Uptake and effect of nano bone char in *Syngonium podophyllum* under hydroponics and soil systems

By

Saher Shahid

Registration No: 02312113009

Supervised by: Dr. Mahtab Ahmad

Department of Environmental Sciences

Faculty of Biological Sciences

Quaid-i-Azam University, Islamabad

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A Dissertation Submitted in Partial Fulfillment of Requirement for the Degree of Master of Philosophy in Environmental Science

QUAID-I-AZAM UNIVERSITY
ISLAMABAD

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Registration No: 02312113009

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<u>CERTIFICATE OF APPROVAL</u>

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Saher Shahid (MPhil Scholar) ____________________

<u>Supervisor:</u>

Dr. Mahtab Ahmad ____________________

Associate Professor

Department of Environmental Sciences

Quaid-i-Azam University, Islamabad

<u>Chairperson:</u>

Dr. Abida Farooqi __________________

Associate Professor

Department of Environmental Sciences

Quaid-i-Azam University, Islamabad

Date

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LIST OF ABBREVIATIONS

NBC	Nano bone char
CNP	Carbon nanoparticles
CNTs	Carbon nanotubes
SWCNTs	Single walled carbon nanotubes
MWCNTs	Multi-walled carbon nanotubes
FTIR	Fourier transform infrared spectroscopy
XRD	X-ray diffraction
EDX	Energy dispersive X-ray
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
BC	Biochar
OM	Organic Matter
ANOVA	Analysis of variance
cm	Centimeter
dS/m	Deci Siemens per meter
EC	Electrical conductivity
DW	Distilled water
mg/L	Milligram per liter
mg/kg	Milligram per liter
ml	Milliliter
^{o}C	Degree Celsius
%	Percentage
WHC	Water holding capacity

ABSTRACT

The use of nanotechnology in terms of nanoparticles, carbon nanotubes, quantum dots, etc., when exposed to the environment, including plants, animals, and humans help increase their productivity. Understanding the fate of these nanomaterials naturally present in our environment or introduced by anthropogenic activities is important. Waste bones are largely produced from the precipitously growing meat sector across the globe, which can be converted to bone char through the process of pyrolysis in an oxygen-deficient environment. Bone char is free from heavy metals and organic contaminants and is a rich source of phosphorus, calcium, and magnesium. All these nutrients significantly contribute to the growth of the plant. This study focused on the uptake of nano bone char in the *Syngonium podophyllum* plant, and its impact on the plant growth under both the hydroponics and soil systems. The techniques used for the detection of nano bone char in the plants tissues include compound microscopy, scanning electron microscopy (SEM), and fourier transform infrared spectroscopy (FTIR). The compound microscopy and SEM results confirmed the presence of nano bone char particles in the leaves and roots of the plants under both hydroponics and soil systems. To observe the effects of nano bone char on plants growth, various parameters including fresh weight, dry weight, root length, shoot length, chlorophyll content, leaf count, total calcium, and phosphates were determined. The hydroponics plants show increase in fresh weight (76.90%), dry weight (123.17%), shoot length (25.29%), chlorophyll content (18.41%) and leaf count (100%) in highest treatment of 500 mg/L as compared to control. The EDX results showed higher contents of carbon (61.64%), phosphorus (0.62%) and calcium (0.95%) for 500 mg/L treated hydroponics plant, as compared to control. Likewise, the soil plants also showed increase in fresh weight (82.50%), dry weight (250%), shoot length (32.0%), root length (37.70%), chlorophyll content (17.6%) and leaf count (100%) in treatment dose of 0.50% than control. The EDX results for soil system plants showed higher contents of carbon (65.79%), phosphorus (1.01%), and calcium (3.45%) for 0.50% treatment as compared to control. It is concluded that the utilization of bone waste in the form of nano bone char will not only reduce the risk associated with the waste but also open a new window for the application of nanotechnology.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Background of Nanotechnology

Before preface and definition of nanotechnology, people used different nano-sized objects and nano-level processes in practice. People subconsciously practiced nanotechnology for thousands of years BC in natural fabrics, flax, cotton silk, and wool. The interesting thing about these fabrics was the network of pores of these fabrics possessing a size of 1-20 nanometers, the conventional nano porous materials. For many years secrets of nanotechnology passed on from generation to generation without knowing the reason for their unique properties. Nanotechnology can be understood as a technology that allows a controllable way of designing nanomaterials and operating them i.e., using them according to their required purpose. Nanomaterials are defined as materials possessing the distinguishing characteristic of at least one of three dimensions on a nanometer scale (Tolochko, 2009).

For the first time in history, the term nanotechnology was brought into consideration by a professor at California Institute of Technology R. Feynman. He delivered a session at the American Physical Society in 1959 and used the sentence ―There is a lot of space down there". It was for the very first time that the possibility of using atoms as a building block and the creation of nanosized material was considered seriously. Today this lecture by Feynman is referred to as the origin of the nanotechnology paradigm (Chang et al., 2020). Feynman came across this latest field of research gaining the hold of the scientific community and leading to the development of two approaches for the synthesis of nanomaterials. These approaches are: 1) the top-down approach, and 2) the bottom-up approach. These approaches differ on the level of quality of nanostructure, speed, and cost of production (Bayda et al., 2019). The top-down approach works on the breakdown of the bulk or large material to achieve nanosized materials (Iqbal et al., 2012). The bottom-up approach focuses on designing and strengthening nanomaterials from the base i.e., atom-by-atom or molecule by molecule either through physical and chemical methods within the nanometer range (1 nm to 100 nm) through managed designing of self-assembly of atoms and molecules (Iqbal et al., 2012).

Nanotechnology word specifically was initiated earlier by N. Taniguchi at the international conference on industrial production in 1974. The scientific world came to know about this word to describe the super thin handling materials with nanometer precision and the development of nano-sized mechanisms (Hulla et al., 2015). During the period 1985, Robert Curl, Harold Kroto, and Richard Smalley found out that carbon has the potential to exist in the form of a stable sphere or ball. They named these structures Buckminsterfullerene or buckyballs. These buckyballs of carbon having chemical formula of C60 or C70 are created when graphite is allowed to evaporate in a motionless environment. This unrolled a new carbon chemistry window that gave the option to develop confined metal atoms and discover recently developed organic compounds (Kroto et al., 1985). In 1986, E. Drexler compiled the ideas of nanotechnology from Feynman‘s point of view and mention them in his book ―Vehicles of Creation: the arrival of the nanotechnology era". During the period of the 1980s and 1990s, multiple important discoveries were made that served as grounds for future inventions in nanotechnology. In 1991, the first-ever nanotechnological program ―National Scientific Fund" was operated in the USA (Tolochko, 2009).

The start of the 21st century was seen as the beginning of nanoscience and nanotechnology. Then-president Bill Clinton supported the funding of research in nanoscience and technology in January 2000. Also, in 2001 the National Nanotechnological Initiative was accepted in the USA. Three years later, President George W. Bush approved the law named the 21st Century Nanotechnology Research and Development Act. In 2004, Xu et al. (2004) during the ablution of single-walled carbon nanotubes accidentally discovered an advanced form of carbon nanomaterial known as carbon dots, now commonly known as quantum dots that has a size range below 10 nm. The carbon dots due to their interesting properties soon gained the attention of scientists as a novel carbon member due to their benevolent, ample, and non-costly nature (Baker et al., 2010). The highly significant properties such as low levels of toxicity and great biocompatibility support C- dots as a suitable candidate for bioimaging, biosensors, and drug delivery processes. After graphene was discovered in 2004, carbon-based materials and nanomaterials enhanced the foundation of every field (Bayda et al., 2017).

Scientists are operating to achieve a breakthrough in nanoscience and nanotechnology for making human life more portable, comfortable, and easier. In a very short period, nanotechnology has taken over various sectors from industrial to medical applications such as treating cancers, diagnostic biosensors, and drug delivery systems. In the industrial sector the food industry makes use of nanomaterial in production, packing and increasing shelf life (Hulla et al., 2015).

1.2. Nanotechnology and Agriculture

The agricultural sector plays the role of backbone in every country‘s economy, especially developing countries where a maximum number of people are linked or dependent on the agriculture sector either directly or indirectly for food consumption or economic development. According to reports from the United Nations, the world population will have a scale of 9 billion by 2050. A widely acceptable option is that an increase in agricultural productivity is required to meet the needs of the growing population. Therefore, it is necessary to shift the focus and interest towards modern technology including nanotechnology and nanobiotechnology in the agriculture sector. Nano agriculture focuses on farming practices involving nano-scale materials and particles to boost livestock, crop yield and increase soil fertility for a longer period.

1.3. Nanotechnology and Fertilizers Use

Nano fertilizers are defined as nanomaterials with specific chemical compositions that support the growth of plants and crops allowing them to benefit from the nutrients for a longer period (Raliya et al., 2017). They have the ability for higher nutrient uptake efficiency than their bulk counterpart. Also, they are applied in small amounts and provide higher dissolution rates in soil solutions as compared to mineral fertilizers applied in bulk quantities. Because of their tiny particle size and high definite surface area nano fertilizer possess the ability to enter the plant cell walls through their pores (Nair et al., 2010). The primary uptake mechanism of nano fertilizers suggests after applying it to the soil, nano fertilizer is dissolved in water and soil solution resulting in the absorption of nutrients through the root system of the plants (Ma et al., 2010). One of the significant sources of water pollution is the excessive use of fertilizers in the fields. Water pollution can be avoided significantly by the release of nutrients in the soil in a controlled manner using nano fertilizers. Nano fertilizers have unique properties such as high absorption ability, high yield of crops, increased photosynthetic activity and surface area of leaves, high elemental efficiency, and decreased soil toxicity (Naderi and Shahraki, 2013).

1.4. Nanoparticles and their Interaction with Plants

Recent discoveries considering the differential properties of nanoparticles on plants have supported the use of nanotechnology in plant biotechnology, crop management, the biofuel industry, etc. However, one of the significant issues required in nanotechnology application is the ecotoxicological impacts associated with nanotechnology (Sekhon, 2022). Nanomaterials biotransform themselves during their entry into plants either through an aerial pathway or root pathway and their process of uptake, transformation and magnification through plants is known as biotransformation. The various roots of uptake of nanomaterials by plant hydroponics method are the most compatible with implying nanotechnology methods (Jeyasubramanian et al., 2016).

Unlike animal cells, it is difficult for foreign particles to enter the plant system due to their robust cell wall. Although recent studies in the past few years have shown the capability of engineered nanoparticles to penetrate the cell wall of plants. Nanoparticles having a pore size less than the cell wall diameter or pore size of the roots allow the easy passage of nanoparticles through them. Also, the leaf stomata of the plant offer the route for the entry of nanoparticles when applied through the leaf surfaces (Nair et al., 2012). The synthetic nanoparticles may also pose nano contamination in soil and water, along with plants that easily absorb and accumulate nanoparticles. The two important pathways that cause the increase of micro and nanoparticles load on the plant are the uptake of nanomaterial through foliar and root system that affects food safety with the possibility of food chain transfer posing a risk to human health (Sun et al., 2021). The optimum dose or exposure of nanomaterial to plants positively affects their growth. For example, TiO_2 at the desired concentration could enhance the growth of spinach by activating the photosynthetic activity of the plant (Sozer and Kokini, 2009).

1.5. Carbon Nanoparticles and their Interaction with Plants

Carbon is one of the significant elements required for sustaining life activities. Along with its presence in organic compounds it also actively participates in biochemical processes. The elemental carbon with its exceptional new nano version excludes coal, graphite and diamond has gained extraordinary significance in nanotechnology applications (Saxena et al., 2014). Among the different nanomaterials, carbon-based nanomaterials hold prime importance in biotechnology applications. These carbon-based nanomaterials include single-walled carbon nanotubes (SWCNTs), multiwalled carbon nanotubes (MWCNTs), buckyballs, graphene, etc. (Nair et al., 2012). The effects of SWCNTs or MWCNTS on plants depend on multiple factors including the type of plants studied, the type of CNTs applied, their concentration, the technique used (such as ultra-sonication) for the formation of CNTs, the growth medium and the environmental conditions in which the experiment is taking place (Tiwari et al., 2014). Multiple research groups have reported the positive impacts of carbon nanotubes on plant growth and development. Therefore, carbon nanotube exposure to onion, ryegrass and cucumber resulted in enhancing its growth in roots (Khodakovskaya et al., 2009). The growth in tomato plants was also activated when subjected to MWCNTS along with affecting their gene expression for cell division and plant development. Khodakovskaya et al. (2012) explained that SWCNTS can penetrate the walls and membranes of the tobacco plant. The distinctive trait of carbon nanoparticles penetrating plants has stimulated interest in using nanoparticles as smart treatment delivery systems in plants. The MWCNTs at the optimum levels of 1040 µg/mL penetrated thick seed coats of tomato plants resulting in stimulating growth and activating its growth (Khodakovskaya et al., 2012). Giraldo and his coworkers (2014) explained that SWCNTs comprising of cerium nanomaterials transported themselves passively and localized in an irreversible fashion within the lipid envelope of chloroplasts of plant extracts. This activity resulted in over three times higher photosynthetic activity in comparison to control along with stimulated electron transport rates (Giraldo et al., 2014). Studies have revealed improved growth in rice plants under lab condition when subjected to low concentrations (20 mg/L) of SWCNTs and MWCNTs. There was increased chlorophyll content and overall photosynthesis rate observed in rice plants. Although, at a high concentration of 500 mg/L, degradation effects were observed in rice (Joshi et al., 2020). The negative impacts of CNTs were observed in some previous studies with toxic behavior of CNTs in *Arabidopsis thaliana* even in the low concentrations of 10 mg/L with an accumulation of CNTs inside the cells (Tan et al., 2009). Therefore, plants are the keystone constituent present on the earth. They are the geo-physical-chemical transducer that delivers food and oxygen essential for life. Along with this, they are the receiving end of multiple contaminants from the environment including carbon-based nanotubes. Hence, plants to CNTs interaction must be thoroughly investigated from basic cellular levels to organismic levels to understand the underlying multidimensional complexities (Tiwari et al., 2014). This information will further help in improving the technology of nano agriculture i.e., an advanced area of nanobiotechnology based on improving soil quality and crop yields.

1.7. History of Biochar

The biochar, in the beginning, was discovered in the old Amerindian populations of the Amazon region, labelled as Terra Preta de Indio. In this region, dark earth was structured by means of char and slash methods (Lehmann and Joseph, 2009). The research was conducted on the soils of this region which indicates that biochar plays a substantial role in improving soil quality. There is growing indication that biochar enhances the carbon stability in the soil along with increasing the nutrient accessibility far off a fertilizer effect. Biochar is quite efficient in building up soil eminence compared to other organic soil modifications. Considering the benefits of biochar, the impression of Terra Nova has developed for strengthening soil fertility and properties by advanced alternatives of management practices which shaped the Terra Preta.

1.8. Biochar

The International Biochar Initiative (IBI) defines biochar as ―a solid material obtained from the thermochemical conversion of biomass in an oxygen-limited environment" (IBI, 2015). Biochar is the rich carbonaceous material coming either from organic or carbon-based feedstock because of specific thermal combustion conditions in an oxygen-deficient environment (Lehmann, 2009). Multiple materials are used for biochar including municipal waste, agricultural waste, food waste or other carbon-rich products. The expression biochar is used in soil sciences and is associated with the management of carbon content in soils. Fundamentally, it means a dark and dusky carbon acquired from biomass through pyrolysis which intimately bears a resemblance to activated carbon comprising a medium-to-high surface area and an organized carbon matrix (Ahmed et al., 2013). Biochar, as a solid carbonaceous material, is coming out as a substitute to fortified carbon with lucrative and environmental benefits.

The distinction that exists between biochar and charcoal is in their final utilization. Charcoal is commonly meant for heat generation purposes whereas, a superior quality biochar has high porosity, high nutrient content, efficient water-storage capacity, a comprehensive microstructure and is applied as an adsorbent of heavy metals or contaminants or soil adjustment (Lehmann and Joseph, 2015).

The first step in biochar production is pyrolysis. Pyrolysis takes place in the absence of oxygen with temperature extending out of 300 °C to 900 °C (Jin et al., 2016). Solid, liquid and gases are produced because of the pyrolysis process. Pyrolysis includes two types: 1) fast pyrolysis and 2) slow pyrolysis. The distinctive attributes of fast pyrolysis involve the temperature of the reactor to meet the specific required level and then the feedstock is fed inside the reactor. The retention time normally continues for quite a few seconds. In slow pyrolysis, the feedstock is placed within the reactor at the start of pyrolysis. The retention period stretches from half an hour to several hours. The evaluation of slow and fast pyrolysis indicates that slow pyrolysis typically gives high yields of biochar (Bridgwater, 2012). A fast pyrolysis process that involves little retention time, i.e., less than 2 sec is normally useful to produce bio-oil which normally offers 75% bio-oil from biomass. Slow and medium pyrolysis with a residence period of minutes to several hours or sometimes days, typically favors 35% of biochar yield (Ahmad et al., 2014). When the feedstock undergoes the pyrolysis process, biochar is produced with a sequence of reactions such as isomerization, dehydration, decarboxylation, depolymerization, and charring. Carbon is linked with cellulose and hemicellulose on the heating breakdown into liquid hydrocarbons through the arbitrary breaking of the polymeric backbone. The carbon connected to additional intricated assemblage, i.e., lignin experiences mid-chain dehydration and repolymerization and results in the formation of biochar with high lignin content in which the functional groups such as $-O-CH_3$ and aliphatic constituents remain likely to undergo polymerization intending to ring structure, i.e., charring took place at high temperature (Zhou et al., 2016). Lignin owing its thermal stability also plays a role in pore development on biochar (Kim et al., 2019).

