinder and the volume was made up to 500 mL with distilled water.
4. The top of the cylinder was covered with parafilm and was inverted for several times to re-suspend the soil.
5. The parafilm was removed gently and 25 mL aliquot was taken from the upper 10 cm of suspension at 48 seconds. The aliquot was transferred to a weighed evaporating dish and put in oven at 105 °C.
6. The second 25 mL of aliquot was taken after 40 minutes from upper 5 cm of the suspension and the aliquot was transferred to weighed evaporating dish and put in oven at 105 °C.
7. After 24 hours, the evaporating dishes were removed from oven, cooled and weighed. The net weight of the first evaporating dish was recorded as combined silt and clay in 1/20 of the soil-water suspension. The net weight of the second was assumed to be 1/20 of the clay.
8. The percentages of the separates were calculated as follows:

\[
\% \text{ Clay} = 20 \times \frac{\text{mass of clay in aliquot}}{\text{total mass of soil}} \times 100
\]

\[
\% \text{ Silt} = 20 \times \frac{\text{mass of silt + clay in aliquot} - \text{mass of clay in aliquot}}{\text{total mass of soil}} \times 100
\]

\[
\% \text{ Sand} = 100\% - (\% \text{ silt} + \% \text{ clay})
\]
3.4.2.2. Soil temperature
Soil thermometers were inserted up to 15 and 30 cm soil depth to record the top soil and subsoil temperature respectively.

3.4.2.3. Soil moisture content
Moisture content was estimated using gravimetric method as described in Baruah and Borthakur [3]
Procedure:
1. 10 g (W1) of fresh soil was taken in a pre-weighed container (C).
2. The samples were kept in an oven at 105 °C for 24 hours and the final weight (W) was recorded.
3. The final oven dry weight (W2) was obtained by subtracting the weight of the container (C-W), and the soil moisture content was determined using the following equation:

\[
\text{Soil moisture content (\%) } = \frac{W1 - W2}{W1} \times 100
\]

3.4.2.4. Soil bulk density
Bulk density of soil was estimated using core sampler method as described in Baruah and Borthakur [3]
Apparatus used:
1. Core sampler: 30 cm long and 6 cm diameter which can be divided into two 15 cm tubes.
Procedure:
1. The volume (V) of the empty core was recorded.
2. The core sampler was pressed into the soil up to 30 cm depth
3. The soil from the outer line and ends of the sampler were removed and brought to the laboratory and weights (W1) were recorded.
4. To obtain the final weights (W2), cores were oven dried at 110 °C for 24 hours.
Bulk density (g cc⁻¹) was calculated by the following formula:

\[
\text{Bulk density} = \frac{W1 - W2}{V}
\]
3.4.2.5. Soil water holding capacity

Soil WHC was estimated following the method of Baruah and Borthakur [3]

_Apparatus used:_

1. Keen box
2. Flat bottom soaking tray

_Procedure:

1. The weight (W1) of empty box with a Whatmann No. 42 filter paper at its bottom was recorded.
2. The box was filled (about half of the box) with sieved air dried soil. The weight of the box (W2) with dry soil + filter paper was recorded.
3. The box was then kept on a soaking tray and the tray was filled with water till the level was 1 cm above the base of the box. The tray was covered to prevent evaporation and kept undisturbed for 12 hours.
4. A blank test with only filter paper was also run and the weight was recorded (W3).
5. The box is carefully removed from the tray and water from outside was wiped. The weight of the box (W4) was recorded.
6. The box was then oven dried for 24 hours at 110 °C and the weight was recorded (W5).

_Calculation:

To calculate water holding capacity, the following weights were computed:

1. Total water in wet soil (S1): W4-W1-W3
2. Weight of oven dried soil (S2): W5-W1

\[
\text{Water holding capacity(\%)} = \frac{S1}{S2} \times 100
\]

3.4.2.6. Soil organic carbon and its fractions

3.4.2.6.1. Soil organic carbon

Soil organic carbon was determined using Walkley and Black [4] method with slight modification.

