# CHAPTER 3

## Methodology and planning of the experiments

This chapter gives an overview of the experimental design and methodology obtained during the course of accomplishment of the objectives. Further details of all protocols have been described in each subsequent chapter and in the enclosed publications also.

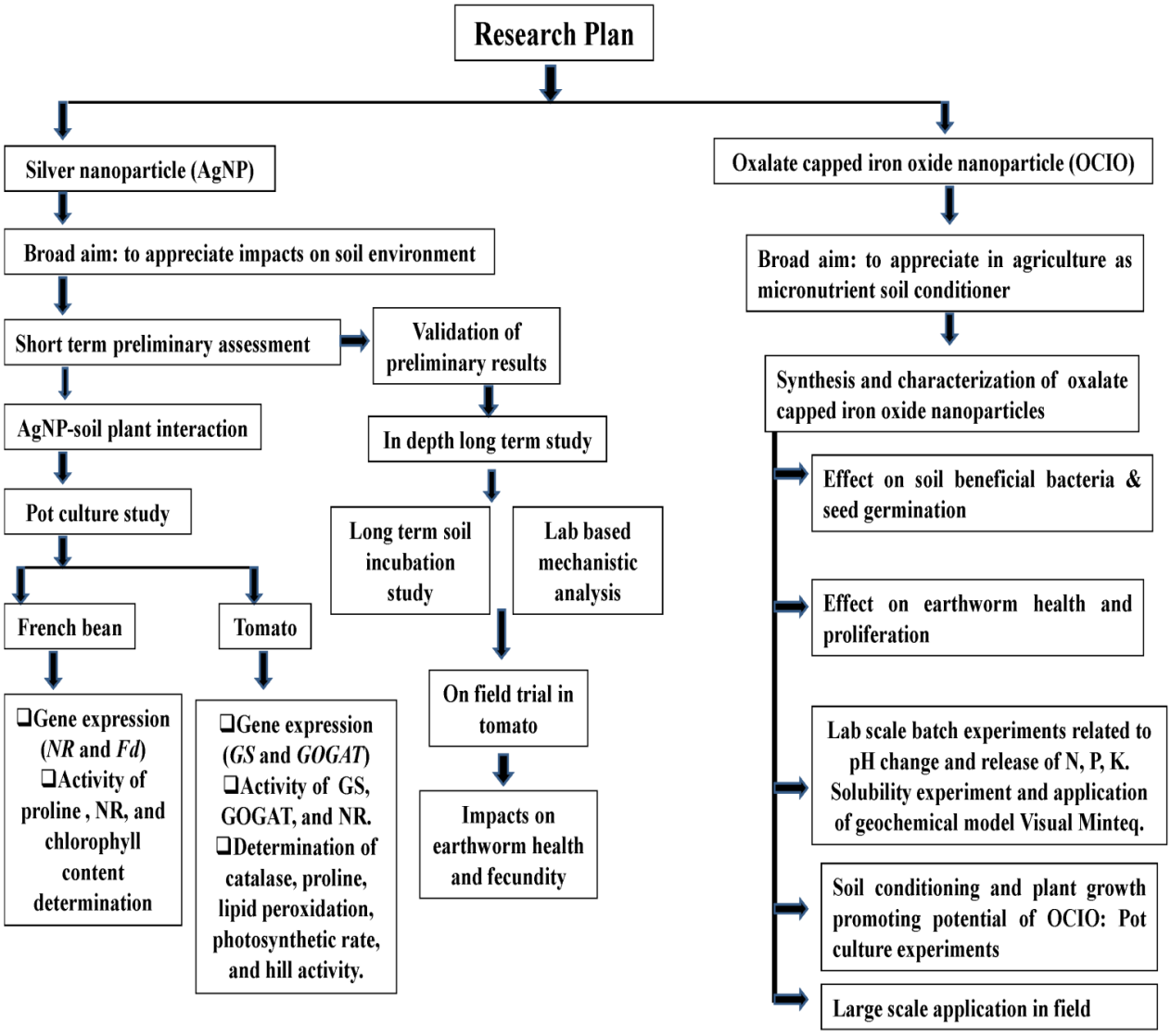


**Fig. 3.1:** Research Plan of the research work

### 3.1. Experiments for silver nanomaterials

*3.1.1. Preparation of the material*

In this study two types of silver nanomaterials have been used. Crude leaf extracts of *Thuja occidentalis* (family *Cupressaceae*) as reducing agent has used for preparation of the green silver nanoparticles (GSNP or AgNP) [1], while the conventional silver nanoparticles (CSNP) was synthesized by using $NaBH_4$ as the reducing agent [conventional silver nanoparticles (CSNP)]. Silver nitrate ($AgNO_3$) and poly ethylene glycol (PEG) were used in the synthesis process of both the SNPs [1].