The main elements that constitute biochar are carbon, hydrogen, oxygen, small amounts of nitrogen, sulfur, and ash. The fundamental configuration of biochar differs based on the conditions in which biochar has been produced and the feedstock considered to produce the biochar (Cha et al., 2016). A biochar that is formed at high temperatures possesses a high surface area along with high carbon content, primarily because of an increase in micro-pore volume as high temperatures cause the exclusion of volatile organic compounds (Chen et al., 2008). Nevertheless, the rise in temperature causes a decline in biochar yields (Xu et al., 2015).

1.9. Feedstock for Biochar

A feedstock is a raw material or biomass which is used to produce biochar. A feedstock is divided into two groups i.e., biomass formation as a biochar or energy source. Secondly, biomass waste as a derivative. All the feedstock differs in amounts of elemental composition and properties. So, the biochar obtained from various feedstock has several application abilities compared to each other. To attain several biochar production objectives such as, enlightening economic side, reduction in environmental emanations and growing multiple materials in formation of biochar (Czajczynska et al., 2017), substantial feedstock sources should be contemplated properly which include type of feedstock, collection of feedstocks, size reduction of feedstock (prior to biochar formation), storage facilities for feedstock, automation in handling and analysis of its composition (Stone et al., 2010). The vagueness based on the price and obtainability of feedstock biomasses diverges all over the world (Speirs et al., 2015). Therefore, preparation of numerous existing feedstocks, linked with chemical pretreatment (Carpenter et al., 2014) and thermochemical procedures, will simplify a consistent, cheap, and high-volume biomass source for developing biochar sector (Yazan et al., 2016). The biochar yield and quality are affected depending on the biomass feedstock selected and the pretreatment methods chosen for biochar preparation (Gai et al., 2014a). Biochar is a practical solution for managing solid waste. It could be obtained in a sustainable manner from the numerous biomass feedstock sources available including agricultural residues, agro-industrial waste, hard-wood forestry biomass, food waste and livestock waste (Gunamantha and Widana, 2018). Waste from agriculture sources, wood, forest residue, animal dung, municipal solid waste, and sludge from sewage are the most common feedstock materials used for biochar production. The feedstock material used for nano biochar production include sugar cane bagasse, rice husk, shell (peanut shells etc.), bamboo, empty fruit bunches, woods, and soya bean stovers. The feedstock has a significant and direct influence on the properties of resulting nano biochar (Ramanayaka et al., 2020).

1.10. Environmental Benefits of Biochar

Owing to unique characteristics, including high carbon content, recalcitrant and catalysis, large surface area, stable structure, and high cation exchange capacity (Rizwan et al., 2016), biochar is extensively used for environmental improvement. The principal usage of biochar covers four major domains, where biochar plays a key role including waste management, energy generation, soil remediation, and climate change mitigation (Ahmad et al., 2014; Zhou et al., 2014a). Biochar is now also being explored in terms of healthcare applications and in industries in terms of uses in electrodes, capacitors, and sensors. The absorptive properties of biochar are enhanced by different pre- and post-activation methodologies (Ramanayaka et al., 2020).

1.10.1. Soil Improvement through Biochar

Biochar contributes significantly to soil conditioning plus has a strong potential in improving the biological and physicochemical properties of soil owing to its rich carbon content. The soil water retention capacity is enhanced due to organic carbon present in biochar. Glaser et al. (2002) reported that soil containing biochar enhanced soil water holding capacity up to 18%. The biochar application not only plays its part in improving soil structure but also soil water retention ability is linked with surface area and hydrophobicity of biochar (Verheijen et al., 2010). Biochar occurrence in soil mixture contributes towards the physical parameters of the soil system including consistency, porosity, depth, packing, particle size distribution, surface area, texture, structure, and pore size distribution. Biochar influences the physical parameters of soil which later affect plant development and productivity. As the accessibility and perforation of air and water in the roots of the plants are evaluated majorly by the physical configurations of the soil (Lehmann et al., 2006; Chan et al., 2008). The surface of biochar comprises of several chemically active groups including hydroxide, carboxyl, and ketones. These chemically active functional groups have great strength for the adsorption of toxic chemicals in soils with heavy metal-contamination (Berek et al., 2011).

1.10.2. Waste Management through Biochar

Waste coming from agricultural and animal sources is becoming a huge environmental challenge that ultimately is responsible for polluting ground and surface water (Matteson et al., 2005). The utilization of this biowaste as feedstock for pyrolysis to produce bioenergy (Bridgwater et al.,

2003) represents an important economic achievement and environmental management. Different waste sources provide chances of recovering energy specifically when a large amount of feedstock is produced at a definite place (Matteson et al., 2005). Hossain et al., (2011) reported that pyrolysis offers beneficial paths for conversion of waste sludge into biochar, thus ameliorating the waste management, dropping its transport costs, and lowering production volumes. The landfilling of organic waste from various agricultural means, food waste and breakdown of animal waste through anaerobic digestion can result in the release of copious amounts of methane along with the oxides of nitrogen. Therefore, reduction in greenhouse gas emissions along with effective waste management strategy could be implemented using the waste coming from these sources for biochar production, which will also reduce waste management cost linked with traditional waste management methods (Kwapinski et al., 2010). Biomass feedstocks of exceptionally low quality, sometimes byproduct waste, is used for gaining energy and biochar through slow pyrolysis process (Kwapinski et al., 2010), in that way, converting the carbon confined in these constituents as of short-term carbon storage to long-term carbon sequences. The movement of carbon in the form of biochar back to the soil confirms a positive feedback mechanism by enhancing the crop growth (Downie et al., 2011).

Environmental pollutants discharge through industrial facilities, residential, municipal, and commercial sources damage adjacent ecosystem and environment. Soil and water resources in the environment are regularly exposed to pollution by organic and inorganic contaminants released because of human activities. Modern methods are being introduced to mitigate polluted soil and water issues. The most vibrant methods are to diminish the bioavailability of contaminants, therefore, decreasing their existence and toxicity in animals and plants. Biochar is an emergent method for reducing the bioavailability of contaminants in the environment with supplementary advantage of soil improvement (Sohi, 2012).

1.10.3. Biochar for Wastewater Purification

Different anthropogenic activities result in the release of heavy metals from different industries such as mining, metal fishing, textiles, electroplating, ceramics, glass, and storage batteries. Heavy metals possess serious toxic effects when they are discharged into the water bodies resulting in water pollution. Latest research has revealed that biochar being a bio sorbent is a valuable substance to adsorb heavy metal ions from contaminated water along with halting the movement of metal ions in soils polluted with metal ions (Chen et al., 2011; Inyang et al., 2012). The effective adsorption capacity of biochar is linked with high porous structure and surface area of biochar that consist of numerous functional groups including hydroxyl, carboxyl, and phenolic groups.

1.10.4. Carbon Sequestration

A process in which carbon is stored and apprehended to prevent its release into the environment is known as carbon sequestration (Duku et al., 2011). The carbon is transferred from reactive and active carbon cycle to stable, inert, or passive carbon cycle, to decrease the carbon emissions in the atmosphere. Biochar provides an efficient and simple path to active carbon system towards the passive carbon system. The biochar application makes it hard for the sequester carbon to be emitted as CO_2 providing an effective method for carbon sequestration (Shafie et al., 2012) (Lehmann et al., 2009). It is projected that approximately 20% of the overall carbon biomass can be apprehended as biochar. The soils comprehend over 80% of the terrestrial stock of organic carbon (Watson et al., 2000). The soil is a fixed carbon sink, the modification of biochar into soils supports the reduction of carbon releases and sequestering carbon for soil remediation (Freibauer et al., 2004) (Lal et al., 2004).

1.11. Definition of Nano Biochar

The biochar ranging in particle size from micrometer or greater is named macro-biochar, and from micrometer to 100 nm as colloidal biochar. A biochar having a particle size of less than 100 nm is categorized as nano biochar. The major difference between bulk biochar and macro or colloidal biochar is the particle size of this biochar. It is also worth mentioning that changes in the particle size of the biochar not only affect the physical appearance of the biochar but also the chemical characteristics are significantly affected (Liu et al., 2018). The properties of nano biochar are similar in fashion to that of carbon-based material (CNTs, SWCNTs, MWCNTS etc.) (Chen et al., 2011). The properties depend majorly on the class of feedstock used and circumstances during the synthesis of biochar. The existence of phenolic and hydroxyl extending and bending in the structure of nano biochar has been observed by Naghdi et al. (2017). Furthermore, nano biochar possesses different characteristic compared to bulk char in terms of particle size, specific surface area, elemental organization (C, H, N, S), EC, presence of trace metals and X-ray diffraction peaks (Rajput et al., 2022).

1.12. Nano Biochar Production

Different methods are available in the literature to produce nano biochar from bulk biochar (Guo et al., 2020; Kumar et al., 2020). Out of all these, mechanical grinding is the most common one (98% used). The conversion of woody bulk biochar into graphite nano biochar through mechanical grinding has been reported by Ramanayaka et al. (2020). The disc milling technique with ethanol is a top-down approach used to produce nano biochar synthesis from bulk oven-dried biochar. Nath et al. (2019) reported oven-dried rice husk biochar conversion into nano biochar through the milling technique. The milling took place in a closed container with 5 mm rounded steel balls striking with the biochar and each other and the walls of the container under high revolution (500 rpm) resulting in the conversion of bulk biochar into nano biochar. According to Naghdi et al. (2017b) ball milling technique for the formation of nano biochar from bulk char is the most efficient green technology though which the smallest size (60 nm) of nano biochar can be obtained. Regardless of the ball milling technique commonly practiced, biochar can be converted into nano biochar directly by a flash heating technique that resulted in the formation of graphitic nanosheets (Ramanayaka et al., 2020). Also, the ultrasonic vibrator was used by Olszczuk et al. (2016) for suspended biochar material converting them into nano-sized biochar through sonication. Double disc milling is also a biochar to nano biochar conversion approach, but it is not practiced frequently due to its high operational cost. Although, double disc milling is highly efficient in the production of nano biochar with improved consistency in terms of size and shape compared to the common ball mill approach (Bayram et al., 2007). The size of the nano biochar is maintained and depends on different conditions including milling, duration, and temperature. Therefore, the desired size of nano biochar particles could be achieved by controlling pyrolytic temperature and grinding time (Rajput et al., 2022).

1.13. Bone Waste Management as Bone Char

Worldwide, the high demand for the meat industry leads to a large amount of bone waste generation. According to OECD 2018 report, meat production will be around forty million metric tons within the next decade with poultry contributing 13% and more than 21% meat from sheep meat (OECD, 2018). A major increase will be seen in terms of bone waste globally. One of the safe disposal methods for animal waste is thermal treatment. This process takes place through co-ignition in a cement kiln or by a standalone ignition plant and ensures maximum destruction of pathogens. However, it is important to consider the energy-efficient methods through which waste management and environmental benefits could be achieved, thereby providing clean energy supply, as a substitute to fuels from fossil sources (Uson et al., 2013).

During the past few years, the production of bone char from bone waste through pyrolysis and gasification is practiced for soil remediation application and environmental remediation. Bone char has been used as an adsorbent for discoloration in sugar manufacturing units for the past many years (Alkurdi et al., 2019). The organic content present in livestock bones consists of 75% of dry weight and 20% of the wet weight of bones. According to Patel et al. (2015), animal bones majorly constitute 70% inorganic compounds whereas, 30% of organics. These materials in bone waste give them distinctive qualities to be used in biogas, biomedical application, livestock, and fertilizer source for plants (Oyejide et al., 2022). The textural properties and the hydroxyapatite content of bone char make them efficient ingredients for pollutant removal. Bone char consists of 70-76% hydroxyapatite content, calcium carbonate 7-9%, and amorphous carbon 9-11% (Mendoza-Castilo et al., 2015). However, there are no well-defined percentages and may vary in some cases with 10% amorphous carbon and 80-90% hydroxyapatite (HAP). HAP is an inorganic material having multiple benefits (Goodman et al., 2013) such as working as a catalyst and for environmental mitigation (Li et al., 2018). Mechanically ground and milled bones are used by farmers as organic fertilizers. They contain enough minerals, vitamins (vitamin B12) and essential amino acids (Silva et al., 2019). The slow application of bones to the soil serves as a source of phosphorus and nitrogen for plants. Phosphorus to plants from bone meal provides support to the plants specifically in soil having low pH i.e., pH below 7 and acidic soil (Oyejide et al., 2022).

The pyrolysis of ground animal bones to produce bone char involves heat treatment in oxygen-deficient environments ranging from more than 500 °C to 700 °C resulting in pyrolyzed porous material (Cheung et al., 2001a). Also, the elevated pyrolization temperature above 700 °C changes the physical characteristics of bone char as increasing the temperature from 650 °C to

1000 °C resulted in a slow color change of bones toward white that is an indicator of complete removal of organic matter from the bone char structure (Rojas-Mayorga et al., 2015a) and degradation of bone char functional groups (Kawasaki et al., 2009). Under the carbon dioxide condition, the increase of calcination temperature from 650 to 700 °C resulted in an increase of total pore volume of cow bone char from 0.2 to 0.23 cm^3/g and specific surface area from 62 to 69 m^2/g (Rojas-Mayorga et al., 2015a). During heating or thermal treatment of animal bones, the optimal weight loss takes place at 500 °C. At the thermal temperature up to 400 °C, the hydroxyapatite sustains its physical state (Kubisz and Mielcarak, 2005). According to Figueiredo et al. (2010), at 600 °C organics is removed from bones and carbonate apatite is formed. A temperature above 600 °C leads to the destruction of apatite structure in bones. However, the temperate range from 500 °C to 600 °C results in changes in the microstructure of bones, and the removal of organics resulting in pore formation in bones. The porosity in the bone char is reduced at elevated temperatures during thermal treatment due to close structure interlocking (Kaseva, 2006). According to Alkurdi et al. (2019), the surface area of bone char is reduced in comparison to carbon-based biochar or activated carbon. The inorganics in the bone char including calcium and phosphate make them an active and attractive adsorbent for environmental contaminants.

1.14. Hydroponics System

Soil is one of the most significant and readily available media for plants to grow. It is responsible for providing nutrients, air, and water and holds to the successful growth of plants (Sardare and Admane, 2019). Sometimes soil also poses serious threats to plant growth. The existence of pathogenic microorganisms and nematodes, unwanted chemical reactions, unrequired soil compaction, and poor degradation capacity due to erosion are a few of them. The conventional methods for maintaining soil and growing crops are difficult due to the large labor force required, high and uninterrupted amounts of water, and large areas. Nowadays, more land is being converted into urban land or metropolitan land, so the soil is not actively available for crop production. Also, some areas are not so fertile topographically and geographically to be utilized for agricultural activities. Under these circumstances, the soil-less system comes to the rescue. The soil-less culture includes techniques of hydroponics and aeroponic. The word hydroponics is derived from the Greek word ‗hydro' means ‗water' and ‗ponos' means ‗labor.' It is a method of growing plants in a nutrient solution without the presence of soil. Plants are grown in a soil-less medium with their root suspended in a nutrient solution (Maharana et al., 2011). In the hydroponic system, the pH of the medium constantly fluctuates as the plants grow. Therefore, pH maintenance is necessary for this system. The pH ranges from 5.5 to 6.5 is the optimum pH range of nutrient solution for nutrient availability of plants. However, the conditions of pH also vary from species to species as some species may also grow well beyond this pH range (Kreij et al., 2003). The water-based system supports mitigating climate change issues along with the utilization of natural resources and solving concerns about food security.

1.14.1. Advantages of Hydroponics System

- Gardening practices become extremely simple and easy, with quick cultivation times and growth environments (Awad et al., 2017).
- As the nutrient solution is in direct contact with roots so roots grow faster as compared to the soil system.
- There is quite a less chance for soil-borne pathogens attack, pests‘ infestation, or other disease attacks on the plants.
- It provides efficient nutrient regulation, with higher planting density hence, giving more production of crops by consuming small land.
- Best suitable for regions of the world that are less fertile for carrying out agricultural activities (Sonneveld, 2000).

1.14.2. Limitation of Hydroponics System

Although hydroponic systems offer multiple benefits, they also have some limitations that must be investigated in detail when practicing this system.

- The algal growth in hydroponic system is possible due to recirculation of nutrient solution. Microalgae can be easily grown in the hydroponics system under nutrient recirculation leading to crop damage and low yields (Awad et al., 2017).
- Algal growth not only creates bad nuance and odor, but releases toxins that are damaging to plants as well as human health when such crops are being consumed by them (Corbel et al., 2014).

- Therefore, it is necessary to develop methods for controlling algal growth in hydroponics systems for achieving sustainable crop production (Kaudal et al., 2016).
- According to Kuzyakov et al. (2014), the stable and highly resistive ability of biochar for microbial damage due to the recalcitrant or persistent nature of biochar can effectively be used as a growth substratum in hydroponics systems.