_Reagents:

1. 1 N K₂Cr₂O₇
2. 0.5 N ferrous ammonium sulphate (FAS)
3. Diphenylamine indicator
4. Conc. H$_2$SO$_4$
5. Conc. H$_3$PO$_4$

*Procedure:*
1. 1 g of dry soil was weighed and put into a conical flask.
2. 10 ml of K$_2$Cr$_2$O$_7$ and 20 ml of conc. H$_2$SO$_4$ was added.
3. The flask was then heated at 150-170 °C for 20 minutes for complete oxidation.
4. After 30 minutes, 200 ml of distilled water was added to it.
5. 10 ml of H$_3$PO$_4$ and 1.5 ml of diphenylamine was added and the contents were mixed.
6. The content was then titrated with 0.5 N FAS till brilliant green color was obtained. 7. A reagent blank was run with each set without soil.

*Calculation:*

Soil organic carbon (%)  
\[ = 10 \times \frac{\text{Titrant in blank (mL)} - \text{Titrant in sample (mL)}}{\text{Titrant in blank (mL)}} \times 0.003 \times \frac{100}{0.5} \]

3.4.2.6.2. Particulate organic carbon

Particulate and mineral associated organic carbon was estimated after Cambardella and Elliott [5] as modified by Divito et al. [6]

*Apparatus used:*
1. 0.53 mm screen
2. Reciprocating shaker

*Reagents:*
1. Na-hexametaphosphate (5 g L$^{-1}$)
2. 1 N K$_2$Cr$_2$O$_7$
3. 0.5 N ferrous ammonium sulphate (FAS)
4. Diphenylamine indicator
5. Conc. H$_2$SO$_4$
6. H$_3$PO$_4$
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Procedure:
1. 10 g of dry soil was dispersed in 30 ml of sodium hexametaphosphate in a 100 mL conical flask.
2. The flask was then placed shook on a reciprocating shaker at a speed of 90 rotations per minute for 18 hours.
3. The soil suspension was then poured over a 0.53 mm screen.
4. Water in the slurry was evaporated in a forced air oven at 45 °C and the dried sample was ground with a mortar and pestle and analyzed for organic carbon (mineral associated organic carbon) following the method described in section 3.4.2.6.2.
5. The POC content was calculated by subtracting mineral associated organic carbon from SOC.

3.4.2.6.3. Microbial biomass carbon
Soil MBC was determined using CHCl₃ fumigation-extraction method [7]

Reagents:
1. 0.5 M K₂SO₄.
2. 66.7 mM K₂Cr₂O₇
3. 40 mM ferrous ammonium sulphate (FAS)
5. Ferroin indicator
6. CHCl₃

Procedure:
1. 3 sets of 10 g soil each were weighed.
2. One set was kept in oven at 105 °C for determination of moisture content determination.
3. Second set was fumigated.
4. Third set was kept in refrigerator till analysis.

A. Fumigation
1. 10 g of field moist soil samples were weighed in beaker and placed in a vacuum desiccator with CHCl₃ placed in another beaker.
2. The desiccator was sealed with vaseline to make it free from any leakages and incubated at 25±2 °C for 48 hours.

B. Extraction
1. 10 mL of K$_2$SO$_4$ (extractant) was added to both the fumigated and non-fumigated samples and shook for 10 minutes.
2. The content was filtered with Whatmann No. 42 filter paper.

C. Oxidation
1. 8 mL of the extract was taken in 50 mL conical flask.
2. 2 ml of K$_2$Cr$_2$O$_7$ was added to it.
3. 15 ml of digestion mixture was mixed.
4. The flask was then heated at 150-170 °C for 20 minutes for complete oxidation.
5. After 30 minutes, 25 mL of distilled water was added to it.
6. 2-3 drops of ferroin indicator were added and the contents were mixed.
7. The content was then titrated with 40 mM FAS till brick red color was obtained. A reagent blank was run with each set without soil.

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)

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

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

\[
VS = \text{wet soil weight} - \text{oven dry soil weight} + \text{extractant volume}
\]

4. Determination of extractable C (Ext C in µg mL$^{-1}$)
a. Volume of K$_2$Cr$_2$O$_7$ consumed (Y, mL)

\[
Y = \frac{\text{Normality of FAS} \times \text{Titrant volume}}{\text{Normality of K2Cr2O7}} \times 100
\]
b. Volume of $\text{K}_2\text{Cr}_2\text{O}_7$ consumed for oxidizing easily mineralizable C in 10 mL of extractant = $2-Y$ mL

c. Amount of extractable C (Ext C in $\mu$g mL$^{-1}$)

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

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

\[ \text{CF or CNF} = \text{Ext C} \times \frac{\text{VS}}{\text{MS}} \]

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

\[ = \frac{\text{CF} - \text{CNF}}{K} \]