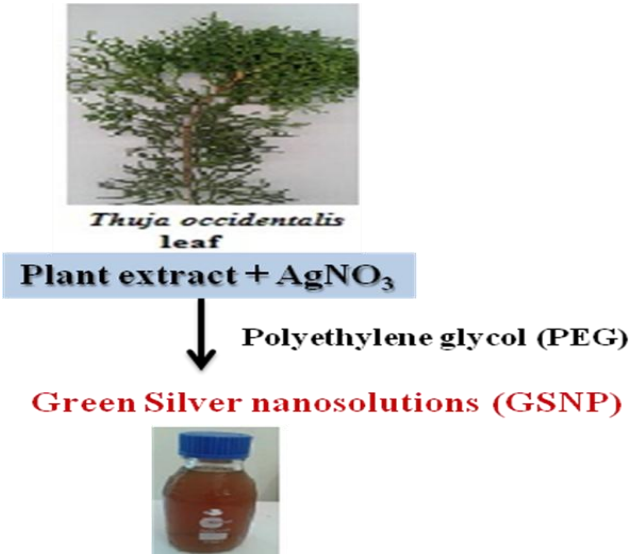


**Fig. 3.2:** Preparation route for green silver nanomaterials

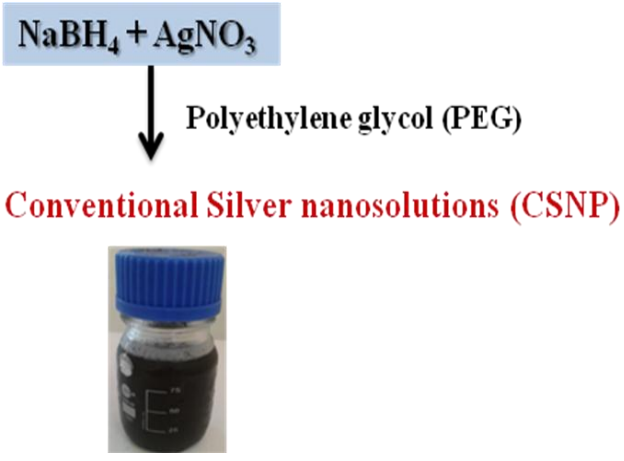


**Fig. 3.3:** Preparation route for conventional silver nanomaterials

### *3.1.2. Fates of silver nanoparticles in soil-plant environment*

#### *3.1.2.1. Preliminary assessments*

Impacts of silver nanomaterials on soil and plant environment were assessed through short term soil-plant experimentations and lab based studies in the preliminary phase.

##### *3.1.2.1.1. Silver nanoparticles-soil and plant interactions*

Composite and representative samples of a typical alluvial soil were collected from a nearby area of Tezpur University, India. The collected soil samples were air dried, sieved, and poured in earthen vessels (2 L volume). Afterwards nanosolutions of different concentrations of CSNP and GSNP were mixed well with the test soil and French bean (*Phaseolus vulgaris*) was grown. Similarly, another experiment was designed with tomato (*Lycopersicon esculentum*). Then soil and plant samples were collected after harvesting the crop and various physico-chemical and biochemical attributes for assessing soil and plant qualities were analyzed in both *P. vulgaris* and *L. esculentum* following established methodologies [2-14]. The minute details of all the protocols and materials are described in chapter 4.

##### *3.1.2.1.2. GSNP-N interactions*

Various lab scale batch experimentations were performed to understand the comprehensive mechanisms of the relationships between the concentrations of GSNP with nitrogen availability. The minute details of the protocol and methodology are described in chapter 4.