1.15. Nanoparticle's Role in Soil System

The distribution of nanoparticles in the terrestrial system either through atmospheric deposition, agriculture application, run-off from surfaces will result in accumulation of carbon-based nanoparticles into the soil in higher concentrations due to their poor migration in soil (Mukherjee et al., 2016; Yang et al., 2017). Plants are an integral part of any ecosystem and hold a central position in the transport and fate of CNPs in the atmosphere either through uptake i.e., root uptake or foliar or bioaccumulation through the food chain (Rico et al., 2011). The amplified concentration of nanoparticles affects plant development and functioning by suppressing seed sprouting, inhibiting plant extension, reducing overall plant biomass, shifting gene countenance along with enhancing the production of reactive oxygen species (ROS). These ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (OH^-). The acceleration in the production of ROS induces oxidation in the nucleic acid, plant protein, lipids, and bio-membranes, and damage to the parts of the plant leading to cell death (Shekhawat et al., 2021). The negative impacts of CNPs are overcome by the plants through their well-developed antioxidants including heme-oxygenase, catalase, guaiacol peroxidase, glutathione reductase, ascorbate peroxidase and superoxide dismutase which can forage the excess ROS. These antioxidants work regularly under normal conditions, but their catalytic activity and reactions are enhanced under the changing environment (Mahawar et al., 2018). Studies have revealed that numerous nanoparticles present toxic effects on plant growth at minimum concentrations and positive impacts at elevated concentrations. This trend may change upon changing the physical appearance, dose, cover, and makeup of nanoparticles (Li et al., 2016). Toxic effects of nanoparticles on plants include inhibition in root growth, increase in peroxidation of membrane lipids, and formation of reactive oxygen species (Rao et al., 2014; Doshi et al., 2008; Lin et al., 2007).

1.16. Problem Statement

With the large consumption of meat throughout the world, a large quantity of bones is produced. These bones become waste. The bone waste formed is a serious environmental problem. There should be a proper management plan for the disposal of this bone waste. The bone waste could be converted into bone char to fix the issue of bone waste. The effectiveness of pristine bone char is minimal for plant enhancement. The pristine bone char could be converted into nano bone char to improve its role in plant development. Also, severe agricultural practices have depleted soil of essential nutrients required for plant growth. Introduction of soil amendment in form of NBC could enhance the uptake of essential nutrients by plants under hydroponics and soil system.

1.17. Objectives of the Study

This research study will cover following objectives:

1) Synthesis and characterization of nano bone char from bone waste
2) Determining the uptake of nano bone char in the *Syngonium podophyllum* plant under hydroponics and soil systems
3) Evaluating the impact of nano bone char on growth parameters of *Syngonium podophyllum* plant

CHAPTER 2

MATERIALS AND METHODS

2.1. Collection of Bone Waste

Cow bones were selected to produce nano bone char. Around 15 kg of bones were collected from a slaughterhouse within the jurisdiction of Rawalpindi. The bones were extensively washed with hot water and then with distilled water for removing flesh and fiber from bones. The bones were then sun-dried and oven-dried at 110 ℃. The metatarsal bone was selected to produce nano bone char due to its less moisture content.

2.2. Bone Char Production from Cow Bones

The dried metatarsal bones were ground into small particle sizes using a mechanical grinder. The mechanical grinder converts the feedstock into smaller particles so that maximum bone waste heating should occur. The bone waste was placed in eight ceramic crucibles. The weight of bone waste was noted (1530 g). The bones were then pyrolyzed in a muffle furnace (Vulcan-550, made in Germany) at a temperature of 500 °C for 2 hours with a ramping time of 5 °C/min (presenting slow pyrolysis). After the bone char was produced, it was weighed, and the percentage yield of biochar was estimated through the following equation:

$$yield = \frac{biochar\ produced}{biomass\ added} \times 100$$

2.3. Conversion of Bone Char to Nano Bone Char

The bone char formed through pyrolysis was subjected to a planetary ball mill (BKBM-V2S, Biobase, China) with a capacity of 2 L. Bone char was filled in four stainless steel containers of the ball mill according to the ball-to-powder ratio. The maximum rotating frequency was set in three cycles with a maximum frequency of 670 revolutions per minute. The time duration was set at 30 minutes to avoid burning and overheating of bone char. The process resulted in the formation of nano bone char (NBC). The NBC was stored in a polyethylene zipper bag to avoid the agglomeration effect due to moisture.

2.4. Characterization of Nano Bone Char

2.4.1. Particle Size Distribution

The particle size distribution and the average size of NBC particles were analyzed by the recommended method of Naghdi et al. (2017). The particle size distribution and the average size of NBC particles were analyzed by the laser scattering technique using the Zetasizer nano S90 apparatus (ZS-90, Malvern instrument, UK). 1 mg of weighed NBC was dispersed in 200 ml of distilled water with 1% ethanol and 0.5% Tween 80 added to it. The solution was placed in an ultrasonicator (S-DS-3, Stalwart instruments, USA) (Ultrasonic power 100 W, frequency 40 kHz) for 60 min. The sample solution was kept cool using ice in sonication.

2.4.2. Proximate Analysis

Proximate analysis of the NBC was performed by following the international standard procedure, including moisture (ASTM D2867- 09), volatile matter (ASTM D5832-98), ash content (ASTM D2866) and resident matter. The proximate analysis begins with determining the moisture content by heating 2.0 g of NBC sample in a drying oven at 105 °C for a time duration of 24 hours without covering the crucibles with a lid or aluminium foil. The weight of the crucibles is to be noted before putting them in the drying oven. To evaluate the amount of moisture removed, the crucibles are again weighed after taking them out of the drying oven. The difference in weights gives information about the moisture content present in the sample. The same samples were then used for determining the volatile matter by placing the samples in a muffle furnace at 450 °C for a period of 1 hour and crucibles covered with lids. The samples were then weighed after taking them out of the furnace to determine the volatile matter. The ash content was evaluated by putting the same samples in a muffle furnace at 750 °C again for 1 hour with open-top crucibles. The crucibles were then weighed for ash content after taking them out of the furnace as soon as it was cooled down. The resident matter or fixed carbon of NBC was determined through the differences in moisture, volatile matter, and ash content according to the following equation:

Resident Matter (%) = 100 - [% (Moisture Content + Volatile Matter + Ash Content)]

2.4.3. Chemical Analysis

2.4.3.1. pH and Electrical Conductivity (EC)

The pH and EC of NBC were measured in a solid-to-water ratio of 1:20 (w/v) based on the method recommended by International Biochar Initiative (IBI-STD-2.0). Almost 2 g of sample was added to 40 ml of deionized water in a falcon tube and was placed on an orbital shaker at 120 rpm for 1.5 hours to ensure proper mixing of biochar. The pH and EC of NBC were then analyzed by using a pH meter and an EC meter (Adwa AD8000, Romania), respectively.

2.4.3.2 Extractable Phosphorus

To determine NBC extractable phosphorus, Olsen‘s method was used (Estefan, et al., 2013). 0.5 g of NBC sample was added in a 10 mL 0.5 M $NaHCO_3$ solution. It was left in a shaking incubator at 150 rpm for 30 min. The solution was then passed through a filter paper, and 5 mL filtrate was taken. Five drops of 0.25% p-nitrophenol indicator were then added. After that drops of 5 N H_2SO_4 were then added until the solution became colorless. It was raised to 40 mL volume and 4 mL of ascorbic acid reagent was added. Blank was also prepared without NBC. Standards were prepared for phosphorus concentrations of 1, 2, 3, 4 and 5 ppm. The absorbance value was measured on a UV visible spectrophotometer (HALO DB-20 UV-VIS Double Beam Spectrophotometer) at 882 nm wavelength. Extractable P was then measured by the formula:

$$Extractable\ P\ (ppm) = ppm\ P\ (calibration\ curve)\ \times \frac{V}{Wt} \times \frac{V_2}{V_1}$$

V is the overall volume of the NBC extract

Wt. is the weight of the NBC taken

V_2 is the volume of the flask taken for measurement.

V_1 is the volume of NBC extract used for measurement.

2.4.3.3. Extractable Calcium

5 g of NBC sample was added to 30 ml of deionized water. It was then placed on a shaker for 8 hours. After that, the mixture was filtered through Whatman filter paper no. 42. One ml of the filtrate was then taken in a test tube, added 4 ml of deionized water into it and 5 ml of lithium

dichloride. It was then analyzed on a flame photometer (FP910, pg. instruments, UK) (Estefan, et al., 2013).

2.4.4. Toxicity Test

NBC was subjected to a toxicity test. Biochar extracts were made using a solid: water ratio of 1:30 for the seed germination test (Rogovska et al., 2012). A toxicity test was performed using lettuce seeds (*Lactuca sativa* L.) due to their preferred sensitivity (IBI, 2015). One gram of NBC was taken, and 30 ml of deionized water was added to it and placed on an orbital shaker for 7-8 hours. The solutions were then filtered out using Whatman filter paper no. 42. The biochar extracts were taken in duplicate with the control group taking one tap water and one deionized water. Filter paper with dimensions of six-by-six inches was used and the weight of each filter paper was also determined. The filter papers were placed in the petri dishes and 2.6 ml of biochar extract per gram of filter paper was sprayed. A total of 6 petri dishes were used duplicates for each type of bone char, and one sample for tap water and deionized water. The extracts were applied on each filter paper after five to seven days to ensure that the seeds are sufficiently moist and in good condition for seed sprouting. The duration of the experiment was 2 weeks, which revealed sufficient germination in all the petri dishes. The seeds in each petri dish were ten and the percentage of germination was determined by using the formula:

$$Germination\ (\%) = \frac{Number\ of\ seeds\ germinated}{total\ number\ of\ seeds} \times 100$$

2.4.5. Morphological Analysis

The surface morphological features of NBC were studied using transmission electron microscopy (JEM -2100 TEM JEOL, Japan) and scanning electron microscopy (SEM*; EFI S50 Inspect Netherland*) well-appointed with energy dispersive x-ray spectroscopy for observing the physical morphology and the elemental composition of nano bone char, respectively.

2.4.6. Functional Chemistry

Fourier transform infrared (FTIR Shimadzu-8400, Technology Links, Japan) spectroscopy was performed on NBC to understand the existence of functional groups on its surface. X-ray diffraction (D/Max-B, Japan) spectrometer was used to determine the crystalline structure and mineralogy of NBC.

2.5. Hydroponics Experiment

The hydroponics experiment was set up by preparing a half-strength Haugland solution comprising potassium nitrate (KNO_3) 202 g/L, calcium nitrate ($CaNO_3$) 472 g/L, magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 493g/L, ammonium phosphate [$(NH_4)_3PO_4$] 80 g/L, potassium dihydrogen phosphate (KH_2PO_4) 136 g/L, iron chelate EDTA ($C_{10}H_{12}FeN_2O_8$) 15 g/L, zinc sulphate ($ZnSO_4$) 0.22 g/L, copper sulphate ($CuSO_4$) 0.08 g/L, manganese chloride ($MnCl_2$) 1.81 g/L, sodium molybdate (Na_2MoO_4) 0.12 g/L and boric acid (H_3BO_3) 2.86 g/L. The pH of the nutrient solution was maintained between 5.6-6.5 with NaOH and HCL. Around eighteen samples of *Syngonium podophyllum* seedlings of the same age and height were taken from a local nursery. They were properly cleansed with deionized water to eliminate all the dirt from the seedlings. Three replicate treatments of 20 mg, 50 mg, 100 mg, 300 mg, and 500 mg of NBC with control (without NBC) were introduced per litre of Haugland solution. The treatment suspensions were dispersed by ultrasonication (100 W, 40 kHz) for 15 min, and then the *Syngonium podophyllum* seedlings were transported instantly into each glass bottle containing one seedling. All glass bottles were casually placed in an indoor lab environment at a temperature of about 28 °C in the daytime with sunlight (16 h) and 18 °C at night (8 h). The seedlings bottles were shaken 2-3 times a day to mix NBC in the solution, allowing them to encounter the root system. The experiment successfully proceeded for 21 days and after that harvesting of plants was done.

2.6. Soil Collection and Characterization

The soil used in the experiment was collected from the agricultural land of district Karak, KPK. An upper 20 cm of soil was collected and brought to the laboratory; the moisture content of the soil was removed by drying the soil in the air for 2 days. To remove the debris, gravel, and unwanted materials from the soil, it was crossed through a 2 mm sieve (mesh no.8). The soil was then stored in plastic bags for experimentation.

Standard methods were followed for soil characterization including pH, EC, organic matter, water-holding capacity, extractable calcium, and phosphates (Estefan et al., 2013). Soil pH and

EC were determined by using 1:5 (w/v) of soil suspension. 2 g soil, and 10 ml distilled water were used. It was left for 30 minutes in an orbital shaker at 150 rpm. Then pH and EC were measured using digital pH and EC meters (Adwa AD8000, Romania). Soil organic matter was analyzed by the most common procedure of Walkley- Black (Estefan et al., 2013) which involves the oxidation-reduction reaction between potassium dichromate and ferrous ammonium sulfate. 1 N potassium dichromate was prepared using 49.04 g of $K_2Cr_2O_7$ in one litre of deionized water. A 0.5 M ferrous ammonium sulphate solution was prepared by dissolving 196 g of $(NH_4)_2.\ FeSO_4.6H_2O$ in Deionized water with 5 mL of conc. H_2SO_4 and the volume was brought to one litre. One g of dried soil was taken into 500 mL of the flask. Using a glass pipette 10 ml of $K_2Cr_2O_7$ was added into soil, followed by 20 mL of H_2SO_4 solution. The flask was shaken properly to mix the solution and 30 min rest was given to cool down the solution. After this, added 200 mL of Deionized water and 10 mL of concentrated phosphoric acid. Around fifteen drops of ferroin indicator were added for colour development. The mixture was then titrated against a 0.5 M solution of ferrous ammonium sulphate. The titration process continued till the color shift was observed from violet-blue to green. A similar process was repeated for the blank.

Calculations:

$$M = \frac{10}{V_{blank}}$$

$$\text{Oxidizable Organic carbon (\%)} = \frac{V_{blank} - V_{sample} \times 0.3 \times M}{Weight\ of\ soil}$$

$$Total\ Organic\ Carbon\ (\%) = 1.334 \times oxidizable\ organic\ carbon\ (\%)$$

$$Total\ Organic\ Carbon\ (\%) = 1.724 \times total\ organic\ carbon\ (\%)$$

where:

M is the Molarity of Ferrous Ammonium Sulphate solution i.e., 0.5M

V_{Blank} is the volume of ferrous ammonium sulphate solution needed to titrate the blank (mL)

V_{sample} is the volume of ferrous ammonium sulphate solution needed for titrating the sample (mL)

To determine soil extractable phosphorus, Olsen's method was used (Estefan, et al., 2013) and for extractable calcium flame photometer method was used, as described in earlier section. The water holding capacity of soil was determined by taking non compacted air-dried soil sample. A filter paper was taken and fitted in a funnel which was then adjusted to ring stand. Soil sample of 100 mL was kept in the funnel, and 100 mL of deionized water was poured on the sample with continuous stirring until the soil became completely saturated. The amount of water drained was collected and measured through a measuring cylinder. Soil water holding capacity was determined by calculating the quantity of water per liter of soil.

Calculations:

$$\frac{\text{Water retained (ml)}}{\text{100 ml of sample}} = \text{water added (ml)} - \text{water drained (ml)}$$

$$\text{Water holding capacity}\left(\frac{\text{ml}}{\text{l}}\right) = \frac{\text{water retained (ml)}}{\text{100 ml of soil sample}} \times 10$$

2.7. Pot Experiment

The soil experiment was set up by applying treatments of 0.1%, 0.2%, 0.5% and 1% wt./wt. of the NBC in three replicates. For this, 0.3 g, 0.6 g, 1.5 g and 3 g of NBC was mixed with 300 g of soil for each treatment with control (without NBC). The field capacity of 300 g of soil was approximately 45 ml which was maintained by watering distilled water throughout the experiment. The NBC was applied in suspension formed by mixing a small amount of distilled water and dispersed by ultrasonication (100 W, 40 kHz) for 15 min. The *Syngonium podophyllum* seedlings were properly cleaned with deionized water to remove all the dirt from the seedlings. Then, the seedlings were placed in the center of the pot and poured sonicated NBC suspension around it. Lastly, the seedling roots were covered with soil from the sides of the pot and stabilized them in the soil. The experiment successfully proceeded for 40 days and after that harvesting of plants was done. This procedure was followed according to standard procedure applied by Khodovoskya et al. (2010).

2.8. Plants Analyses

Several plant growth parameters were measured which are as followed:

Before harvesting, **chlorophyll content** was noted through a SPAD meter (SPAD-502 Plus) for hydroponics and soil plants. Readings of three leaves per plant were taken and then the average was calculated. For one leaf, three readings were taken i.e., one at the center and two from edges. The average was then calculated.

Shoot height was determined by using a measuring scale. **Leaf count** was determined by counting the leaves of a plant. The **fresh weights** of leaves stems, and roots were measured immediately after harvesting. They were washed to remove excess soil and dust.

On the day of harvesting small fragments from the leaves, shoots and roots were taken from both hydroponics and soil plants to form specimens on slides. These fresh samples of plants were observed under a **compound microscope** for detecting the presence of NBC in them. The resolution lens for detection varied between 4x i.e., minimum resolution and 10x for maximum resolution.

After observing plant samples under a compound microscope, the plants were dried. Plants were placed in paper bags for drying in an oven at 55 ℃ till the weight become stable. The **dry weight** was then measured. Microscopic structures and surface morphology of plants were observed through scanning electron microscopy (SEM; EFI S50 Inspect Netherland) coupled with an energy-dispersive X-Ray (EDX) analyzer.