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

3.4.2.6.4. Humic acid carbon, fulvic acid carbon and degree of humification (E4/E6)

The HAC, FAC and E4/E6 was estimated by following the method given by Page et al. [8]

Reagents:
1. 0.1 N sodium pyrophosphate in 0.1 N NaOH solution
2. 0.1 N NaOH
3. Conc. H$_2$SO$_4$

Procedure:
1. 5 g soil was dispersed in 100 mL sodium pyrophosphate solution and shook (5000 rpm) for 30 minutes in a stopper 250 mL conical flask.
2. The flask is kept overnight and filtered with Whatmann No. 1 filter paper.
3. The pH of the filtrate was adjusted between 2-3 using Conc. H$_2$SO$_4$ and kept overnight.
4. The solution was filtered using Whatmann No. 1 filter paper. The filtrate was analysed for fulvic acid carbon.

5. The precipitate in the filter paper was washed with 20 mL 0.1 N NaOH.

6. The absorbance of the solution was recorded in 465 nm (E4) and 665 nm (E6).

7. The solution was analyzed for humic acid carbon.

8. The organic carbon content (HAC and FAC) of the solutions was determined by dichromate oxidation [4] as described in section 3.4.2.6.2.

9. The degree of humification was calculated from E4/E6 ratio.

### 3.4.2.7. Carbon-mineralization

C-mineralization (as CO₂ evolved) was studied in a set of sealed polyethylene bottles with soil collected at harvest of each crop in laboratory condition for a period of 90 days following the method given by Angers and Recous [9]

**Reagents:**

1. 1 N NaOH
2. 0.5 M BaCl₂
3. Phenolphthalein indicator
4. 0.25 M HCl

**Procedure:**

1. Polyethylene bottles of 500 mL capacity with 100 g field moist soil in each were used for this experiment and incubated at 27±3 °C under anerobic condition with water content at 60% of its water holding capacity up to 90 days.
2. A set of 39 polyethylene bottles that included 12 treatments and 3 replications along with 3 bottles without soil (control) were taken for measuring carbon mineralization.
3. Glass vials of 5 mL capacity containing 1 N NaOH were hanged in each bottle to trap CO₂-C respired.
4. Samplings were done periodically at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 and 90 days from the beginning of incubation.
5. On each sampling day, the vials were replaced with another set containing fresh NaOH and bottles were kept back in incubator.
6. The CO$_2$ production was determined by back titration of NaOH solution with 0.5 M HCl in an excess of BaCl$_2$ (5 mL) using phenolphthalein (2-3 drops) as indicator.

7. Throughout the incubation period, at each sampling dates, soil moisture was checked by weighing the bottles and was adjusted with distilled water.

8. The values of CO$_2$ evolved were divided by the mass of the soil samples (on an oven-dried weight soil basis) and were expressed as µg CO$_2$-C g$^{-1}$ dry soil.

The CO$_2$ evolved is calculated as follows:

$$\text{CO}_2 \text{ (mg g}^{-1}\text{ soil)} = \frac{\text{mL of NaOH} \times \text{N of NaOH in blank} - \text{mL of NaOH} \times \text{N of NaOH in sample} \times 22}{\text{Initial soil weight (g)}} \quad (a)$$

$$\text{CO}_2 \text{ (mg kg}^{-1}\text{ soil)} = a \times 1000$$

3.4.2.7.1. Kinetics of C-mineralization

The obtained CO$_2$ evolution data was added to calculate the cumulative CO$_2$ evolution during the incubation period (90 days) and the values were plotted in graphs.

Using the equation $y = mx+c$, the slop of the graph was calculated. Further, first order kinetic equations were used to determine potentially mineralizable C (C$_0$).

$$C_m = C_0 (1 - e^{-kt})$$

Where, C$_m$ is the cumulative C at any specific time t (day) respectively, $k$ is first order rate constant (day$^{-1}$)

3.4.2.7.2. Half-life of C

Half-life of soil C under various treatments was calculated using the formulae

$$\text{Half-life} = \frac{0.693}{k}$$

Where, $k$ is first order rate constant

3.4.2.8. Soil available N

Available soil N was determined using alkaline potassium permanganate method as given by Subbiah an Asija [10].
Equipment:
1. Kjeldahl distillation set (Kelplus-Elite EXVA)