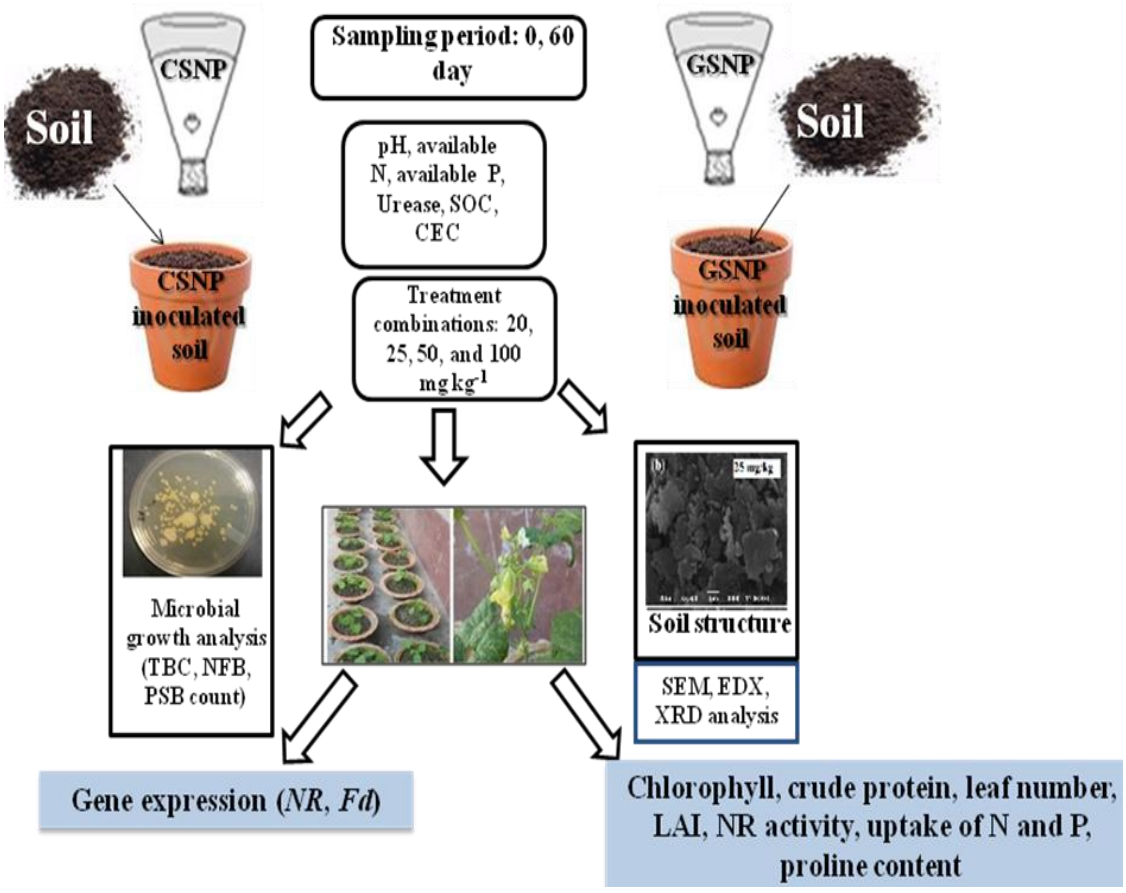


**Fig. 3.4:** Impacts of silver nanomaterials on soil-plant quality attributes in French bean cultivation

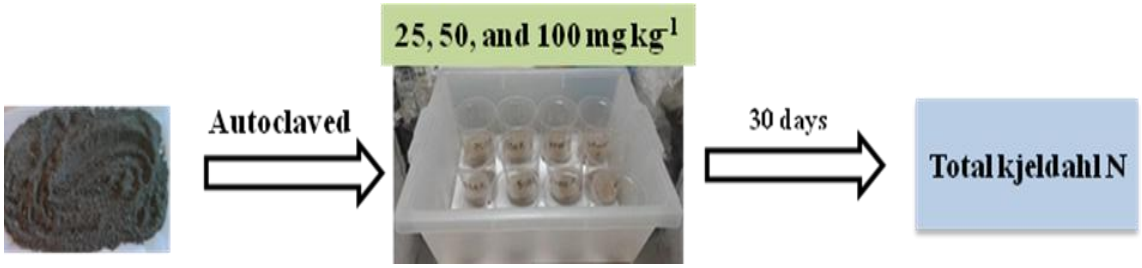


**Fig 3.5:** Impact of green silver nanoparticles (GSNP) on soil TKN status

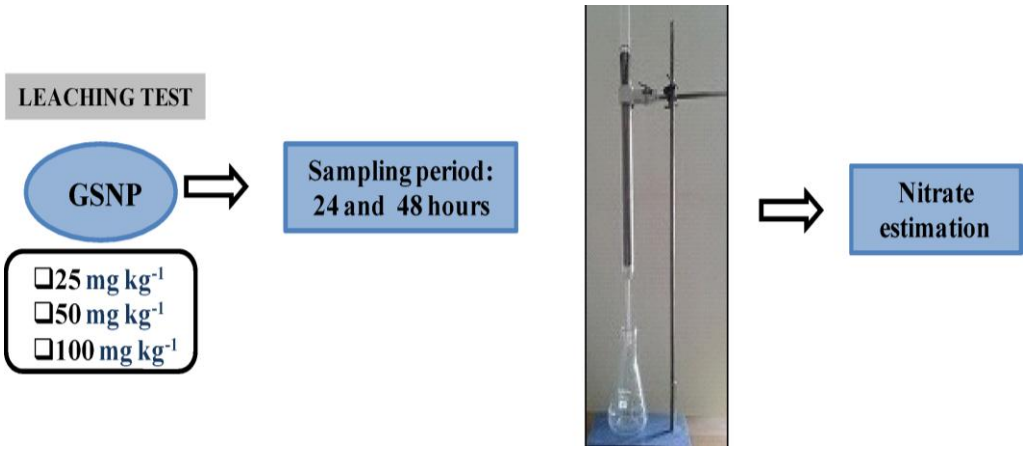


**Fig. 3.6:** Impact of green silver nanoparticles (GSNP) on nitrate leaching of soil

*3.1.2.1.3 Impacts on beneficial soil bacteria*

Microbes play a significant role on nutrient status in a soil environment. Microbial growth in treated soil samples were analyzed adopting standard methodologies [15]. Colony growth of total bacteria, N-fixing and P-solubilizing bacteria were measured. The details of the methodology are described in chapter 4.

*3.1.2.2. In depth and long term study*

On the basis of the results of the preliminary short term studies, the second phases of experimentations were conducted with an aim to validate the previously obtained outcomes and thereby appreciate the impacts of AgNP through more detailed and focused experimentations.

*3.1.2.2.1. Impacts on soil physico-chemical properties*

Long term soil incubation study was carried out with soil obtained from the same locality. Here also, various concentrations of AgNP solutions were applied to the soil samples and the study was continued incubated for 72 weeks. The changes in various physico-chemical attributes were periodically analyzed to comprehend the relationship between concentrations of nanomaterials with pH, surface area, particle size, and availability of N, P, K, S etc. of the treated soil samples [2-5,16-18].