For shoot **elemental analysis**, 0.15 g of plant shoots were grounded and placed in a conical flask. The ratio of the acid mixture was 1:2 for perchloric and nitric acid, respectively. 10 ml of acid mixture was introduced to each sample. They were kept for some period and then digested on the hotplate at 200 ℃ until all particles were completely digested. Samples were then filtered and raised to 50 ml volume. The reagents were prepared for determining the total calcium and phosphorus with ammonium heptamolybdate and ammonium vandate in HNO_3. We took 22.5 g of ammonium heptamolybdate and dissolved it into 400 mL of deionized water. In 300 mL of hot deionized water, added 1.25 g ammonium metavanadate. Both solutions were merged in one liter flask and allowed the solution mixture to cool down at room temperature. Gradually added 250 mL of conc. HNO_3 to the solution mixture and allowed the solution to cool at room temperature. Finally, make the volume of solution up to 1 liter with deionized water. 3 ml of plant sample digestate was taken in a test tube and added 3 ml of reagent prepared previously. Then the solution mixture was mixed on a vortex mixer and analyzed on a UV/Visible spectrophotometer at wavelength at 410 nm for extractable phosphorus and on flame photometer for extractable calcium.

2.9. Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the significant difference between different treatments and application rates.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Nano Bone Char Characterization

3.1.1. Proximate and Chemical Analysis Results

Pyrolysis conditions at which the bone char is produced significantly contribute to its chemical composition (Alkurdi et al., 2019). The temperature of 500 ^oC for bone char production was chosen because a temperature below 500 ^oC results in incomplete removal of organic matter from bone structure, whereas raising the charring temperature may cause the dehydroxylation of the hydroxyapatite structure. The temperature range of 500-600 ^oC is the optimum temperature where organics from the bones get removed (Patel et al., 2015). The results of various chemical and proximate analysis are given in Table 1. The yield of NBC obtained through pyrolysis was 76.00±0.25%. The NBC produced through pyrolysis showed a high yield due to inorganic minerals present in the bone char.

The moisture content, volatile matter, ash content, and resident matter of NBC were 3.07±0.67, 9.10±0.35, 78.69±1.40, and 9.14±1.72%, respectively. The proximate properties of biochar (mobile matter, resident matter, and ash content) also depend on the feedstock type and the pyrolysis temperature. The pyrolysis temperature of 500 ^oC results in decrease of mobile matter in biochar and an increase in the resident matter. The resident matter is also an indicator of fully carbonized organic matter present in biochar (Ahmad et al., 2012). The ash contents of biochar produced from non woody biomass are generally high due to the accumulation of inorganic minerals and organic matter combustion residue (Cao and Haris, 2010). The organic matter of NBC was 3.00±0.43%. This small value of organic matter may be due to the loss of volatile organic carbon during the pyrolysis process.

Table 1: Proximate analysis and physiochemical properties of nano-bone char (NBC).

Parameters	Results
Yield (%)	76.00 ± 0.25
Moisture (%)	3.07 ± 0.67
Mobile matter (%)	9.10 ± 0.35
Ash content (%)	78.69 ± 1.40
Resident matter (%)	9.14 ± 1.72
pH	8.22 ± 0.02
EC (dS/m)	4.01 ± 0.01
Na (mg kg^{-1})	578 ± 0.01
K (mg kg^{-1})	264 ± 0.06
P (mg kg^{-1})	142 ± 0.02
NO_3-N (mg kg^{-1})	3.80 ± 0.01
Organic matter (%)	3.00 ± 0.43
Zeta Size (d.nm)	10.42

The pH of NBC was 8.22±0.02, which indicates its alkaline nature. The pH of biochar depends on multiple factors including the type of feedstock and the pyrolysis condition at which biochar is produced (Ahmad et al., 2016). Biochar produced above 350 °C has alkaline pH. The carbon in biochar starts to become ash along with alkali salts separation from the organic matrix, thus increasing biochar pH (Ahmad and Kanari, 2003). The pH of NBC is parallel with the study of Alkurdi et al. (2019) who confirms the pH of bone char to be in the range of 8.4 when the pyrolysis temperature is 500 ^{o}C. Also, the non-wood biochar has higher pH values due to the presence of salts including carbonates, chlorides, potassium, and calcium in the ash content of the biochar (Montes-Moran et al., 2004).

The EC of NBC was 4.01±0.01 dS/m. Biochars formation at higher pyrolysis temperature (>400 °C) normally indicates higher EC values (Cantrell et al., 2012). This effect has been accredited to the increasing number of residues or ash caused by the loss of volatile material during pyrolysis (Cantrell et al., 2012). The higher EC values of NBC may also be linked to the presence of sodium, potassium, phosphorus, and nitrate nitrogen in NBC present in high amounts of 578±0.01, 264±0.06, 142±0.02, and 3.8±0.01 mg kg^{-1}, respectively.

3.1.2. Particle Size Distribution

The NBC size was analyzed by a zeta sizer and is represented in Fig. 1. As the nanoparticles are materials in the size range of 1-100 nm scale, a maximum amount of bone char particles (72%) were distributed in the range of 10 nm. The other 25% particles were distributed in the range of 306 nm, and about 2% of NBC particles lie in the range of 5400 nm. The particle size distribution confirmed that the NBC synthesized was on nanometer scale. The size of NBC higher than the 100 nm could be due to the agglomeration of nanoparticles (Ghoshal and Singh, 2022).

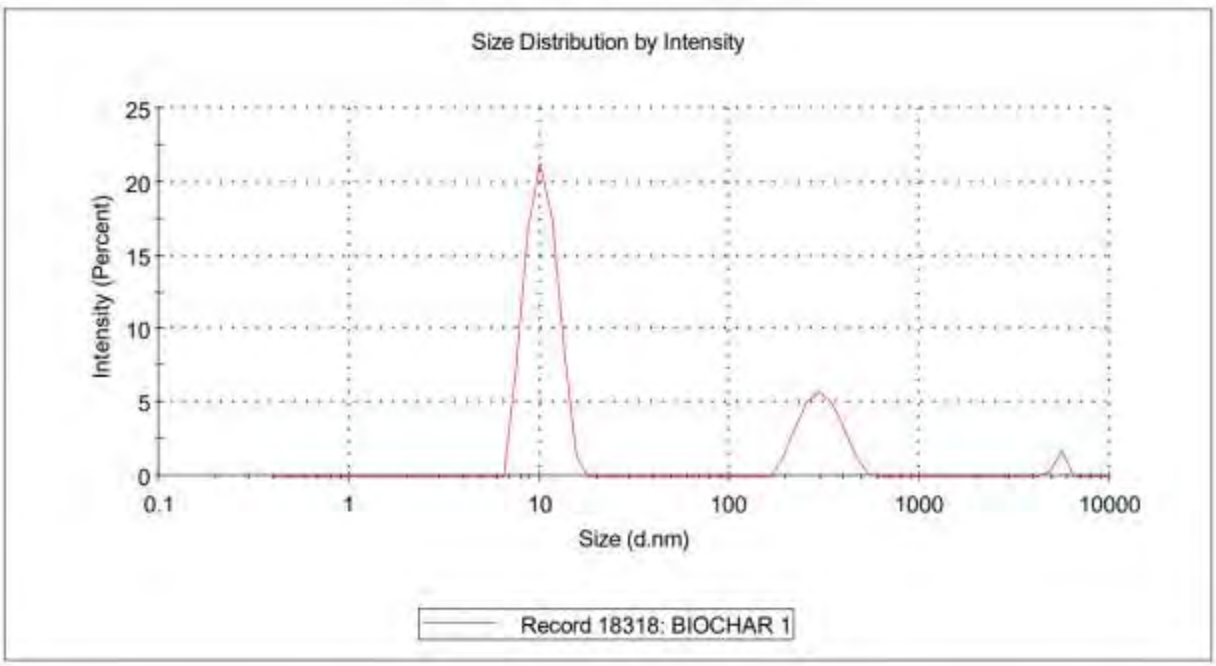

Fig. 1. Particle size distribution of NBC.

3.1.3. Surface Morphology

The SEM-EDX images for morphology and elemental analysis of NBC are shown in Fig. 2. The SEM image of NBC (Fig. 2a) reveals agglomeration of nano bone char particles. As the size of bone char is nanoscale, so particles were not seen as a separate identity but in the form of agglomerated mass. The NBC nanoparticles possess a rough texture with irregular geometry and random size distribution of particles. These images correspond with the literature studies of Arnich et al. (2003) and Camargo et al. (2012). The EDX of NBC (Fig. 2c) shows carbon, oxygen, calcium, phosphorus, sodium, aluminium, magnesium, and chloride as the main constituents present in NBC. The carbon present in NBC is 36.3%, oxygen 41.5%, calcium 12.5%, phosphorus 7.8%, sodium 0.7%, aluminium 0.7%, magnesium 0.4% and chloride 0.1%. The elemental analysis presents a higher percentage for oxygen (41.5%) indicating NBC may have more O-containing functional group and carbon (36.3%) with calcium and phosphorous also contributing a high percentage compared to other elements due to hydroxyapatite present in bone char. The hydroxyapatite composition is also confirmed through the ratio of Ca/P (12.5/7.8 = 1.60) which is found in agreement with the previously reported data (Shahid et al., 2019).

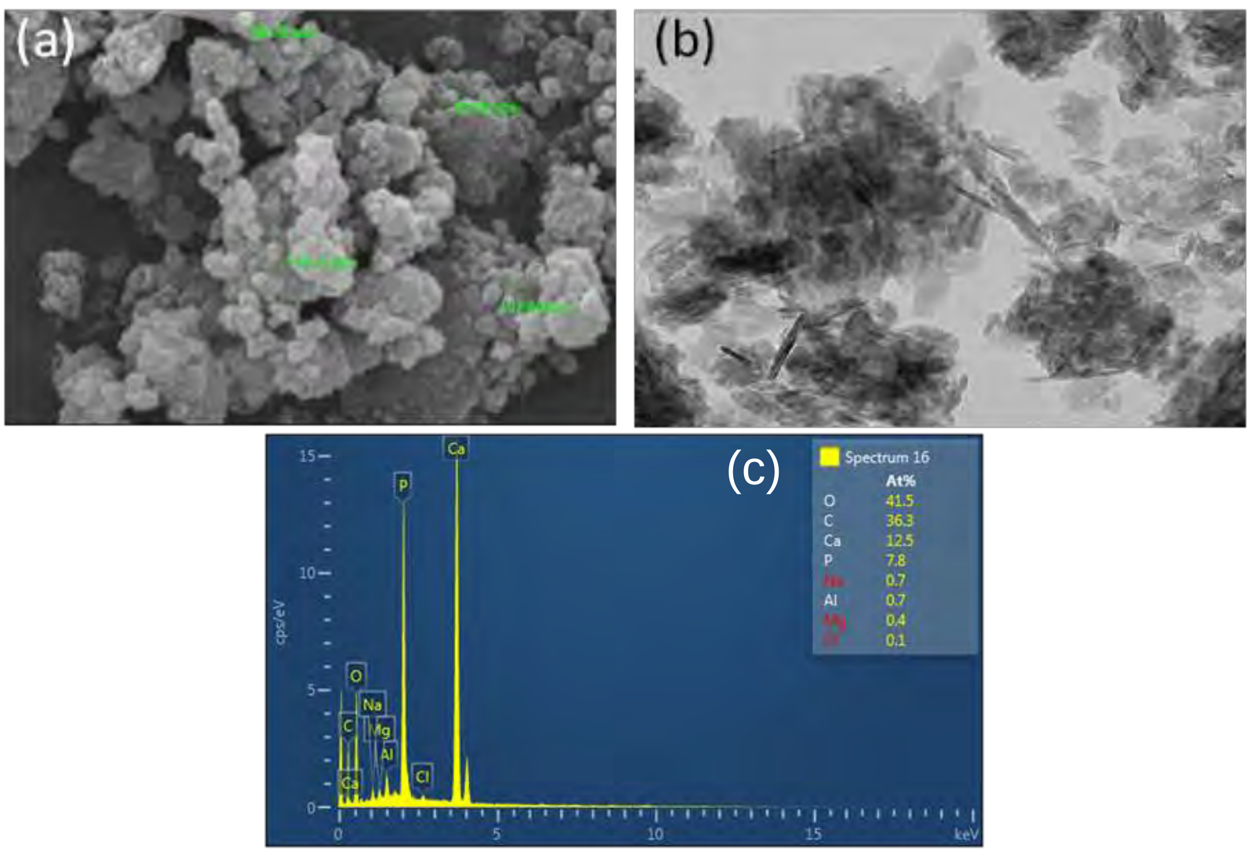

Fig. 2. SEM (a), TEM (b) and EDX spectrum (c) of NBC.

It can also be observed that NBC has a nano-granular structure (Fig. 2b) that could provide a larger surface area and would favour the complex reactions of NBC (Shahid et al., 2019). The TEM photograph showed that the particles of NBC were polycrystalline and irregularly polygonal in shape.

3.1.4. Surface Chemistry

Fig.3a represents the FTIR spectroscopy results of NBC. Peaks observed at different wave numbers give an idea about the functional groups present on the surface of NBC.

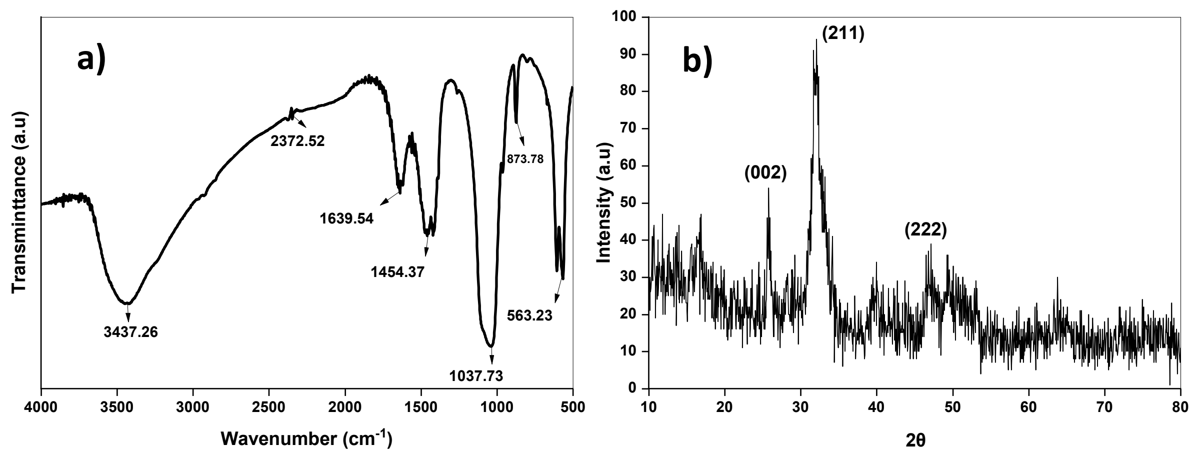

Fig. 3: FTIR (a) and XRD (b) spectra of NBC.

The specific characteristics of hydroxyapatite are represented by bands at 3437.26, 2372.52, 1639.54, 1454.37, 1037.73, 873.78, 563.23, and 467 cm^{-1}. The band at 3437.26 cm^{-1} presents the stretching mode of vibration of the hydroxyl (-OH) group. The band at 2372.52 cm^{-1} indicates the presence of both organic phases of vibrational alkyne C≡C and C=C aromatic bond. The band at 1639.54 cm^{-1} represents the symmetrical bending mode of vibration of a hydroxyl group (-OH). The bands at 1454.37 and 873.78 cm^{-1} represent the presence of the CO_3^{-2} group. These findings correspond with the previous study of Mayorga et al. (2015). The band at 1037.73 cm^{-1} presents the major band in spectra with a symmetrical stretching mode of vibration between the phosphate and oxygen group (PO_4^{3-}). The peak for the phosphate group is deep indicating high concentrations of phosphate ion present in bone char. The peaks at 563.23 cm^{-1} represent the asymmetrical bending mode of vibration between the phosphorus and oxygen. The band observed at 476 cm^{-1} also indicates the mode of vibration for the PO_4^{3-} group. The obtained spectra correspond to the study by Shahid et al. (2019), Bedin et al. (2017) and Jia et al. (2018).

Fig. 3b shows the X-ray diffraction pattern of bone char. The XRD provides information about whether the bone char possesses a crystalline or amorphous structure. The diffraction peaks observed at the 2θ (degree) of 25.8°, 31.7°, and 46.5° appeared in correspondence with the crystal plane of hydroxyapatite (002), (112), and (222), respectively. The diffraction peaks obtained at 2θ (degree) are consistent with the standard crystalline hydroxyapatite pattern. These results are in correspondence with Shahid et al. (2019) and Mayorga et al. (2013). As the nano bone char is composed of hydroxyapatite mineral, it is observed that the peaks identified are following the crystalline phase of hydroxyapatite. It is to be noted that the hydroxyapatite phase is stable, and no decomposition phase was observed.

3.2. Uptake of NBC by *Syngonium podophyllum* under Hydroponics System

Fig. 4 shows the compound microscopy results for different parts of *Syngonium podophyllum* plants treated with NBC in the hydroponics system. The compound microscopy images were taken for the control (0 mg/L), 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L and 500 mg/L NBC concentrations. No black NBC particles were observed in the plants growing in control treatment. The leaves and roots of these plants were clear from any effect of NBC. Whereas, the treatments of 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L and 500 mg/L showed the presence of NBC black particles in their leaves and roots.