Reagents:
1. 2 N KCl
2. 4% Boric acid
3. 2.5% NaOH
4. 0.32% KMnO$_4$
5. 0.02 N H$_2$SO$_4$
6. Mixed indicator (bromocresol green and methyl red in ethanol)
7. MgO powder and Devardas alloy

Procedure:
1. 100mL of 2 N KCl solution was added into the bottle, and kept in a shaker for 1 hour and then allowed to stand.
2. 20 mL of clear aliquot was pipetted out in a 100 mL distillation flask.
3. To it 25 mL of 0.32% KMnO$_4$, 2.5% NaOH and 0.5 g each of MgO and Devardas alloy was added.
4. 25 mL of boric acid with 3-4 drops of mixed indicator was pipetted in a conical flask and placed at the end of the delivery tube to trap the N distilled.
5. Distillation was continued till a distillate of about 100 mL was collected in the conical flask.
6. The trapped N (ammonium-N+ nitrate-N) was titrated with 0.002 N H$_2$SO$_4$ (Titrant).
7. A reagent blank without soil was also run.

Available N was calculated as follows:

$$N \text{ (ppm)} = \frac{(\text{mL of titrant in sample} - \text{blank})}{\text{Soil weight (g) x 1000}} \times 0.02 \times 100 \times 14 \quad ---- \ (a)$$

$$\text{Available N (kg ha}^{-1}) = a \times 2.24$$

3.4.2.9. Soil available P
Reagents:
1. Bray’s extractant (NH₄F in HCl)
2. Molybdate reagent (Ammonium molybdate in HCl)
3. Stannous chloride solution (stock solution) (10g of SnCl₂ was dissolved in 25mL of conc. HCl)
4. Working Solution: 1ml of stock solution was diluted freshly to 66.0 mL with distilled water before use.
5. Standard phosphate solution: 0.439 g KH₂PO₄ was added to about 500 mL of distilled water. To it, 25 mL of 7 N H₂SO₄ was added and the volume was made up to 1000 mL with distilled water. This is the 100 ppm standard stock solution. From this 2 ppm of solution was prepared made by diluting it 50 times.

Procedure:
1. 2.5 g of soil was weighed in a flask to which 25 mL of Bray’s extractant was added.
2. The solution was kept in the shaker for 5 minutes and then centrifuged for 10 minutes at 6000 rpm.
3. 10 mL of extractant was taken in 50ml of volumetric flask.
4. 10 mL of molybdate reagent was added to the volumetric flask and the solution was diluted to about 40 mL with distilled water.
5. 2 mL stannous chloride (SnCl₂) solution was added to the volumetric flask and volume was made up to 50 mL with distilled water.
6. After 10 minutes absorbance was observed at 660 nm.
7. One blank was prepared without soil sample.
8. To prepare the standard curve, 1, 2, 3, 4, 5 and 10 mL of 2 ppm solutions were taken in a 50 mL volumetric flask and step 4, 5 and 6 was carried out. After getting Once the linear calibration curve was established, the slope of the curve was determined and then the concentration of the unknown solution was calculated by using the equation y = mx + c.

\[
\text{Available P (kg ha}^{-1}\text{)} = \text{Sample concentration (ppm)} \times 2.24
\]

3.4.2.9. Soil available K
Available K was estimated following the method given by Jackson [12]
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Equipment:
1. Flame photometer with K filter (Systronics BD-MSI)

Reagents:
1. 1 N ammonium acetate (pH 7)
2. KCl solution (1000ppm stock solution)
3. Standard curve for K: 1, 2.5, 5, 7.5 and 10 mL of 1000 ppm solution was extracted in a 100 mL volumetric flask and the volume was made up to 100 mL to obtain solution of 10, 25, 50, 75 and 100 ppm. The graded standard solutions were used to calibrate the flame photometer.

Procedure:
1. 2 g of soil sample was taken in a conical flask and 20ml of ammonium acetate was added to it.
2. The solution was shook for 30 minutes and then filtered using Whatmann No.1 filter paper followed by taking the reading in flame photometer in K filter.

$$\text{Available K (kg ha}^{-1}) = \frac{R \times \text{volume of extractant} \times 2.24}{\text{Soil weight (g)}}$$

Where, R is the ppm of K in extract (photometer reading).

3.4.2.10. Soil dehydrogenase activity
Dehydrogenase activity was determined following the method modified by Garcia et al. [13].