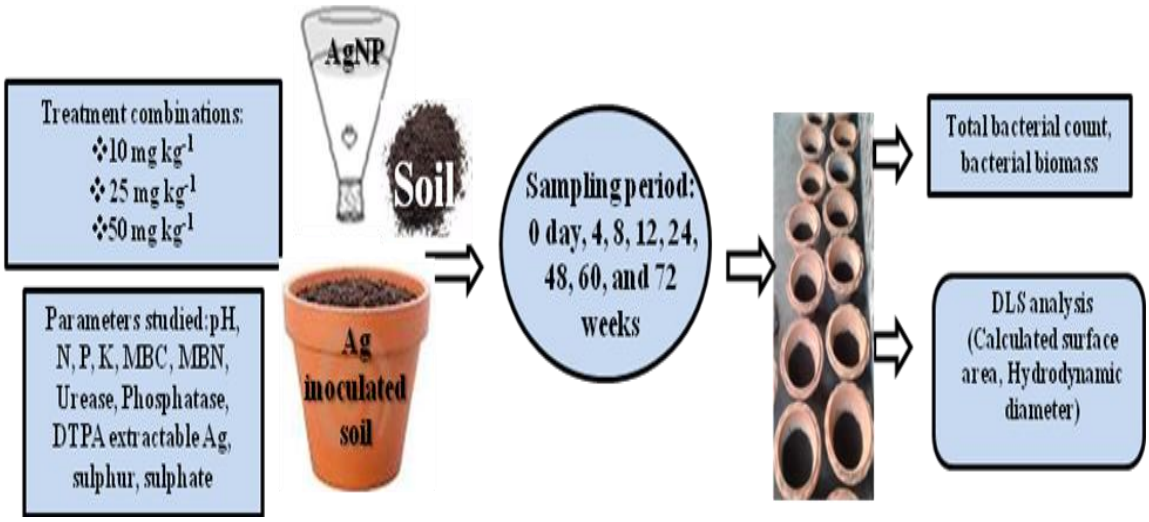


**Fig. 3.7:** Impact of AgNP on soil quality attributes in long term incubation study

*3.1.2.2.2. Behavior of AgNP in aqueous media*

Various lab scale batch experimentations were performed to understand the comprehensive mechanisms of the relationships between the concentrations of AgNP with pH and changes in N ($NH_4$-N), Available P, Available K, $SO_4^{2-}$, and $S^{2-}$ were recorded at different time intervals following standard protocols [10]. The details of all the protocols and methodology are described in chapter 4.