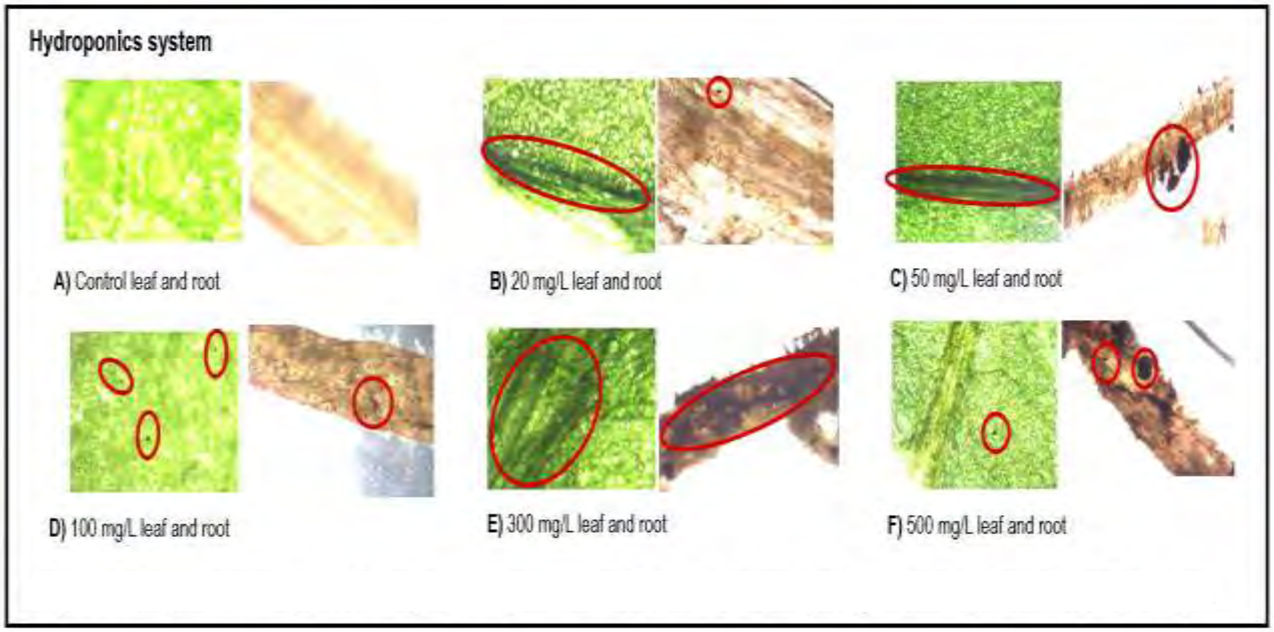

Fig. 4: Compound microscope images of leaves and roots of *Syngonium podophyllum* plants grown under hydroponic system treated with various concentrations of NBC.

At low concentrations of 20 to 100 mg/L NBC, the uptake is identified in the form of black particles in the main veins of the leaves. The images of roots were taken through cross-sectional areas of the roots which showed the NBC black particles attached to the inner region of the roots. Likewise, at high concentrations of 300 and 500 mg/L of NBC, black particles were observed in the leaves of these plants distributed through the leaf surface. In roots, the NBC particles were seen separately along with their high agglomeration at distinct locations. It was observed that the roots of the plant seedlings were covered with NBC and were darker with increasing treatment concentration. When biochar was introduced in the nutrient solution the biochar particles having larger size settled down at the bottom of the bottles and there was a possibility that they might have taken some nutrient elements away from the solution due to adsorption. The colloidal form of NBC remained suspended in the nutrient solution stably and interacted with the roots of the plants, thereby resulting in high chance of absorption and/or uptake of NBC particles through the root system. Similar results were observed by Ristroph et al. (2017). The average size of the roots is approximately 1000 times higher than the diameter of carbon nanoparticles i.e., 20-50 nm (Sonkar et al., 2012). Therefore, the nanoparticles or their agglomerates have a diameter smaller than the pore diameter of the cell wall of the plant and could conveniently pass and reach the plasma membrane of the plant. The internalization of nanoparticles can also take place through endocytosis by forming a cavity-like structure around nanoparticles by a plasma membrane. Uptake of nanoparticles may also occur through embedded transport carrier proteins or ion channels (Nair et al., 2010). Our results of compound microscopy showing uptake of NBC coincide with electron microscope studies of Liu et al. (2009) that show that carbon nanoparticles can penetrate the plants and be embedded into the xylem vessels. Inside the cytoplasm, these carbon nanoparticles interact or may bind with different cytoplasmic organelles and interfere with metabolic processes taking place inside the plant (Jia et al., 2005).

3.3. Fresh Weight, Dry Weight, and Shoot Length of *Syngonium podophyllum* Plants under Hydroponics System

The fresh weight of the plants grown in the hydroponics system after 21 days is shown in Fig. 5a. The fresh weights of the plants in control, 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L and 500 mg/L were 4.33±0.57, 4.66±0.57, 5.66±0.57, 6.66±0.57, 7.33±0.57, 7.66±0.57 g, respectively. It was observed that the fresh weight of the plants increased with the increasing NBC concentrations in the hydroponics system. The high value of fresh weight was observed in plants at the treatment dose of 300 mg/L and 500 mg/L i.e., 7.33±0.57 and 7.66±0.57, respectively. The per cent increase in fresh weight of hydroponics plants exposed to 300 mg/L and 500 mg/L was 69.23% and 76.90%, respectively, as compared to control (without NBC). The control group showed the minimum value for the fresh weight i.e., 4.33±0.57. According to Tripathi et al. (2011), it is presumed that carbon nanoparticles can incorporate within the xylem based on the concept of the formation of large capillaries that increases the water uptake mechanism of plants. Thus, the channels formed in the presence of carbon nanomaterials inside the xylem support water uptake and transportation either through the channel process or the adsorption process. As fresh weight is an indicator of moisture content in the plant. Our results indicate that high concentrations of NBC increase the moisture content in the plants in comparison to the control.

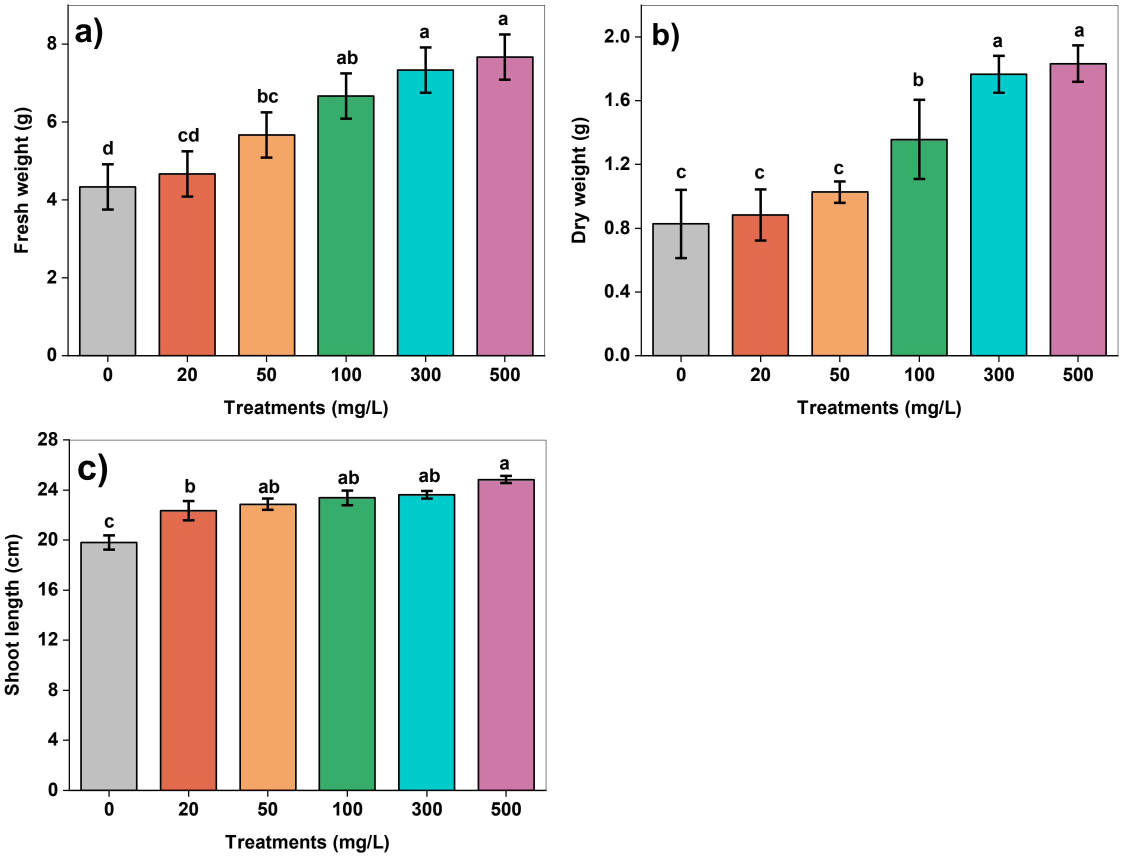

Fig. 5: Fresh weight (a), dry weight (b), and shoot length (c) of *Syngonium podophyllum* plants grown under hydroponics system treated with various concentrations of NBC.

The dry weight of plants grown in a hydroponics system is shown in Fig. 5b. The values for dry weight of plants in a hydroponics system for control, 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L and 500 mg/L were 0.82±0.21, 0.88±0.16, 1.02±0.06, 1.35±0.24, 1.76±0.11, and 1.83±0.11 g, respectively. The dry weight of the plants with NBC treatment showed significantly high values for the dry weights in comparison to the control at 100, 300 and 500 mg/L NBC treatments, which indicates the positive impact of NBC on plants growth under hydroponics system. However, up to 50 mg/L treatment, no significant increase in dry weight of plants was observed in comparison to the control. The percent increase in the plant dry biomass at 300 mg/L and 500 mg/L was 114.63% and 123.17%, respectively as compared to control. These results correspond with the studies of Khodvoskya et al. (2012) according to which the CNTS exposure to the plants results in an increase in its total biomass as compared to the control.

The shoot lengths of plants in hydroponics system are shown in Fig. 5c. The values of shoot lengths for control, 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L, and 500 mg/L were 19.81±0.57, 22.35±0.76, 22.86±0.46, 23.36±0.57, 23.622±0.28, and 24.82±0.28 cm, respectively. The maximum shoot length was achieved for the plant receiving treatment of 500 mg/L NBC. The per cent increase in the shoot length of hydroponics plants at high concentrations of 300 mg/L and 500 mg/L of NBC was 19.23% and 25.29% as compared to the control. Our results are also in accordance with Saxena et al. (2014) who reported that carbon nanoparticles give higher values for shoot length in the wheat plant as compared to the control.

It can be concluded from our results that the fresh weight, dry weight and shoot length of the plants generally increased with the increasing NBC concentrations in the hydroponics system. The high value for these parameters was observed in plants at the high treatment dose of 300 mg/L and 500 mg/L. The presence of nanoparticles results in the formation of new capillaries or channels that increases the water uptake mechanism of plants. Thus the channels formed in the presence of carbon nanomaterials inside the xylem support water uptake and transportation resulting in enhanced growth of plants.

3.4. Chlorophyll Content and Leaf Count of *Syngonium podophyllum* Plants under Hydroponics System

The chlorophyll content of plants in hydroponics system is shown in Fig. 6a. The values of chlorophyll contents for control, 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L, and 500 mg/L at the time of harvesting were 42.53±0.97, 44.69±0.64, 45.1±0.25, 46.1±0.35, 48.65±2.4, 50.36±2.5, respectively. Chlorophyll is an important pigment involved in the process of photosynthesis that converts light energy into chemical energy. The increase in chlorophyll content of the plant support light absorption and synthesis of carbohydrates during photosynthesis. Chlorophyll content in leaves gives an estimation of the photosynthetic potential of plants. Greater concentration indicates that more nitrogen is incorporated into the leaves of a plant (Gitelson et al., 2002).

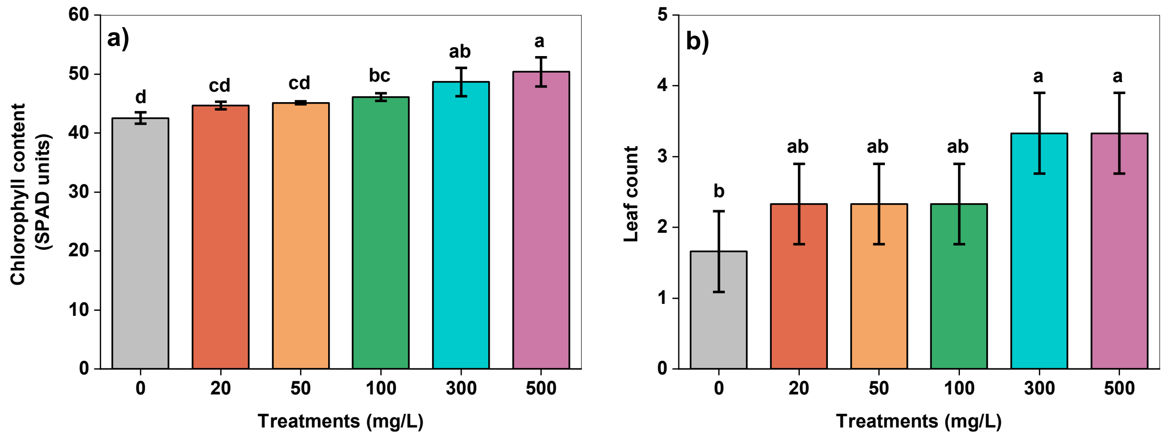

Fig. 6: The chlorophyll content (a) and leaf count (b) of *Syngonium podophyllum* plants grown under hydroponics system treated with various concentrations of NBC.

The maximum chlorophyll content was recorded in the leaves of the plants exposed to higher concentrations of NBC, i.e. 300 and 500 mg/L. The values of chlorophyll content were observed to increase with increasing NBC concentrations. It means higher concentrations of NBC result in increasing the photosynthetic activity in plants by increasing the chlorophyll content. The per cent increase in chlorophyll content of hydroponics plants at 300 mg/L and 500 mg/L concentrations was 14.38% and 18.41%, respectively, as compared to control. These results are following the studies of Wang et al. (2018) who reported that the mung beans when exposed to carbon dots showed higher values of chlorophyll content in the plant as compared to the control. It was observed that the carbon dots speed up the electron transfer rate which enhances the photosystem activity of plants.

The leaf count of plants in hydroponics is presented in Fig. 6b. The values of leaf count for control, 20 mg/L, 50 mg/L, 100 mg/L, 300 mg/L and 500 mg/L at the time of harvesting were 1.66±0.57, 2.33±0.57, 2.33±0.57, 2.33±0.57, 3.33±0.57, 3.33±0.57, respectively. The number of leaves is an important indicator of plant growth. The higher the number of leaves in a plant the more photosynthetic activity resulting in higher plant yield. The plants in hydroponics treated with NBC showed an increase in the number of leaves as compared to the control. The per cent increase of leaf count at 300 mg/L and 500 mg/L was 100% as compared to the control. However, it was difficult to predict how nanomaterials interact with plants resulting in a modification in their genetic and molecular mechanism (Nair et al., 2010). The studies have revealed that the effect of CNTs on plant growth and development depends on multiple factors including plant species, type of nanomaterial, concentrations in which nanomaterials are exposed to plants and particular conditions of the experiment such as the method for nanoparticle uptake into the plant system (Wang et al., 2013; Khodovoskaya et al., 2012).

To sum up, maximum chlorophyll content was recorded in the leaves of the plants exposed to higher concentrations of NBC. The higher concentrations of nano bone char result in increasing the photosynthetic activity in plants followed by an increase in chlorophyll content. It was proved through literature that the carbon dots speed up the electron transfer rate which enhances the photosystem activity of plants. The higher number of leaves in a plant refers to more photosynthetic activity by the plant giving a higher plant yield. The plants in hydroponics show an increase in the number of leaves in plants given NBC treatment as compared to the control.

3.5. Changes in Surface Morphology and Elemental Composition of *Syngonium podophyllum* Leaves under Hydroponics System

Figure 7 represents the SEM images of leaf samples of *Syngonium podophyllum* in a hydroponics system for control, 100 mg/L, 300 mg/L, and 500 mg/L at a 20-micron meter scale. In the leaves of control treatment, no discrete appearance or agglomeration of carbon nanoparticles was seen. Whereas, in the remaining treatments at all concentrations from 100 mg/L to 500 mg/L the embedment of carbon nanoparticles of different shapes and sizes could be seen. These embedded particles represent the uptake of NBC in leaf samples. As NBC is rich in calcium and phosphorus content due to hydroxyapatite, they appear as white agglomerated particles under the SEM.

