3. MATERIALS AND METHODS

The field investigations pertaining to the “Studies on effect of glyphosate on biochemical attributes of tea [Camellia sinensis (L.) O. Kuntze]” were conducted at the Research Farm of Department of Tea Husbandry and Technology, CSK HPKV, Palampur during 2008 and 2009. Laboratory investigations were carried out in Department of Agronomy, Forages and Grassland Management Department of Chemistry and Biochemistry, CSK HPKV, Palampur and Institute of Himalayan Bioresource Technology (IHBT, CSIR) Palampur. The details of material used and experimental procedures adopted in the study are described in this chapter.

3.1 Experimental site

3.1.1 Location

The experimental farm is situated at 32°6’ N latitude, 76°3’ E longitude, and at an altitude of 1291 m above mean sea level. The area falls in mid hills sub-temperate zone of the mid hills of Himachal Pradesh.

3.1.2 Climate and weather conditions

The mean weekly meteorological observations of cropping season recorded at meteorological observatory of Department of Agronomy, Forages and Grassland Management, College of Agriculture, CSK HPKV, Palampur have been depicted in Fig. 3.1(a) and 3.1(b) appended in Appendix-I and II. A critical resume of meteorological data during 2008 showed that total rainfall of 1299.4 mm was received during the season. The average relative humidity ranged from 68 to 88 per cent and mean minimum and mean maximum temperature values were 11.1 and 19.8°C, respectively. During the year 2009, the total rainfall of 1388 mm was received. Average relative humidity ranged from 47 to 91.5 per cent and mean minimum and mean maximum temperature was 16.4 and 27.2°C, respectively.

3.2 Experimental details

A field experiment consisting of four treatments replicated five times was laid out during 2008-2009. The details are given below:
Fig. 3.1(b) Mean weekly meteorological data for the period 2009 at Palampur
Treatment detail

\[ T_1 = \text{Control} \]
\[ T_2 = \text{Glyphosate 0.5 kg ha}^{-1} \]
\[ T_3 = \text{Glyphosate 1.0 kg ha}^{-1} \]
\[ T_4 = \text{Glyphosate 2.0 kg ha}^{-1} \]

Details of field layout plan are indicated in Fig. 3.2

### 3.3 Field operations

The details of the field operations during the period of experimentation are given in Table 3.1.

#### Table 3.1 Schedule of field operations

<table>
<thead>
<tr>
<th>Operation</th>
<th>Year</th>
<th>Date</th>
<th>Details of operations/ treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbicide spray</td>
<td>2008</td>
<td>July 10, 2008</td>
<td>Glyphosate was applied at different dose as mentioned in treatments</td>
</tr>
<tr>
<td></td>
<td>2009</td>
<td>July 24, 2009</td>
<td></td>
</tr>
</tbody>
</table>

### 3.4 Herbicide used

<table>
<thead>
<tr>
<th>Common name</th>
<th>Trade name</th>
<th>Chemical name</th>
<th>Active ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyphosate</td>
<td>Glycel</td>
<td>[N-(phosphonomethyl) glycine]</td>
<td>41 per cent</td>
</tr>
</tbody>
</table>

### 3.5 Physico-chemical characteristics of soil

Prior to the commencement of the experiment, soil samples were collected from about twenty spots in experimental area with the help of tube auger. These samples were thoroughly mixed, air dried, ground in a mortar with pestle, passed through 2 mm sieve and used for studying physico-chemical parameters (Table 3.2).
Building Department of Tea Husbandry and Technology

1. $T_1 =$ Control
2. $T_2 =$ Glyphosate 0.5 kg ha$^{-1}$
3. $T_3 =$ Glyphosate 1.0 kg ha$^{-1}$
4. $T_4 =$ Glyphosate 2.0 kg ha$^{-1}$

Fig. 3.1 Layout Plan of the Experimental Area
Table 3.2  
Physico-chemical characteristics of soil

<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>Content</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Mechanical fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand (%)</td>
<td>26.2</td>
<td>International pipette method (Piper, 1966)</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>41.1</td>
<td></td>
</tr>
<tr>
<td>Clay (%)</td>
<td>29.8</td>
<td></td>
</tr>
<tr>
<td>Textural class</td>
<td>Silty clay loam</td>
<td></td>
</tr>
<tr>
<td>B. Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (1: 2.5 :: soil : water)</td>
<td>5.21</td>
<td>Glass electrode pH meter method (Jackson, 1967)</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>2.44</td>
<td>Walkley and Black’s Rapid titration method (Piper, 1966)</td>
</tr>
<tr>
<td>Available nutrients (kg ha⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>407.68</td>
<td>Alkaline potassium permanganate method (Subbiah and Asija, 1956)</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>54.41</td>
<td>Olsen’s method (Olsen’s et al., 1954)</td>
</tr>
<tr>
<td>Potassium</td>
<td>353</td>
<td>Neutral normal ammonium acetate method (Jackson, 1967)</td>
</tr>
</tbody>
</table>