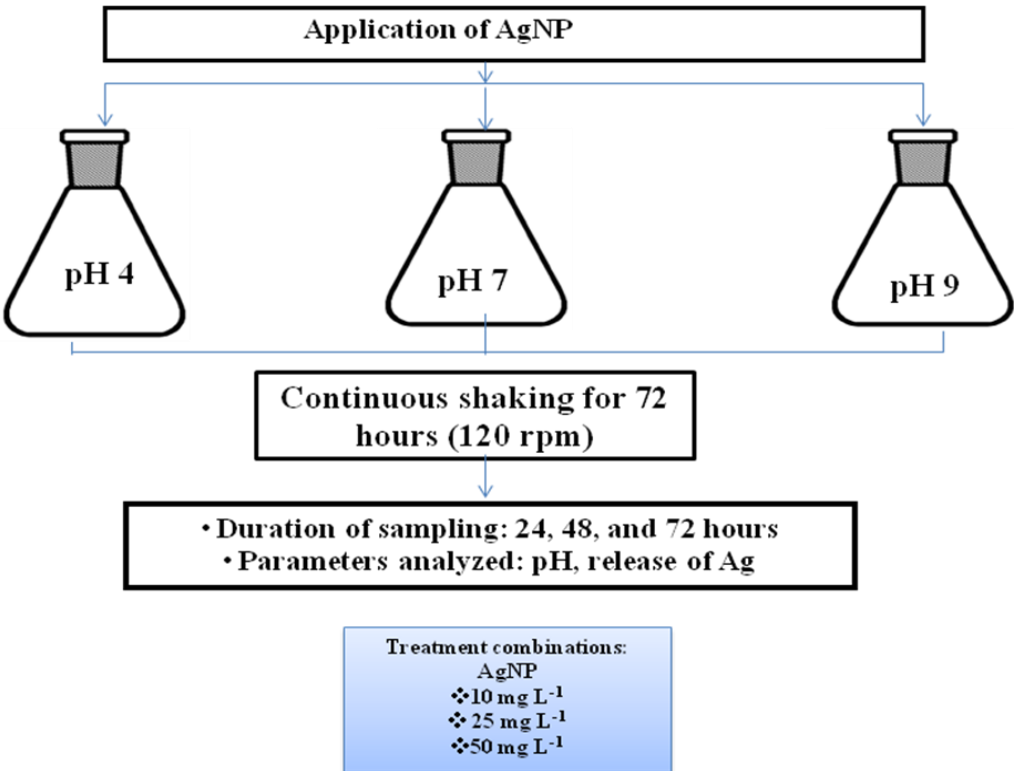


**Fig. 3.8:** Effect of AgNP on various pH conditions

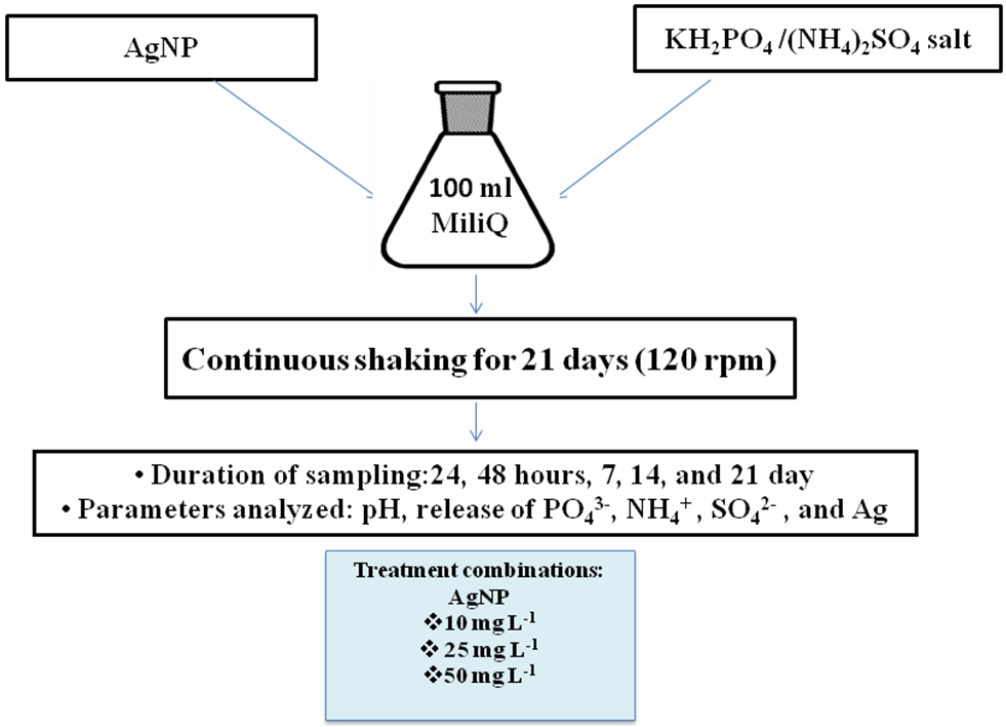


**Fig. 3.9:** Effect of AgNP on P and N solubility in aqueous medium

*3.1.2.2.3. On field trial with tomato*

Based on the outcome of the preliminary and in depth long term pot culture study, a field experiment was conducted in a farmer's field nearby Tezpur University campus for two consecutive years with tomato (*Lycopersicon esculentum* c.v. Badshah F1 hybrid) as the test crop. The detail about experimental part of this study is described in chapter 4.

Soil sampling was done after harvesting of the crop and various physico-chemical parameters were analyzed following established methodologies [2-5,10,17]. The yield and shelf life of tomato were enumerated after harvesting of plant samples.

*3.1.2.2.4. Impacts on earthworm health*

Earthworms (*Eisenia fetida*) were exposed to AgNP for 120 days and their growth and fecundity were monitored. Ag accumulation in earthworm body was also determined along with histological assay to appreciate the effect of AgNP on internal tissue structure [10,19]. The changes in the levels of oxidized stress enzymes catalase [20], reduced glutathione (GSH) [21], glutathione peroxidise (GPx) [22], and glutathione S transferase (GST) [23] were also studied following standard methods. The details are given in chapter 4.

## 3.2. Experiments for oxalate capped iron oxide nanomaterials

The oxalate capped iron oxide nanomaterials (hereafter OCIO) were synthesized by adopting a novel route abiding green chemistry principles. We used ferrous sulphate ($FeSO_4$) as iron source and oxalic acid as a capping agent [24]. The novelty of this material has also been claimed for protection under Indian patent act (Indian patent, Application Number: 201631010727) and a PCT application (International patent, Application Number: PCT/IN2017/50114) has also been filed recently. The adopted pathway for synthesis is schematically represented as below:

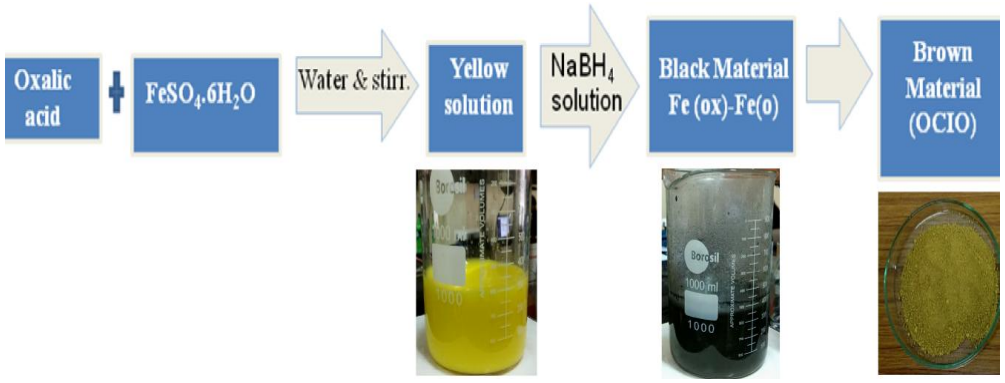


**Fig. 3.10:** Preparation route of oxalate capped iron oxide nanomaterial (OCIO)

The oxalate capped iron oxide nanomaterial (OCIO) was synthesized with the major aim to utilize the material in agriculture as a slow release iron supplying soil conditioner.

*3.2.1. Effects on soil beneficial bacteria (N-fixing and P-solubilizing) and seed germination*

*Rhizobium Sp.* and *Serratia marcescens* were selected as the test species and their growth pattern in response to OCIO exposure assessed by following disc diffusion method [25].

Seed germination assay was conducted using healthy seeds of *Vigna radiata* and *Vigna mungo* and RSG (relative seed germination), RRG (relative root growth), and GI (germination index) were calculated following Karak et al. [26].

*3.2.2. Impacts of OCIO on earthworm health and proliferation*

Earthworm incubation study was conducted similarly with the silver nanomaterials using *Eisenia fetida* as the test species and incubation was carried out upto 60 days. The details are provided in chapter 5.