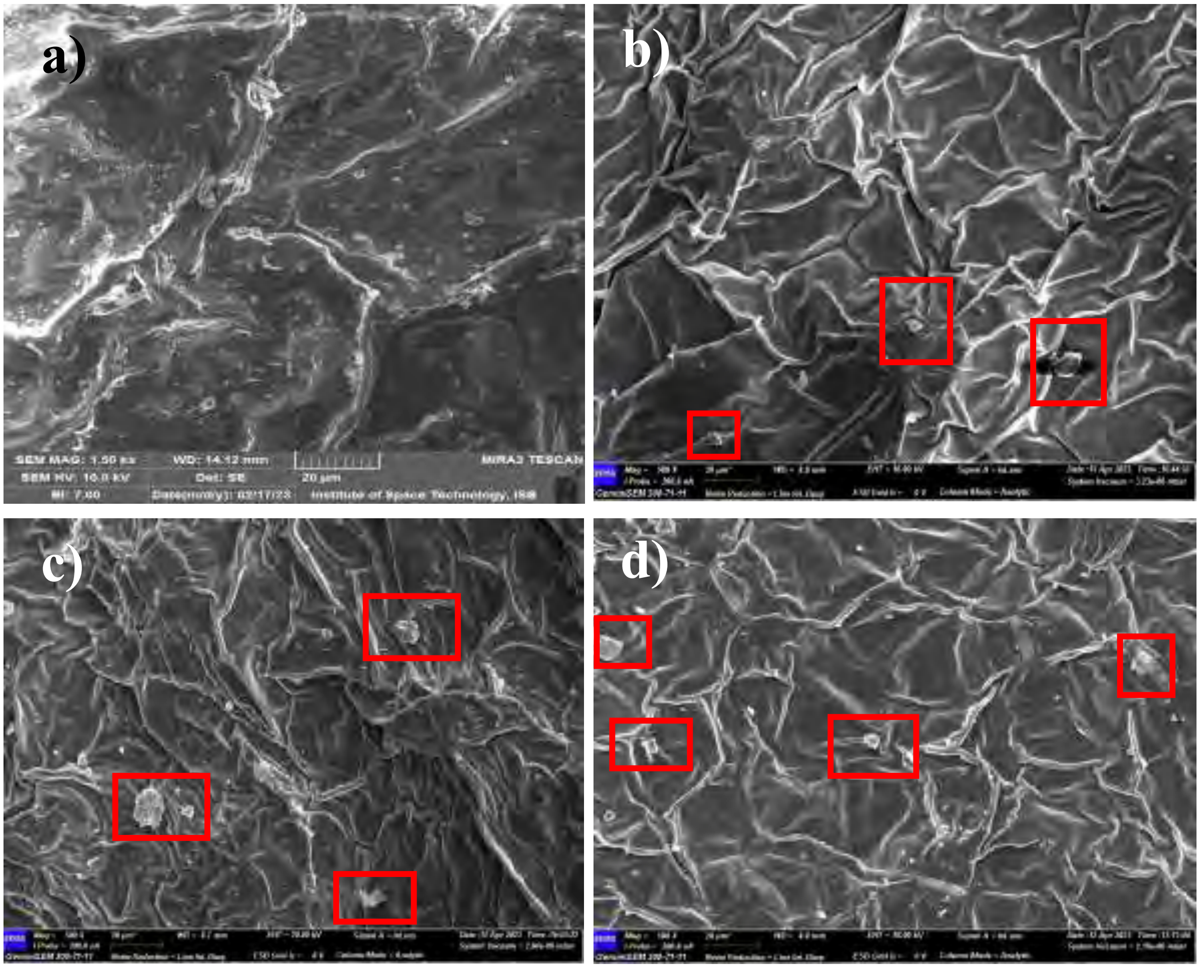

Fig 7. The SEM images (at 20-micron meter scale) of leaves of *Syngonium podophyllum* plants grown under hydroponics system treated with 0 mg/L (control) (a), 100 mg/L (b), 300 mg/L (c), and 500 mg/L (d) of NBC.

The control group and 500 mg/L were further observed at a 2-micron meter scale (Figure 8 (a)). The images show the embedded particles of NBC at the high concentration dose of NBC in the leave samples, whereas no such agglomeration was observed in the control group. Figure 8 (b) represents SEM images at a high magnification of 500-nanometer scale. It differentiates the control group and the 500 mg/L treated group. The high concentration of NBC in the hydroponic solution resulted in its uptake from roots to shoots and finally to the leaves. Although the samples passed through rinsing and dehydration processes, still NBC particles were able to be detected in its leaves‘ surfaces. Our results correlate with previous studies where carbon nanomaterial penetrated the hard rice husk with a thickness of around 25-micron meter and was observed under the SEM showing nanoparticle settlement in the rice plant (Nair et al., 2012). According to Joshi et al. (2020), the shoots and roots of wheat plants were analyzed for carbon nanomaterials detection through SEM images and it detected the carbon nanomaterial inside the wheat plant. The nanoparticles when interacting with plants in an aqueous medium result in their absorption, uptake, translocation and accumulation in the plant tissues (Zhu et al., 2008). Therefore, we conclude that NBC was detected on the surface of the leaves of the *Syngonium podophyllum* plant, which indicates the active role of nanomaterials in changing anatomical structures.

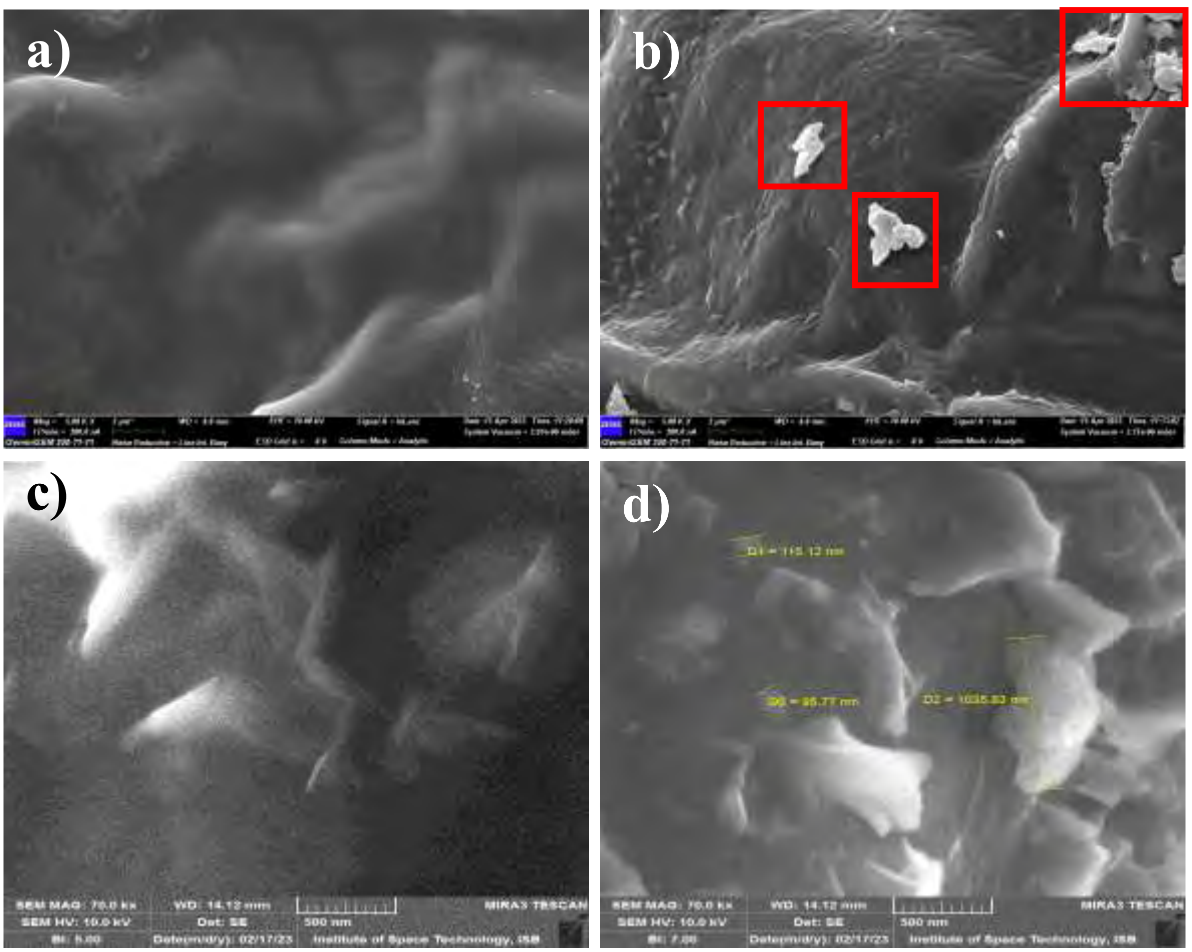

Fig 8. The SEM images of leaves of *Syngonium podophyllum* plants grown under hydroponics system treated with 0 mg/L (control) (a) and 500 mg/L (b) of NBC at 2-micron meter scale, and 0 mg/L (control) (c) and 500 mg/L (d) of NBC at 500-nano-meter scale.

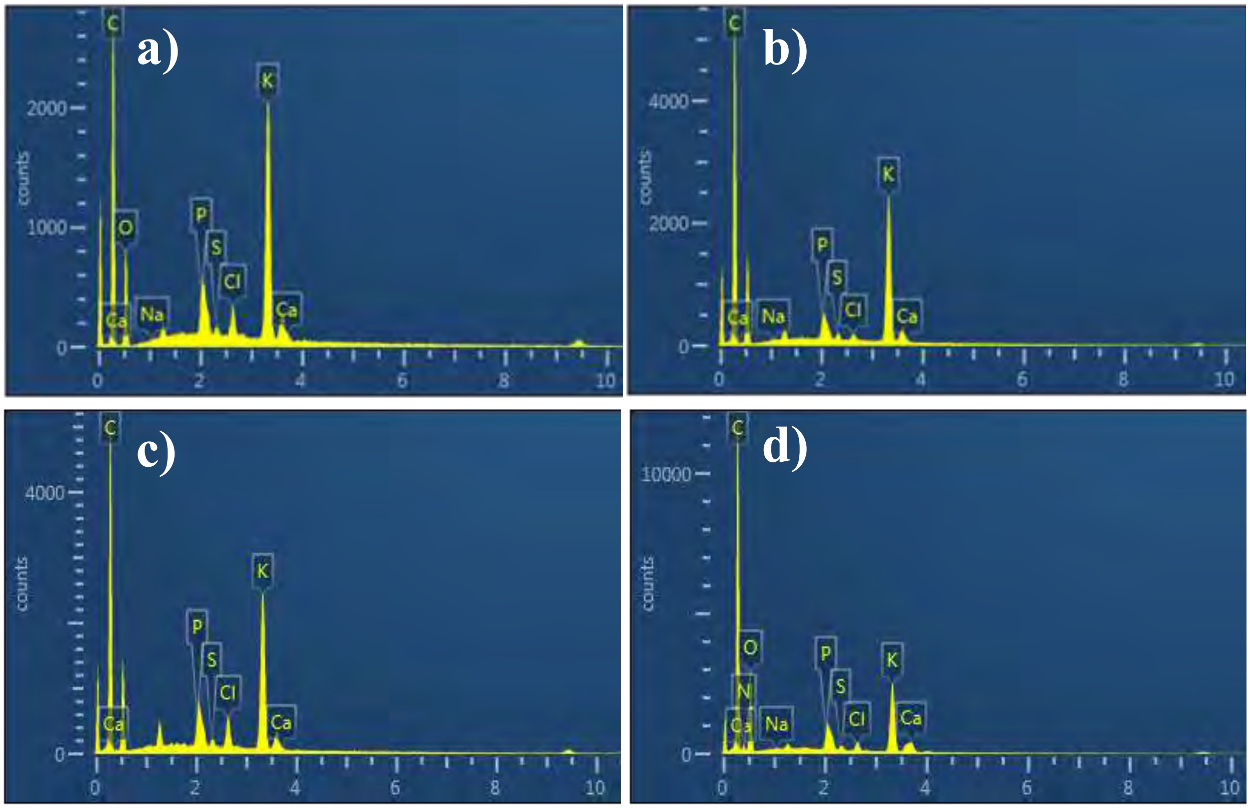

Fig 9. The EDX spectra showing elemental distribution in the leaves of *Syngonium podophyllum* plants grown under hydroponics system treated with various concentrations of NBC.

Table 2: Elemental distribution (%) in leaves of *Syngonium podophyllum* plants grown under hydroponics system treated with various concentrations of NBC.

Treatment	C	P	Ca	O	K	S	Cl
Control	55.93	0.48	0.48	25.20	15.13	0.50	1.34
100 mg/L	58.79	0.72	0.19	27.79	11.58	0.46	0.35
300 mg/L	59.77	0.98	0.35	25.51	11.41	0.46	1.65
500 mg/L	61.64	0.62	0.95	28.18	5.89	0.35	0.55

The elemental distribution of NBC in the leaves of *Syngonium podophyllum* is represented in Figure 9 and Table 2. The elemental distribution showed a high value of carbon in the NBC-treated leaves as compared to the control group. The carbon content in the control group, 100 mg/L, 300 mg/L, and 500 mg/L was 55.93%, 58.79%, 59.77%, and 61.64%, respectively. Also as NBC is rich in calcium and phosphorus due to the presence of hydroxyapatite, the EDX results show their high concentrations in the leaves of *Syngonium podophyllum* with increasing NBC concentration as compared to the control group. The calcium content in the control group, 100 mg/L, 300 mg/L, and 500 mg/L was 0.48%, 0.19%, 0.35%, and 0.95% with phosphorus content showing the value of 0.48%, 0.72%, 0.92%, and 0.62% respectively. According to Xin et al. (2022), biochar exposure to corn seedlings in a hydroponics system result increase in the calcium and phosphorus content in plants as compared to control without any biochar treatment. Also, the biochar exposure resulted in an increase in the phosphorus content in the leaves of the *Syngonium podophyllum* plant as compared to the control (Zulfiqar et al., 2019). As the bone based biochar contains a distinctive amount of plant nutrient element present in it including calcium, phosphorus and magnesium (Zimmer et al., 2018). Therefore, these nutrients are observed in higher amounts in treated plants as compared to control.

3.6. Physiochemical Properties of Soil

Physical and chemical analysis of the soil was performed and the results are presented in Table 3. The soil textural class is sandy loam representing 9% clay, 4.34% silt, and 86.65% sand. The water holding capacity and the bulk density of the soil are 27.43±2.79% and 1.61 g cm^{-3}. The pH and EC of the soil are 8.29±0.01 and 0.06±0.00 dS/m. This indicates that the soil is alkaline in nature. The organic matter in soil is 1.04±0.23%. The concentrations of extractable phosphates and calcium were 4.11±0.15 and 3.80±0.01 mg/kg, respectively. The texture of the soil is sandy loam. It means the soil is porous and well-drained. Porosity increases the availability of oxygen to roots in the soil. The low organic matter in soil may cause soil fertility issues (Imran et al., 2010). Organic carbon is also lower in soil i.e., 0.61±0.13%. However, the addition of NBC in soil may fulfil the organic matter requirements of the soil.

Table 3: Physiochemical properties of soil.

Parameters	Results
Texture	Sandy Loam
Sand (%)	86.65
Silt (%)	4.34
Clay (%)	9.00
Bulk density (g cm^{-3})	1.61
Water holding capacity (%)	27.43±2.79
pH	8.29±0.01
EC (dS m^{-1})	0.06 ±0.00
Extractable phosphate (mg/kg)	4.11±0.15
Extractable calcium (mg/kg)	177±8.98
Organic matter (%)	1.04±0.23
Total organic carbon (%)	0.61±0.13

3.7. Physiochemical Parameters of Soil after Harvesting

The pH of the soil was determined after 40 days of pot experiment (Figure 10). The pH value of the soil treated with 0.0% (control), 0.1%, 0.2%, 0.5% and 1.0% NBC was 7.2±0.1, 8.5±0.06, 8.4±0.06, 8.7±0.1, 8.7±0.06, respectively. The pH significantly increased in all treatments in comparison to the control due to NBC‘s alkaline nature owing to high ash contents and carbonates of Ca, K, and Mg (Yuan and Xu et al., 2011). According to Song et al., (2018), the pH of soil amended with biochar gives a higher pH value due to the high surface area and porous nature of biochar increasing the cation exchange capacity of the soil.

The EC value of the soil in control, 0.1%, 0.2%, 0.5% and 1.0% treatment with NBC was 0.12±0.004, 0.10±0.012, 0.10±0.012, 0.11±0.006, and 0.11±0.001 dS/m, respectively (Fig. 10 (b)). It was observed that the EC of the NBC-treated soils decreased in comparison to the control after 40 days of the pot experiment. According to Burrell et al. (2016), the porous and irregular morphology of biochar enhances the water-holding capacity of soil and dilutes the concentration of salts including Na^{+}, Ca^{+2}, Mg^{+2}, Cl^{-}, and SO_4^{-2} therefore, reducing the osmotic pressure of soil.

The biochar has abundant calcium and magnesium content present in it therefore, these elements can be exchanged with Na^+ and lower the overall salinity of the soil (Xiao et al., 2022). As NBC is rich in calcium and magnesium so it has the potential to undergo exchange with the sodium salt present in the soil lowering the value of EC after harvesting.