3.6 Sampling procedure

3.6.1 Green tea shoots

The samples of green tea shoots (two leaves and a bud) were collected from Research Farm of Department of Tea Husbandry and Technology, CSK HPKV, Palampur at two hours after herbicide spray and at fifteen days intervals throughout the plucking season during 2008 and 2009. The details of sampling for both the years are given in Table 3.3.
Table 3.3  Date of collection of green tea shoots

Ist year (2008)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Date of sample collection (Days)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10\textsuperscript{th} July, 2008 (0)</td>
<td>Freshly plucked tea shoots collected under ice cold conditions estimated for polyphenol oxidase activity and remaining samples of green tea shoots were subjected to heat treatment within 20 minutes of their plucking in a microwave oven for 2 minutes and finally dried in a hot air oven at 60 ± 0.5°C for 48 hours.</td>
</tr>
<tr>
<td>2</td>
<td>25\textsuperscript{th} July, 2008 (15)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9\textsuperscript{th} August, 2008 (30)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24\textsuperscript{th} August, 2008 (45)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8\textsuperscript{th} September, 2008 (60)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>23\textsuperscript{rd} September, 2008 (75)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8\textsuperscript{th} October, 2008 (90)</td>
<td></td>
</tr>
</tbody>
</table>

IInd year (2009)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Date of sample collection (Days)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24\textsuperscript{th} July, 2009 (0)</td>
<td>Freshly plucked tea shoots collected under ice cold conditions estimated for polyphenol oxidase activity and remaining samples of green tea shoots were subjected to heat treatment within 20 minutes of their plucking in a microwave oven for 2 minutes and finally dried in a hot air oven at 60 ± 0.5°C for 48 hours.</td>
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<td></td>
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<tr>
<td>4</td>
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<tr>
<td>7</td>
<td>22\textsuperscript{nd} October, 2009 (90)</td>
<td></td>
</tr>
</tbody>
</table>

The dried samples of green tea shoots were crushed into powder in a blender and stored in sealed polythene bags which were stored in dessicator. These samples were used for biochemical constituents i.e. shikimic acid, total amino acid, polyphenols, catechins.
3.6.2 Soil

Soil samples (0-15 cm depth) were collected at different intervals of time i.e. 0, 15, 30, 45, 60, 75 and 90 days after herbicide application from each of the treated plots along with control. Collected samples were mixed thoroughly, air-dried, processed and analyzed for the herbicide residues as given in section 3.8

3.7 Biochemical analysis

3.7.1 Shikimic acid

Shikimic acid in dried sample was estimated by the method of Mossor and Schramm (1972).

Reagents

p-hydroxybenzaldehyde (0.2 per cent):
Dissolved 0.2 g of p-hydroxybenzaldehyde in 100 ml of hot water
2. Concentrated sulphuric acid (98 per cent)
3. Shikimic acid (Acros)

3.7.1.1 Preparation of standard curve

Stock solution (10 mmoles) of shikimic acid was diluted to working solution (0.1 to 0.7 μmoles) and standard solutions were dispensed in test tubes (triplicate). Concentrated sulphuric acid (3 ml) was added to each test-tube and the contents were vortexed on vortex-mixer for proper-mixing. Added 1 ml of 0.2 per cent aqueous solution of p-hydroxybenzaldehyde with constant stirring and heated for one hour. An intense purple-violet colour developed. The absorbance of coloured solution against blank was measured at room temperature at 590 nm on Thermospectronic UV-1.

3.7.1.2 Estimation

Dried tea sample (100 mg) was taken in 20 ml hot water and filtered through Whatman No. 1 filter paper. Filtrate was centrifuged for 15 minutes at 10,000 rpm. Supernatant (100 μl) was taken (triplicate set) in test-tubes. Shikimic acid in samples was measured by following the similar steps as given under section 3.7.1.1.
3.7.2 Amino acid profile

Amino acid profile in dried sample was estimated by the method given by Gulati et al. (1999).