*3.2.3. Lab scale batch experiments related to precipitation/dissolution dynamics in soil, pH change and release of N, P, and K*

pH variation and release pattern of Fe from OCIO treated solutions under different chemical conditions was studied by incorporating known levels of the nanoparticle in aqueous solution of different pH (4, 5, 6, 7, 8, and 9). The changes in pH and Fe

release were measured in the filtrates [2,27]. The protocol has been detailed in chapter 5.

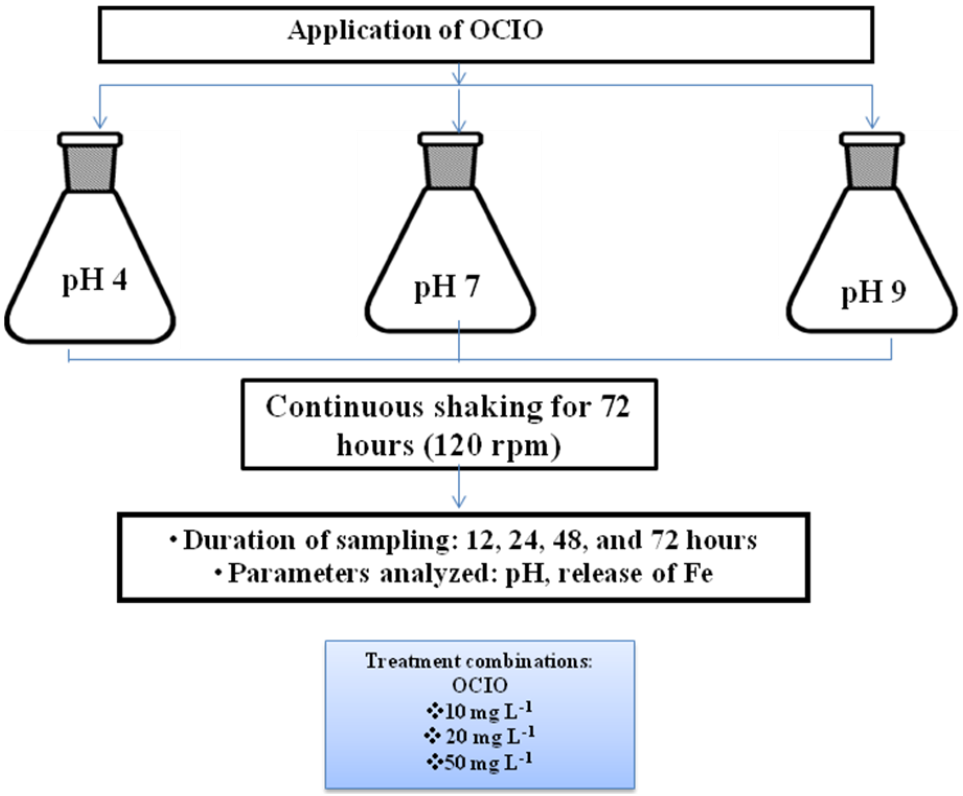


**Fig. 3.11:** Effect of OCIO on various pH conditions

The dissolution/precipitation dynamics of phosphate and Fe was studied by dissolving known levels of $KH_2PO_4$ in known levels of OCIO, $FeSO_4$, Fe-EDTA, and Fe-oxalate solutions respectively. The changes in pH, P, and Fe release was periodically studied [2,27]. A similar study was conducted by mixing the known levels of OCIO and $(NH_4)_2SO_4$ and dynamics of pH, N, and Fe was monitored at 24, 48 hours, 7, 14, and 21 days. In chapter 5 the description of this mechanistic study has been described in detail.

Solubility experiment was conducted to determine the water soluble concentrations of different elements present in the nanomaterial treated soil. Alluvial soil samples were mixed with deionized water and reacted at 120 rpm (rotation per minute) for 7, 14, and 21 days. A portion of each filtrate was analyzed for Fe, Mn, Ca, Mg, $PO_4^{3-}$, $NO_3^-$, $SO_4^{2-}$, $Cl^-$, total alkalinity, and pH following standard methods as referred earlier [10].