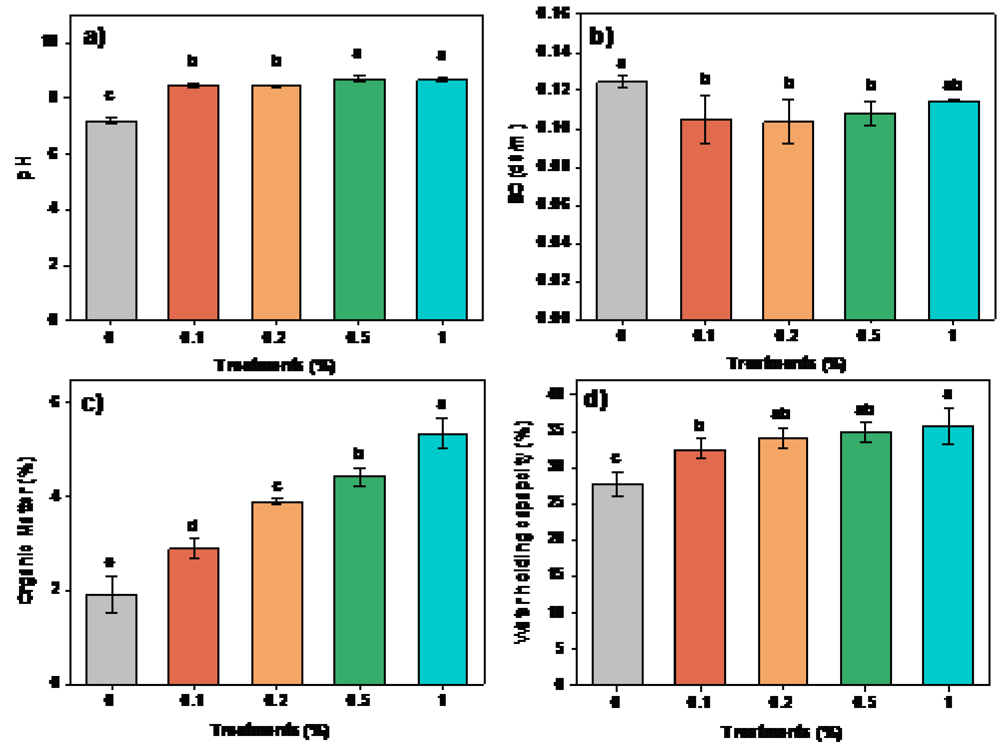

Fig 10: Soil pH (a), EC (b), organic matter (c) and water holding capacity (d) after harvesting *Syngonium podophyllum* plants grown under soil system treated with various application rates of NBC.

The organic matter value of the soils treated with 0% (control), 0.1%, 0.2%, 0.5% and 1.0% was 1.93±0.38, 2.9±0.2, 3.89±0.07, 4.43±0.19, and 5.35±0.33%, respectively. It was observed that the organic matter increased with an increase in the NBC application rate (Fig. 10 (c)). According to Li et al. (2017), the introduction of organic amendments to soil enhances the soil's organic content and results in an improvement in soil quality. The organic matter content in the soil increased due to organic matter from the NBC.

The water holding capacity of the soil in control, 0.1%, 0.2%, 0.5% and 1.0% was 27.77±1.68, 32.53±1.37, 34.12±1.37, 34.92±1.37 and 35.71±2.38%, respectively. It was observed that the water-holding capacity of the soil risen with the increase in NBC dose (Fig. 10 (d)). Biochar could increase water retention in soil due to its porous structure, which also influences nutrient retention as it could hold nutrient-rich water within the pores (Gryze et al., 2010). Biochar works as a soil conditioner by strengthening the physical, chemical, and biological properties of soil including water-holding capacity and soil nutrients retention and improving plant growth (Oguntunde et al., 2008). Fertilizing the soil with biochar has a positive impact on the water-holding capacity of soil as biochar has the potential to absorb five times more water than its weight (Gasior and Tic, 2017).

3.8. Uptake of NBC by *Syngonium podophyllum* under Soil System

Figure 11 shows the compound microscopy images of leaves and roots of the *Syngonium podophyllum* plants grown in the soil treated with various application rates of NBC. It was observed under the microscope that no carbon particles were detected in the plants of the control group. The leaves and roots of these plants were clear from black NBC particles. However, the plants grown under NBC treatments showed high concentrations of NBC particles in their leaves and roots at all treatments.

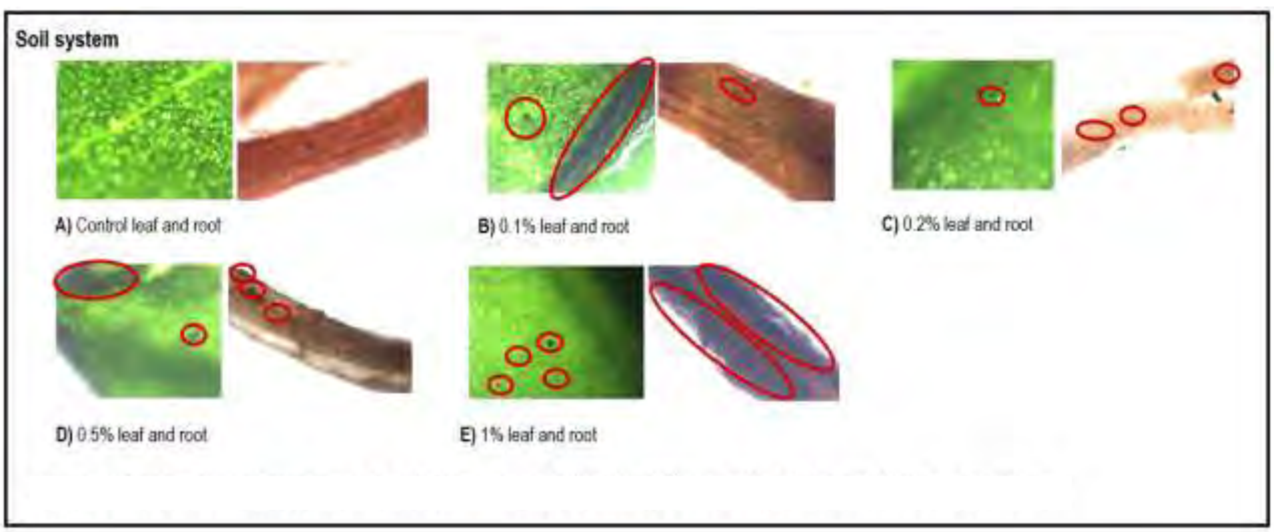

Fig. 11: Compound microscope images of leaves and roots of *Syngonium podophyllum* plants grown in soil treated with various application rates of NBC.

The particles were traced at different points along the leaves. The main veins of the leaves were dark enough to indicate the presence of NBC in them. Similarly, the roots of these plants show the presence of particles at various locations along with agglomeration of NBC at different points in the root system. However, it was observed that at low treatment doses i.e., 0.1% and 0.2%, the effect of NBC in leaves and roots was slightly less in comparison to leaves and roots of the plants at high treatment doses i.e., 0.5% and 1%. The physical characteristics including diameter, length, and solubility of carbon nanomaterials play a significant role in penetrating plant tissue. If the diameter of the roots is smaller than the particle size of CNTs, they will be unable to penetrate inside the root tissues. According to Yuan et al. (2011), carbon nanoparticle penetration inside the cells can take place through endocytosis. Uptake of nanoparticles may also take place through embedded transport carrier proteins or ion channels (Nair et al., 2010). Similar results were observed with nano-sized biochar particles in the roots of rice plants, with the diameter of nano biochar present in the root cells in the range of 10 to 23 nm (Yin et al., 2023). These results of the absorption of biochar particles through the root system are also consistent with previous studies (Zhang et al., 2020; Sun et al., 2020). According to Lv et al. (2019), the absorption and uptake of nanoparticles (only 40-50 nm) in the root cells is possible by either the apoplastic or symplastic pathways. There might be another pathway for the uptake of nano biochar through root cells, specifically particles having a size larger than 50 nm. Lui et al. (2009) studied the uptake of single-wall carbon nanotubes (SWCNT) in tobacco cells (with a diameter greater than 500 nm) through endocytosis. The small size of carbon nanoparticles improves their penetration into the plant cells and tissues as the penetration completely be determined by the size and the concentration of carbon nanoparticles (Ahmed et al., 2019).

3.9. Fresh Weight, Dry Weight, and Shoot Length of *Syngonium podophyllum* Plants under Soil System

The fresh weight of the plants grown in the soils amended with different application rates of NBC is shown in Figure 12 (a). The values for the fresh weight of plants in control, 0.1%, 0.2%, 0.5%, and 1.0% are 5.33±0.57, 7.66±0.57, 9.33±1.15, 10.33±0.57, and 9.33±0.57 g, respectively. It was observed that a high value of fresh weight was achieved in the plants with 0.5% treatment i.e., 10.33±0.57 g, while the minimum value was observed for the control group i.e., 5.33±0.57

g. Overall, it was observed that the fresh weight of the NBC-treated plants was higher than the control group.

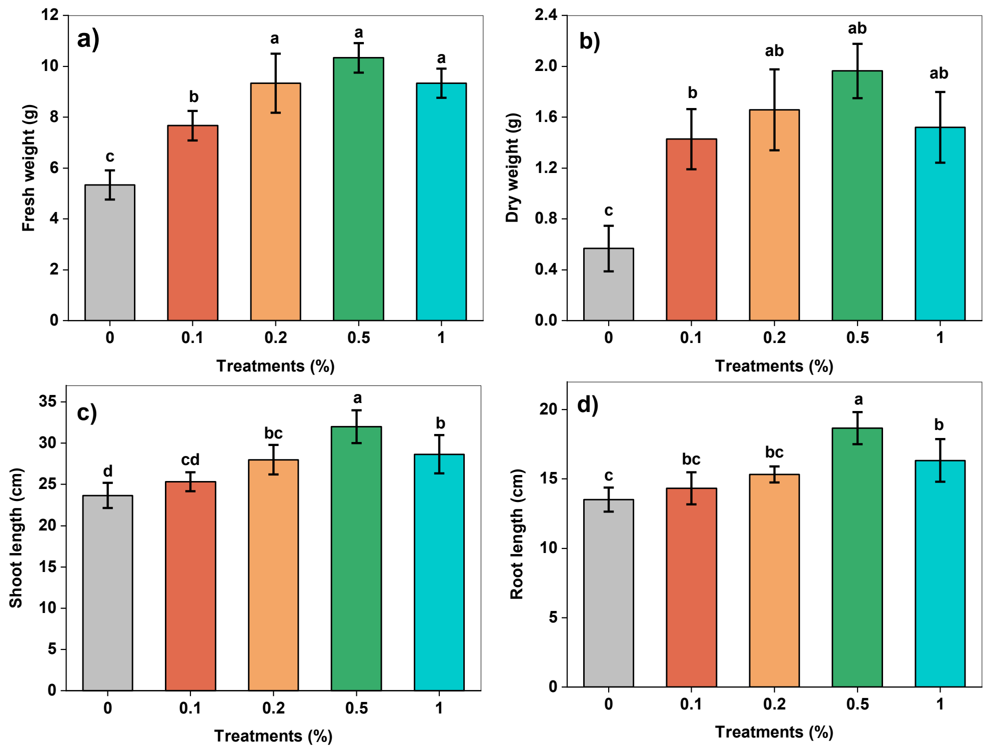

Fig. 12: Fresh weight (a), dry weight (b), shoot length (c) and root length (d) of *Syngonium podophyllum* plants grown in soil treated with various application rates of NBC.

The percent increase in fresh weight of plants for 0.5% and 1.0% treatments was 82.50% and 64.84%, respectively, as compared to control. Similar results were observed in the previous studies by Khodakovskaya et al. (2012) and Wang et al. (2012). The probable reason for the increase in water uptake by plants subjected to carbon nanoparticles could be either length of the roots, i.e., the longer the length of the roots the more they will facilitate more water movement towards aerial parts of the plant, or due to the higher number of stomata present in leaves. It is also possible that CNTS can create new pores for water regulation and support the capillary action of water in plants. Also, molecular channels can be formed by carbon nanomaterials which will result in more water uptake in plants (Joshi et al., 2020).

The dry weight of the plants grown in the soil system is presented in Figure 12 (b). The values for the dry weight of plants grown in control, 0.1%, 0.2%, 0.5%, and 1.0% NBC treatments are 0.56±0.17, 1.42±0.23, 1.65±0.31, 1.96±0.21, and 1.52±0.27 g, respectively. A high value of dry weight (1.96±0.21 g) was achieved in the plants for 0.5% treatment, followed by 0.2% treatment (1.65±0.31 g). The minimum value of the dry weight for the plants was observed in control treatment (0.56±0.17 g). The per cent increase in dry weight of plants grown in soil observed in 0.2% and 0.5% was 194.64% and 250%, respectively, as compared to the control. The nano bone char comprises carbon, calcium, phosphorus, potassium, magnesium, and organic matter present in it. These constituents are taken up by the plants through the roots, supporting the growth of plants. Biochar is a source of several important nutrients, its intricate relationship with soil results in the release of nutrients, making them available for plants to uptake (Bista et al., 2019).
The shoot length of the plants grown in the soil system is presented in Figure 12 (c). The values for the shoot length of plants in control, 0.1%, 0.2%, 0.5%, and 1.0% were 23.66±1.52, 25.33±1.15, 28.00±1.80, 32.00±2.00, and 28.66±2.30 cm, respectively. Maximum increase in the shoot length was observed for the plants in soil at the treatment level of 0.5%. The percent increase in the shoot length of soil plant at 0.2%, 0.5% and 1.0% was 18.34%, 35.24% and 21.13%, respectively, as compared to the control. However, a slight decrease in shoot length was observed when the plants were exposed to a high treatment dose of NBC (1.0%). This trend of shoot length corresponds with the study of Joshi et al. (2020) in which the shoot length of rice plants increased proportionally at lower doses of single-walled carbon nanotubes (until 80 ug/ml) in soil system after that decrease in the growth of rice plants was observed.

The root length of the plants grown in the soil system is presented in Figure 12 (d). The values for the root length of plants in control, 0.1%, 0.2%, 0.5%, and 1.0% are13.5±0.86, 14.33±1.15, 15.33±0.57, 18.66±0.15, and 16.33±1.52 cm, respectively. An increase in the root length was observed with an increase in the treatment levels of 0.10%, 0.20% and 0.50%. However, at 1.0% of NBC treatment, the plants showed a slightly lower growth in root length as compared to other treatments except control. The per cent increase in the root length of the plants in soil at 0.2%, 0.5% and 1.0% was 13.13%, 37.70% and 20.51%, respectively, as compared to the control. Our results correspond with the results of Wang et al. (2018), where mung beans exposed to carbon dots showed a positive effect on the root length from lower to higher concentrations i.e., 0.02

mg/ml to 0.12 mg/ml. However, the maximum concentration i.e., 0.12mg/ ml of carbon dots did not stimulate the growth in the sprouts of mung beans. Root systems play a significant role in the growth of plants because of their water-holding capacity, which transports water and nutrients to various parts of plants. The definite impacts of NBC on root length, shoot length, and biomass were caused by the increase in the water-absorbing capacity of roots.

The enhanced growth in *Syngonium podophyllum* plants at different NBC concentrations is because the NBC treatment improves the phosphorus, potassium, and nitrogen content in the plant's organ, which is useful for the growth of the plant. Our results correlate with previous studies where CNP improved the growth of tobacco plants by increasing their height, leaf area, dry matter, and chlorophyll content (TaiBO et al., 2013).

The decrease in the plant growth at high treatment level of 1.0% might be owing to the buildup of NBC particles on the roots as they are in direct contact with the nanoparticles and might detain the uptake of minerals by the crops and therefore limit the plant growth at higher concentration of NBC. A decline in the growth of the *Vigna radiata* plant was observed at a higher concentration of 200 µM as a limitation in the uptake of minerals by the crops due to the accumulation of CNPs in the root system of the plant (Shekhawat et al., 2021; Ghoto et al., 2020).

3.10. Chlorophyll Content and Leaf Count of *Syngonium podophyllum* Plants under Soil System

The chlorophyll content of plants in soil system is presented in Figure 13 (a). The value for chlorophyll content in control, 0.1%, 0.2%, 0.5%, and 1.0% NBC treatments was 39.61±0.25, 42.31±2.36, 44±1.71, 46.6±1.25, and 44.91±1.88, respectively. The plants with the treatments of 0.1%, 0.2%, and 0.5% showed increasing values for chlorophyll content. The per cent increase in chlorophyll content for 0.2%, 0.5%, and 1.0% was 11.08%, 17.6% and 13.38%, respectively, as compared to the control. One possible explanation for the increase in chlorophyll content is that the electron transport rate is enhanced when plants are treated with carbon nanoparticles as the plants exposed to carbon nanoparticles capture more photons of light and transfer light energy to enhance the transport rate of electrons that ultimately enhance the photosynthesis rate (Gilardo et al., 2014; Deng et al., 2017).

Figure 13 (b) indicates the leaf count for plants in the soil system. The leaf count in control, 0.1%, 0.2%, 0.5%, and 1.0% treatments was 1.66±0.6, 2.66±0.57, 2.66±0.57, 3.33±0.57, and 2.33±0.57 respectively.