Reagents

1. Acetone
2. Ethyl acetate
3. Petroleum ether
4. n-butanol
5. Iso-butanol
6. Iso-propanol
7. Ethanol
8. Methionine (1 mg/ml)
9. 0.5 per cent copper sulphate in ethanol: Dissolved 0.5g of copper sulphate in 10ml of distilled water. After sonication for 3-4 minutes added ethyl alcohol dropwise to make up the volume (100 ml)
10. 2 per cent nin-hydrin in 80% acetone

3.7.2.1 Estimation

Dried sample (400 mg) was taken in centrifuge tube along with 80 per cent acetone (3 ml). Vortexed sample on vortex-mixer for proper mixing and centrifuged for 10 minutes at 10,000 rpm. Concentrated supernatant was taken in 30ml capacity separating funnel. Added to it 2 ml each of ethyl acetate, petroleum ether and n-butanol. The aqueous layer was collected in vial.

The collected aqueous layer was loaded on to the Whatman No. 3 paper and were chromatographed using isobutanol/isopropanol/water/acetone (4:3:2:1 v/v) as the solvent front. Amino acids resolved into six zones were eluted following nin-hydrin spray (2 per cent) and colour was developed with ethanolic copper sulphate (0.5 per cent) and read absorbance at 520 nm on Thermospectronic UV-I. Standard curve was prepared by using methionine (1 mg/ml).
3.7.3 Polyphenols

Total polyphenols in dry tea powder were estimated by the method as outlined by Bray and Thorpe, 1954.

Reagents

1. **Folin ciocalteu’s reagent (1N)** Folin Ciocalteu phenol reagent (Sisco Research Laboratories) was diluted in the ratio of 1:1 with double distilled water. The reagent was stored at 4°C.

2. 35 per cent sodium carbonate [E. Merck India] solution

3. 0.2 per cent gallic acid solution

3.7.3.1 Preparation of standard curve

Standard curve for polyphenols was prepared by taking gallic acid (0.2 %) in 25 ml volumetric flask in the range of 0.8 to 8.0 µg/ml. Folin ciocalteu’s reagent (0.5 ml) was added followed by 1 ml of 35 per cent sodium carbonate solution. The solution was mixed thoroughly with the help of vortex mixer and absorbance at 725 nm was recorded after 40 minutes incubation at 30°C with the help of Thermospectronic UV-I.

3.7.3.2 Extraction

Powdered tea (100 mg) samples were taken in centrifuge tube. 5 ml of acetone (60%) was added to each tube and vortexed for 10 minutes on vortex mixer. The aqueous contents were centrifuged for 15 minutes at 10,000 rpm and filtered.

Estimation

Prepared extract (100 µl) was taken (in triplicate set) in volumetric flask. The samples were analyzed by similar steps as adopted for standard curve for polyphenol under section 3.7.3.1.

3.7.4 Catechins

Catechins in dry tea power were estimated by the method of Singh *et al.* (1999).
**Reagents**

**Preparation of diazotized sulfanilamide**

Diazotized sulfanilamide was prepared by taking 1 g of sulfanilamide. The diazotization reaction was carried out in ice-cold conditions (<10°C) in the presence of sodium nitrite (1 ml of 1% NaNO₂) followed by the addition of 0.8 ml of hydrochloric acid. The precipitate (pale yellow colour) formed were filtered through Whatman No. 1 filter paper, dried and kept in deep freezer for further use.

1. **Reagent A**
   
   Prepared diazotized stock solution (1% w/v) in acetone.

2. **Reagent B**
   
   Dilute hydrochloric acid
   
   Concentrated hydrochloric acid (35.4 per cent of specific gravity 1.18) was diluted (30:100 v/v) with double distilled water.

3. **Stock solution**
   
   D(+) catechin (Sigma, USA) solution (1 mg/ml) was prepared in distilled water.

**3.7.4.1 Preparation of standard curve**

Stock solution (1 mg/ml) was taken in 25 ml volumetric flask in the range of 0.8 to 12 μg/ml. 1 ml of reagent A (1% diazotized sulfanilamide in acetone w/v) was added; this was followed by addition of 1 ml of reagent B (30% hydrochloric acid v/v). Solution was allowed to react at room temperature for one hour. At the end of incubation period, the volume was made upto the mark with distilled water and mixed well. Absorbance was recorded at 425 nm on Thermospectronic UV-1.

**3.7.4.2 Estimation in tea shoots**

**Extraction**

Extraction for the catechins was done in the same manner as described in section 3.7.3.2
3.7.4.3 Estimation

Aliquots (100 µl) of prepared extract in triplicate set were taken in 25 ml volumetric flasks and estimated by similar steps adopted for the standard curve of catechins as described under section 3.7.4.1.