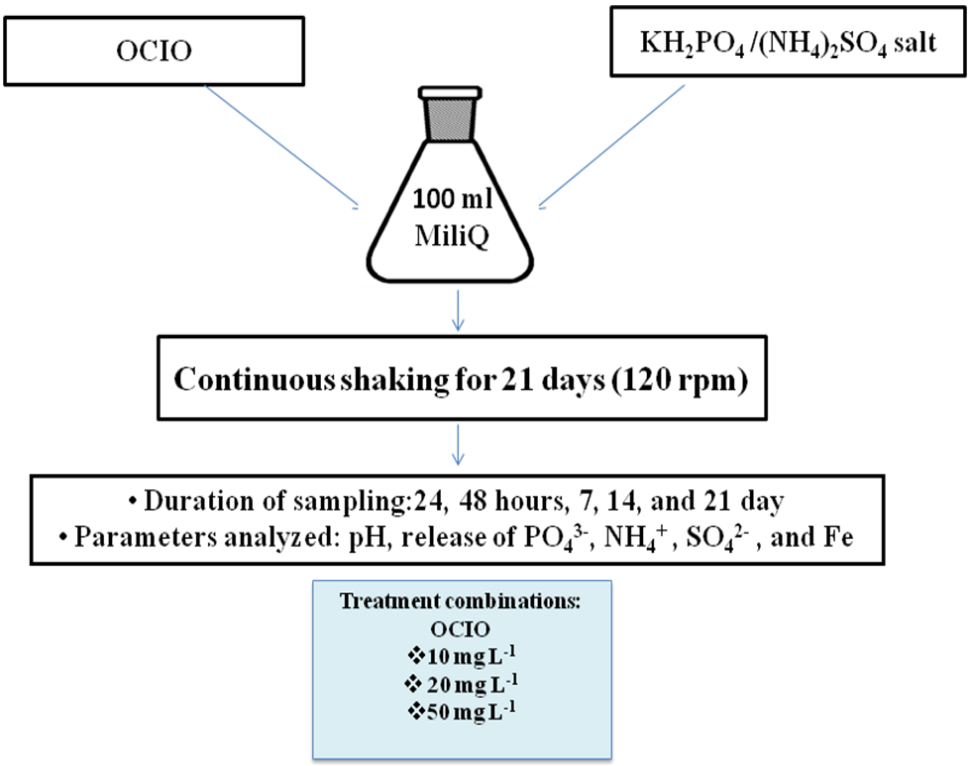


**Fig. 3.12:** Effect of OCIO on P and N solubility in aqueous medium

*3.2.4. Soil conditioning and plant growth promotion potential of OCIO: pot culture experiment*

The experimental samples of a typical alluvial soil was collected from nearby vicinity (Napaam, Tezpur) and processed subsequently. Earthen pots of 2 L volumes were filled with the prepared test soil and incubated with the various concentrations of OCIO, $FeSO_4$, Fe-EDTA, and Fe-oxalate for 90 days. The changes in soil pH, Bulk density (BD), water holding capacity (WHC), soil organic carbon (SOC), nutrients (N and P), urease and phosphatase activities were periodically (0, 45, and 90) assessed [2-5]. In addition, surface area, charge, and hydrodynamic diameter (HDD) of OCIO incorporated soil samples were determined by dynamic light scattering (DLS) and BET counter [16].

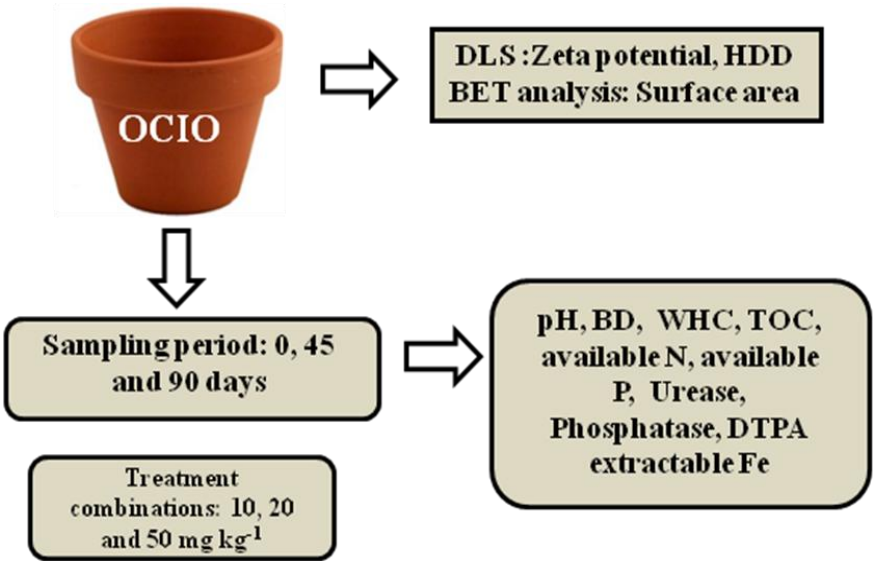


**Fig. 3.13:** Effect of OCIO on soil quality attributes in pot scale study

The competence of the synthesized compound to accurate Fe deficiency was estimated in highly leached soil. Nutrients were artificially leached by passing deionized water for a period of 96 hours through the soil samples (Fig. 3.14). Afterwards tomato seedling was transplanted into each pot and the deficiency recovery potential of OCIO was assessed by studying attributes (total chlorophyll content, yield, uptake of P, Fe, and leaf chlorosis indicative in plants) [7,10]. The detail of protocols and methodology has been described in chapter 5.