Reagents:
1. 0.4% 2-p-iodo-nitrophenyl-phenyltetrazolium chloride (INT)
2. 95% ethanol
3. Nitrophenyl Formazan standard solution: 100mg of Nitrophenyl Formazan was dissolved in 80 mL ethanol and the volume was made up to 1000 mL with ethanol.

Procedure:
1. 1g of soil was weighed into which 0.2 ml of 0.4 % INT was added and incubated for 20 hours at 22°C.
2. After 20 hours 10ml of ethanol was added.
3. Vigorous shaking was done for 1 hour.
4. The mixture was filtered through Whatmann no.1 filter paper.
5. Absorbance of the red colour of iodo-nitro-tetrazolium-formazan (INTF) was observed at 490 nm.
6. For preparation of the standard curve, 0.1, 0.2, 0.3, 0.5, 0.8 and 1mL of the standard solution was taken in 50 mL volumetric flask and the volume was made up with ethanol.
7. Dehydrogenase activity was calculated from the standard curve and expressed as mg INFT g⁻¹ soil 24 h⁻¹.

Calculation:
\[
\text{Dehydrogenase activity (mg INFT g}^{-1}\text{h}^{-1}) = \frac{\text{Amount of INFT from standard curve (mg)}}{\text{Soil weight (g)}}
\]

3.4.2.11. Soil phosphatase activity
Phosphatase activity was determined following the method given by Tabatabai and Bremner [14]

Reagents:
1. 20 mM p-nitrophenyl phosphate
2. 0.5 M acetate buffer
3. 1 M NaOH
4. 0.5 M CaCl₂
5. p-nitrophenol (PNP) Master Solution (1000µg mL⁻¹)
6. p-nitrophenol working Solution (10µg mL⁻¹)

Procedure:
1. 1 g of soil sample was taken.
2. 4 mL of acetate buffer was added to the soil samples.
3. Into the mixture 1 mL of p-nitro phenyl phosphatase was added.
4. Samples were kept in BOD incubator at 37±1°C for 1 hour.
5. After 1 hour, the reaction was stopped by adding 4 mL of 0.5 M NaOH and 1mL of 0.5 M CaCl₂.
6. Then the mixture was centrifuged at 400 rpm for 10 minutes and absorbance was measured at 400nm.
7. Calibration graph was prepared with standards containing 10, 20, 30, 40 and 50 µg PNP and the enzyme activity was expressed as µ mol PNP g\(^{-1}\) h\(^{-1}\).

*Calculation:*

\[
\text{Phosphatase activity} = \frac{\text{Amount of PNP from standard curve (µg)}}{\text{Soil weight (g)}}
\]

**3.4.2.12. Soil urease activity**

Urease activity was determined by hydrolysis reaction as described by Tabatabai and Bremner [15].

*Reagents:*
1. Urea solution (0.2 M)
2. Tris-HCl buffer (pH 9.0)
3. Phenol-Pentacyano-nitrosyloferate solution (Phenate solution)
4. Potassium chloride-silver sulfate solution
5. Alkaline hypochlorite solution
6. Toluene
7. Standard ammonium solution: 10 g of NH\(_4\)Cl was dissolved in 100 mL ammonium free water which forms a 28 µg of NH\(_3\)-N of stock solution.

*Procedure:*
1. 5 g of fresh soil was weighed in a 50 mL volumetric flask.
2. 0.2 mL of toluene was added to the volumetric flask followed by addition of 9 mL of Tris-HCl buffer.
3. Samples were kept in the shaker for few minutes to mix the contents and then 1 mL of 0.2 M urea was added to the mixture.
4. Flask mouth was covered by a stopper and kept in the incubator at 37 °C.
5. After 2 hours the volume was made up to 50 mL with Potassium chloride-silver sulphate solution.
6. The contents were centrifuged and then supernatant was used for the estimation of ammonia.
7. 1 mL of supernatant was taken into which 1 mL of phenate solution was added, followed by 1 mL of alkaline hypochlorite solution.
8. The reaction was kept for 5 minutes at 37 °C and 7 mL of water was added.
9. Absorbance was measured at 625 nm.
10. In the controls (without soil), 1 mL of 0.2 M urea was added after adding KCl-Ag₂SO₄ solution.
11. Ammonium chloride was used as a standard.
12. Enzyme activity was calculated from the standard curve and expressed as µ mol g⁻¹ h⁻¹.