3.7.5 Estimation of polyphenol oxidase (PPO) activity

Polyphenol oxidase activity of fresh tea shoots were estimated in freshly prepared acetone powder by the method of Singh and Ravindranath (1994) as described below:

(a) **Preparation of acetone powder**

Weighed 5 g of fresh tea shoots were macerated in chilled acetone (-20°C) in pre-chilled pestle-mortar. Added chilled acetone enough to cover the shoots and ground them for 2-3 minutes at low speed and then at high speed for 3-5 min. Filtered the residues through Whatman No. 1 filter paper and washed the residue with chilled acetone repeatedly until residues become colourless. After this the powder was air dried and stored in air tight sealed vials at -4°C.

(b) **Preparation of enzyme extract**

Weighed 0.1 g white part of acetone powder and transferred into cleaned, pre-cooled centrifuge tube. In centrifuge tube, added 4 ml double distilled water (mixed properly) and centrifuged the contents at 4,000 rpm for 10 minutes at 4°C. The supernatant containing water soluble enzyme was filtered through cotton and collected into a test-tube already placed in ice-box. Residues contained bound enzyme which was re-extracted with a 5.0 ml of 0.2 M Na₂SO₄ solution and again, centrifuged at 4,000 rpm for 10 minutes at 4°C. Once again, supernatant was collected in previously pooled test tube and mixed properly. This was termed as enzyme extract.

(c) **Enzyme assay with (+) catechin as substrate**

For the estimation of PPO activity, 0.1 ml of enzyme extract was taken into a test tube (maintained cold condition) with 0.4 ml double distilled water and 1.5 ml (+) catechin (5 mM) as substrate and was assayed spectrophotometrically (Singh, 1994) against control. Absorbance of test solutions was measured at 380 nm at 30 sec interval for five minutes with the help of Thermospectronic UV-I spectrophotometer.
3.8 Quantitative analysis of glyphosate

Glyphosate was analysed by modifying the method given by Simenson et al. (2008).

3.8.1 Preparation of working standard

Stock solution of glyphosate was prepared by dissolving 10 mg of accu standard glyphosate (100% purity) in 10 ml of sodium tetraborate buffer (1M pH=9.0). The working standard 1.0, 5.0, 10.0, 20.0, 40.0 and 100 ppm were prepared from stock solution.

3.8.2 Derivatization of glyphosate

2 ml working standards of each concentration were taken in separate vials and 1.0 ml of sodium tetraborate was added to each vial followed by addition of 1.0ml of 9-fluoro enylmethyl chloroformate (0.002M in acetone). These solutions were incubated for 30 minutes at room temperature for complete derivatization and were analysed by method as discussed under section 3.8.4.3.

Percent recovery test

To ascertain the extraction efficiency of glyphosate recovery experiments were carried out with soil and tea leaves. The samples were fortified with glyphosate at 0.5 and 1.0 µg g<sup>-1</sup> respectively. The fortified samples were extracted as per methods mentioned under section 3.8.4.1 and extracted samples were derivatised and analysed for herbicide concentration as given in section 3.8.2 and 3.8.4.3.

3.8.4 Glyphosate analysis

Three important steps involved in residue analysis are extraction, cleanup and estimation.

3.8.4.1 Extraction

The representative sample (50g soil/ 50g tea) was taken in 500ml stoppered conical flask. Distilled water (200ml) was added to it. Flasks were shaken for 3 hours in
a reciprocating shaker and filtered through whatman filter paper No.1 using activated charcoal. Extraction was repeated with 100 ml of distilled water and the combined filtrate was dried on hot plate and 2ml of sodium tetraborate (1M pH 9.0) was added to it. The procedure given under section 3.8.2 was adopted for derivatization.

3.8.4.2 Cleanup

This step was not required in present methodology.

3.8.4.3 Estimation

The residue of glyphosate in derivatized extracts were quantified on HPLC equipped with photo-diode (PDA) detector.

Column: 25 cm × 4.6 mm id. zorbax NH₂

Mobile phase: 50% acetonitrile 50% water 0.05M KH₂PO₄

pH adjusted to 6.0 with 7N KOH.

Flow rate: 0.8 ml/ minute

Column temperature: Ambient

Injection volume: 20 µl

Detectors: UV 206 nm.

Statistical analysis

The data on various parameters recorded during the course of investigation were subjected to statistical analysis as per method given by Gomez and Gomez (1989).