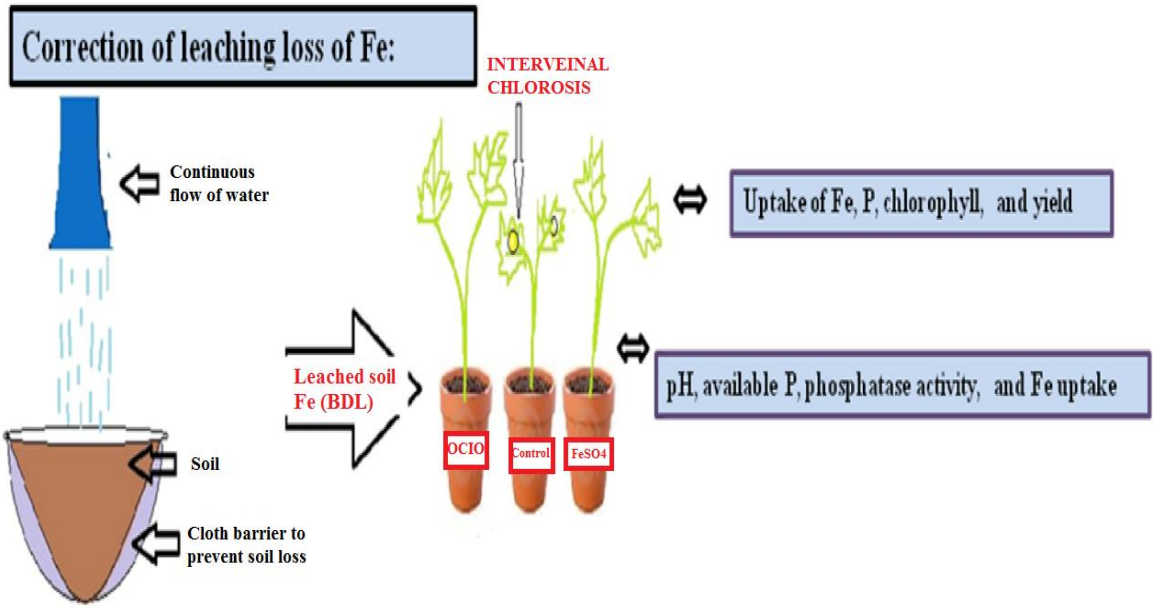


**Fig. 3.14:** Fe deficiency recovery potential of OCIO in nutrient leached soil

*3.2.5. Field experiment- Impact of large scale application*

Field trial of OCIO was conducted in a nearby farmer's field in the same manner as described for AgNP. The details of the analytical tools have been described in chapter 5.

**3.3. Quality assurance and quality control**

The general quality control guidelines (QC) provided by Tezpur University was rigorously followed during performance of all analysis in different experimental designs. Quality assurance and quality control are defined as below:

Quality Assurance is defined as "a set of coordinated actions such as plans, specifications, and policies used to assure that a measurement program can be quantifiable and produce data of known quality".

Quality control is defined as "The routine use of procedures designed to achieved and maintain a specified level of quality for a measurement system". A monitoring system without adequate QA/QC runs the risk of not being able to control the quality of data, and not being able to assure accuracy and precision.

*3.3.1. Sample storage and preservation*

Sample collection, preparation (air drying, grounding, and sieving) and preservation was done as per the recommendations of AOAC. Prepared samples were preserved in cleaned, air tight plastic containers with proper name and collection date accordingly. Most of the sample analysis was done within 2-3 days after collection of samples. However, for some experimental analysis samples were stored at 4°C maximum upto 28 days post collection.

*3.3.2. Calibration procedure*

All analytical instruments were calibrated beforehand and properly verified at least once a day or before sample analysis. One blank and several standard solutions were required for instrument calibration procedure. However, for some instruments (e.g., pH meter), blank is not required; hence several standard solutions were used regularly. In order to ensure precision in the sample analysis standard reference materials (SRMs) were used during the experiment period.

*3.3.3. Initial demonstration of performance*

The initial demonstration of performance mostly deals with characterization of analytical instruments and laboratory performance. Determinations of linear calibration range and method detection limits (before analysis) were the issues considered under this.

*3.3.4. Linear calibration range (LCR)*

The linear calibration range was established for all important instruments (like Kjeltec analyzer, UV-VIS Spectrophotometer, Flame photometer, ICP-OES, and AAS) in the initial period and this was verified at least twice in a year or whenever a major change was noticed in the instrument response. Generally in the verification process 1 blank and 3 standards were used, however this may vary depending on the specific protocols for specific instruments. If the verification in linearity was exceeded by ±10% than the initial data, then linearity in calibration range was re-calculated.

*3.3.5. Method detection limit (MDL)*

Establishment of method detection limits were obtained through analyzing different replicates of a standard solutions or reagent water keeping the whole analytical methods identical.

The MDLs were calculated using the following formula: MDL=t×S

Where t=student's t value for a 99% confidence level a standard deviation estimate with n-1 degree of freedom [t=3.14 for seven replicates]. S=standard deviation of the replicate analyses. MDLs should be estimated at least twice in every six months period.

*3.3.6. Instruments and equipments*

*3.3.6.1. Operations and maintenance*

All the analytical instruments were maintained judiciously in proper condition. Proper records were maintained for instrument calibration, correct operations, and trouble-shooting etc. Maintenance of all analytical instruments and equipments were done on a daily basis by following some guidelines mentioned below:

**Table 3.1: Guidelines followed for calibration and daily maintenance of the laboratory equipments**

| Equipment | Calibration | Maintenance |
|---|---|---|
| pH Meter & probes | After analysis of every 90 samples pH probe was calibrated at pH 4, 7, and 9 with standard buffer solutions. | The probe was regularly cleaned, checked and ensured that the electrode was properly dipped into standard solutions. |
| Weighing Balances | Balances were calibrated every day before use. Authentic weights were used to check the precisions of the balances. | Regular cleaning was done with ethanol water after every use. Air tight cabinets were maintained. At least once in a year specialized persons were called for cleaning and certification of accuracy of the instrument. |
| UV-VIS Spectrophotometers | Calibration was done after initial warm up and verified after analysis of every 40 samples. | Maintenance was done by assigned professionals once in a year. |
| ICP-OES | Calibration was done after initial warm up of the instrument and verified after every 40 samples analyzed. | Maintenance was done yearly by assigned professionals once in a year. |
| Flame photometer | Calibrated after initial warm up. Verified after every 40 samples analyzed. | Maintenance was done yearly once by professionals. |
| Automated Pipettes | Calibrated twice in a month. | Cleaning after every use. |

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