Calculation:

\[
\text{Urease activity} = \frac{\text{Amount of NH₄} + \text{from standard curve (µg)}}{\text{Soil weight (g)}}
\]

3.4.3. Plant analysis

3.4.3.1. Plant height

Plant height (cm) was measured using a meter ruler in the field. For wheat, the entire length (for soil surface to tip) was taken into account whereas for okra and green gram, the length from soil level to the top fully opened node of the main shoot was considered to measure the plant height.

3.4.3.2. Leaf number and leaf area

Total number of leaves (fully opened) was counted manually at each sampling dates. Leaf area was recorded by using a laser leaf area meter (model CI-203, USA).

3.4.3.3. Leaf area index

Leaf area index was calculated by the formula as given by Moosavi [16].

\[
\text{LAI} = \frac{\text{Leaf area per plant}}{\text{Ground surface area per plant}}
\]

3.4.3.4. Shoot and root biomass

1. The plants were uprooted and brought to laboratory to measure the root and shoot biomass.
2. The plants were washed properly to remove the soil particle and then separated into shoot and root.
3. After recording the fresh weights, the plants were oven dried at 80 °C for 72 hours and dry weight of shoot and root biomass were recorded.

3.4.3.5. *Net photosynthesis rate*
Net photosynthesis was recorded under ambient environmental condition using an infrared gas analyzer (LI-6400 portable photosynthesis system; LI-COR, USA). Photosynthetic rates were measured between 10:00 to 12:00 hours of the day.

3.4.3.6. *Yield and yield components*
The crops were harvested from 1 m² area and biological yield (total aboveground biomass) and economic yield was recorded.

**Wheat:** Wheat was harvested when all the panicles became brown. After harvest, the grains were separated from the chaff and the grain yield (economic yield) was recorded.

**Okra:** Young 3-4 days old pods were harvested manually for 10 times starting from 50 to 90 days after sowing at 4 days interval and weights were recorded. Cumulative yield was calculated by adding all the harvests from each treatment.

**Green gram:** Harvesting was done when 75% of the pods matured indicating full darkish pod and brittle on slight pressure. After harvest the grains were separated from the pod and the weights were recorded.

3.4.3.6. 1. *1000 Grain Weight*
1000 grain weight of green gram and wheat was calculated manually.

3.4.3.6. 2. *High Density Grain*
High density grain in wheat was quantified based on specific gravity.

*Procedure:*
At first, solution of specific gravity 1.20 Mg m$^{-3}$ was prepared by dissolving 270 g of common salt in 1L of distilled water. Then, 100 grains were put into each solutions prepared at four beakers. The floaters and sinkers were recorded. The sinkers at specific gravity 1.20 Mg m$^{-3}$ were categorized as good grains or high density grains.

3.4.3.6. 3. Estimation of Harvest Index

Harvest index was calculated by dividing economic yield by biological yield obtained from one meter square area.

Harvest Index = (Economic yield/biological yield) ×100

3.5. INDICES

3.5.1. Carbon pool index (CPI)

The CPI was estimated after the method given by Blair et al. [17]

$$\text{CPI} = \frac{\text{Organic carbon of treatment}}{\text{Organic carbon of reference soil}}$$

The organic carbon of control plots was considered as the reference soil

3.5.2. Sensitivity index (SI)

The SI of different SOC fractions was calculated by the formula given by Yang et al. [18].

$$\text{SI} (\%) = \frac{\text{SOC fraction in treatment} - \text{SOC fraction in control}}{\text{SOC fraction in control}} \times 100$$

3.5.2. Agronomic efficiency

To assess the agronomic performance of different treatments, the following calculations were made according to Guarda et al. [19]

$$\text{Agronomic efficiency} (\text{kg/kg}) = \frac{\text{Yield of fertilized treatment} - \text{Yield of non fertilized treatment}}{\text{Fertilizer N content}}$$

3.6. STATISTICAL ANALYSIS

All the statistical analyses were performed in SPSS for windows 16.0. The mean values and the standard errors (SE) were calculated for the entire data set. Data, at the end of
first and second year, were analyzed by ANOVA to determine the effect of treatment, year of cultivation and interaction of treatment × year for the objective 1. For the objective 2, the effect of fertilizer treatment (F), water regimes (W), year (Y), interaction of F×W, W×Y, F×Y and F×W×Y were analyzed using three-way ANOVA with the data obtained at the end of first and second year. Duncan’s multiple range tests (DMRT) was performed at p ≤ 0.05. Pearson’s correlation study was also done to find the linear relationship among the studied parameters.

References