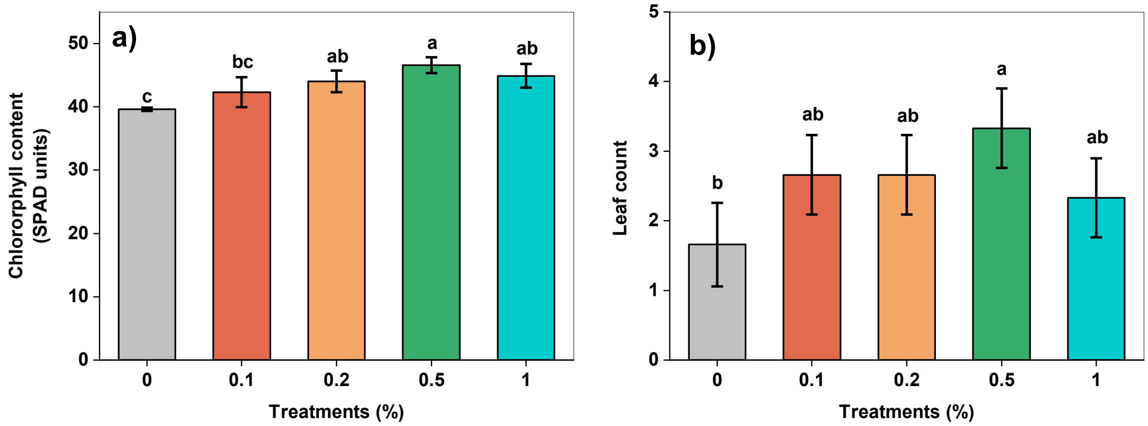

Fig. 13: Chlorophyll content (a) and leaf count (b) of *Syngonium podophyllum* plants grown in soil treated with various application rates of NBC.

A high number of leaves was detected in plants at the treatment dose of 0.1%, 0.2%, and 0.5% application rates of NBC. The per cent increase in the leaf count for 0.2%, 0.5% and 1.0% was 60.2%, 100% and 40.36%, respectively, as compared to the control. The results indicate that NBC amendment to the soil presents an enhancement in the photosynthetic activity of the plant and an overall increase in plant yield. However, the amount of NBC introduced in the system also plays a significant part in regulating plant yield. The optimum dose of NBC sufficiently supports plant growth, whereas the higher dose does not significantly affect plant yield or may negatively influence plant yield. The results of our study correlate with Liu et al. (2014) and Zhang et al. (2012) where the introduction of biochar as an amendment to soil results in the increase in plant yield in rapeseed, sweet potato, and rice crops. The chlorophyll content in the *Vigna radiata* seedlings was also high at initial treatment when exposed to CNPs (Shekhawat et al., 2021) indicating the fact that CNPs facilitate chlorophyll biosynthesis. The CNPs improve the photosystem activity by speeding up the electron transfer rate (Wang et al., 2018). The slight decrease in the chlorophyll content at a high treatment dose might be due to oxidative damage that occurs in the cell plasmids because of the apparent linkage of CNPs with chloroplasts, which eventually interrupts the chlorophyll biosynthesis or chlorophyll reduction in the leaves when exposed to elevated concentrations of CNPs (Ghoto et al., 2020).

3.11. Changes in Surface Morphology and Elemental Composition of *Syngonium podophyllum* Leaves under Soil System

Figure 14 shows the SEM images of leaf samples of *Syngonium podophyllum* grown in soil system for control, 0.5% and 1.0% NBC treatments at a 20-micron meter scale.

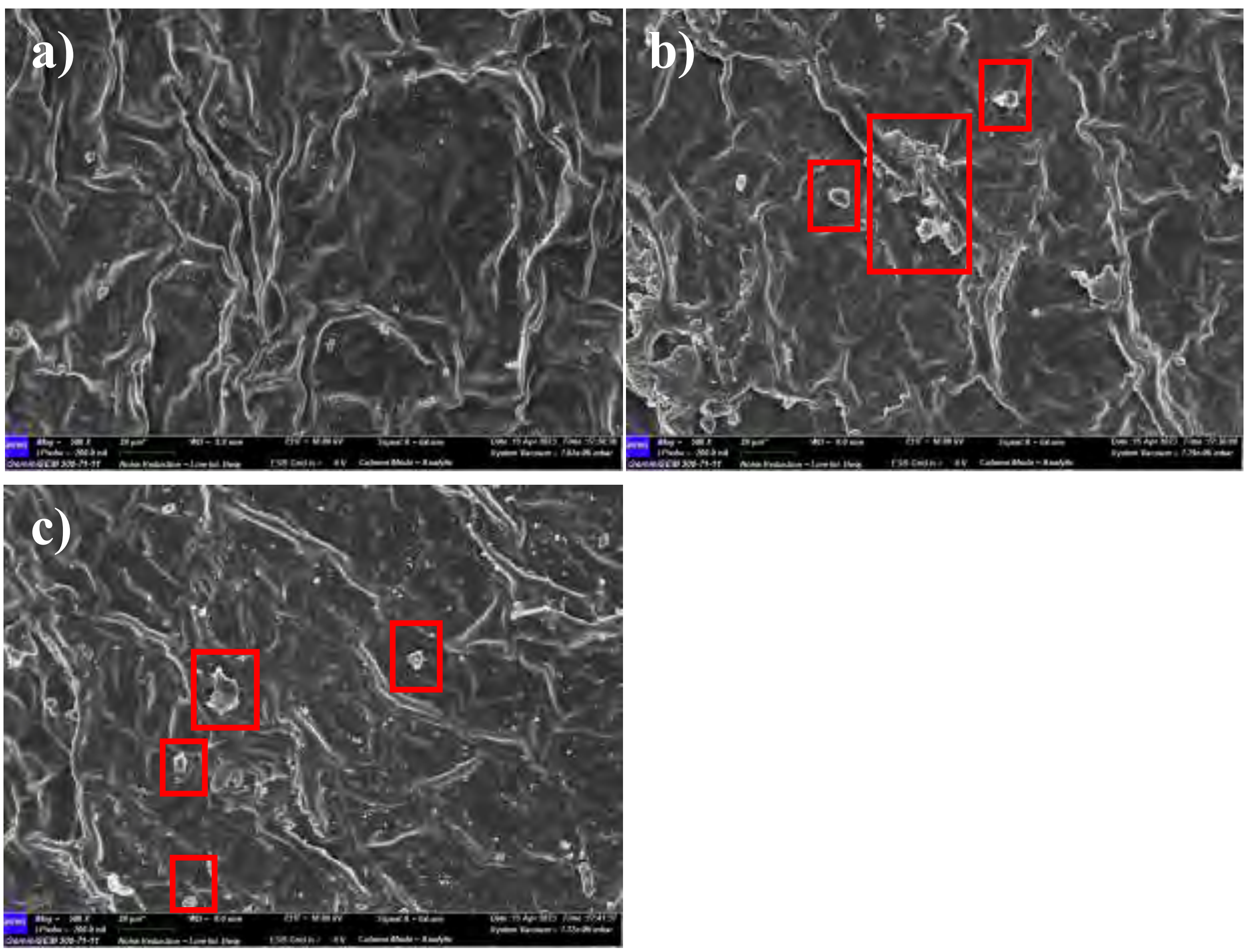

Fig. 14: The SEM images (at 20-micron meter scale) of leaves of *Syngonium podophyllum* plants grown under soil system treated with 0.0% (control) (a), 0.5% (b), and 1.0% (c) of NBC.

Figure 15 shows the SEM images of the same leaf samples at a 2-micron meter scale. The control group did not show discrete carbon particles or agglomeration of carbon particles. The remaining treatments (0.50% and 1.0%) showed the embedment of carbon particles of different shapes and sizes. These embedded particles represent the uptake of NBC from roots to the leaves. As NBC is rich in calcium and phosphorus content due to hydroxyapatite so they appear as white agglomerated particles. The high concentration of NBC in *Syngonium podophyllum* resulted in its uptake from roots to shoots and finally to the leaves.

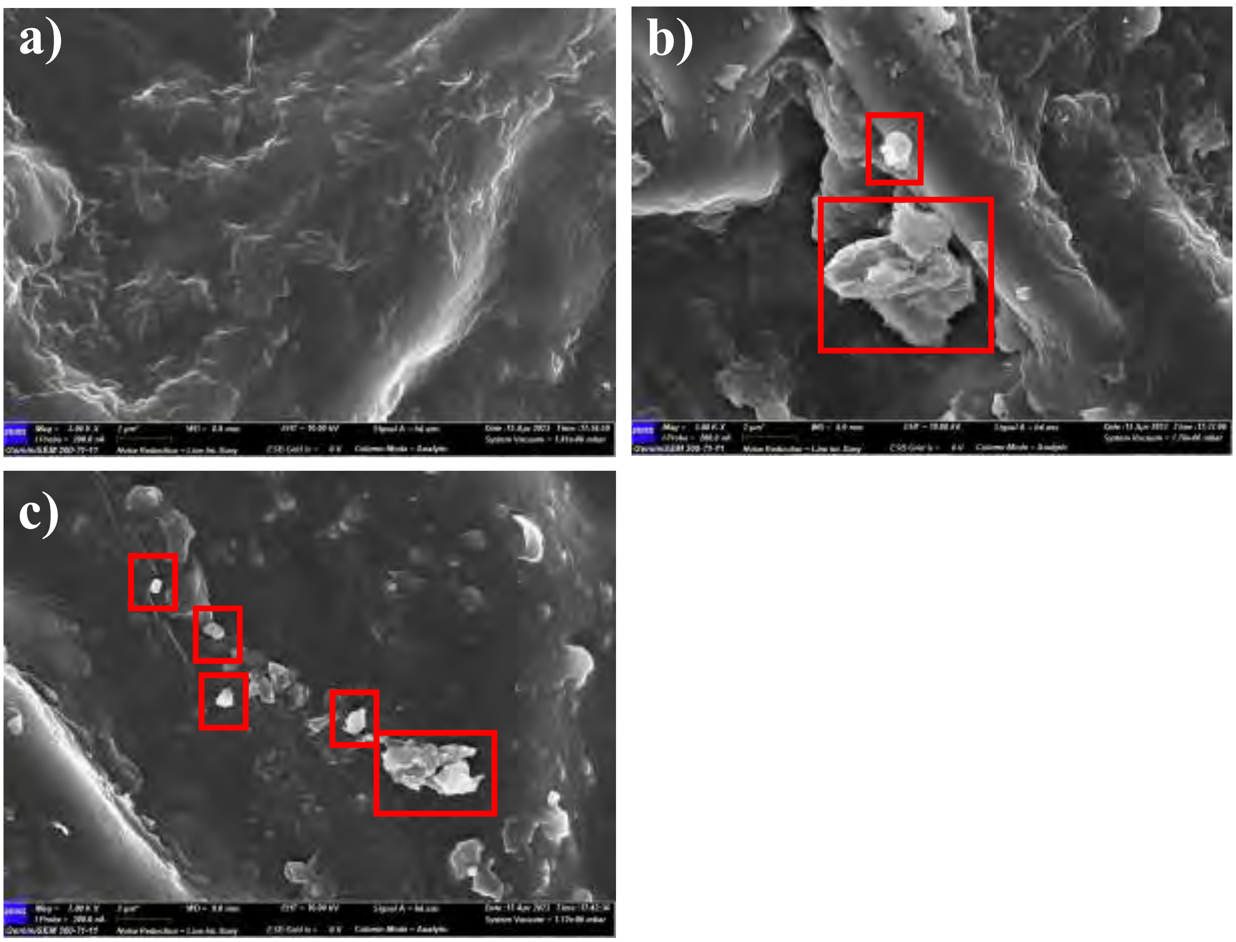

Fig. 15: The SEM images (at 2-micron meter scale) of leaves of *Syngonium podophyllum* plants grown under soil system treated with 0.0% (control) (a) and 1.0% (b) of NBC.

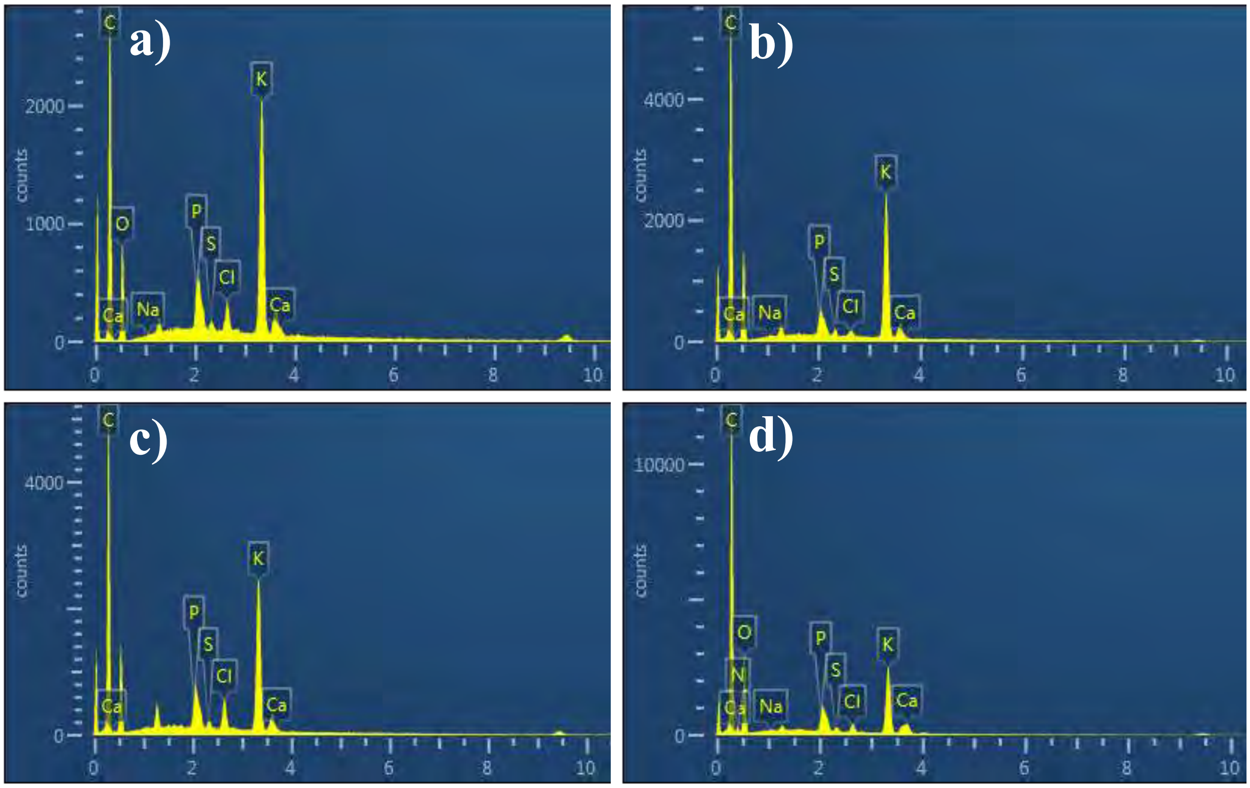

Fig. 16: The EDX spectra (at 20-micron meter scale) of leaves of *Syngonium podophyllum* plants grown under soil system treated with 0.0% (control) (a), 0.5% (b), and 1.0% (c) of NBC.

Table 4: Elemental distribution (%) in leaves of *Syngonium podophyllum* plants grown under soil system treated with various applications of NBC.

Treatment	C	P	Ca	O	K	S	Cl
Control	46.29	0.58	0.37	47.91	4.03	0.42	1.34
0.5%	65.79	1.01	3.45	17.58	10.10	0.85	1.82
1.0%	69.52	0.58	0.28	18.19	8.21	0.26	2.79

The elemental distribution of NBC in the *Syngonium podophyllum* leaves is represented in Fig. 16 and Table 4. A high value of carbon in the NBC-treated leaves was observed as compared to the control. The carbon content in the control, 0.5% and 1.0% was 46.29%, 65.79%, and 69.52% respectively. According to Lin et al. (2009), the carbon nanotubes are absorbed from the soil through the roots and consequently distribute or spread inside the plants to reach the leaves and fruits. They analyzed these results based on the study of tomato plants. The nanomaterials either natural or man-made when subjected to soil or any medium could easily be absorbed by the plants and accumulate in their structure and tissues (Khodovoskya et al., 2012). Also, the EDX results present their high concentrations in the leaves of *Syngonium podophyllum* with increasing NBC concentration as compared to the control group. The calcium content in the control group, 0.50 and 1% was 0.37%, 3.45%, and 0.28% with phosphorus at 0.58%, 1.01%, and 0.58% respectively. According to Shahid et al. (2019), the EDX spectra of NBC show a high percentage of calcium, phosphorus and magnesium as the main component of biochar. Also, the uptake of nutrients elements including phosphorus and calcium was enhanced in the soil drenched with carbon nanoparticles with increasing application rate of CNPs in corn seedlings (Xin et al., 2022). The slight decrease in calcium percentage in 1% treatment with the increasing application may be linked with the explanation that CNPs hold the nutrients and slowly release these mineral nutrients for plant uptake which enhance the overall efficiency of nutrient uptake (Xin et al., 2022).

3.12. Comparison of NBC Uptake by *Syngonium podophyllum* plants under Hydroponics and Soil Systems

The comparison of hydroponics and soil systems for the uptake of carbon nanoparticles reveals that the plants grown in the hydroponics system give a high value of the fresh weight, dry weight, shoot length, chlorophyll content and leaf count with increasing treatment dose. The nutrient uptake was more efficient in the water-based hydroponics system due to direct contact with carbon-based suspended particles. The efficient uptake of NBC at 500 mg/L in a hydroponics system enhanced the physiological parameters including fresh weight, dry weight, shoot length, chlorophyll content, leaf count and nutrient uptake (C, Ca, and P) of the *Syngonium podophyllum* plant, thereby increasing plant yield. The plant‘s growth was higher in the hydroponics system with an increase in the dose of NBC due to particle uptake and nutrient uptake by *Syngonium podophyllum.* As particles were easily taken up by the roots through the xylem system through water transportation channels.

In the soil system, the plants showed a higher value of the physiological parameters as compared to the control. The nutrient uptake of soil plants also increased with an increase in NBC treatment at 0.50%. The efficient uptake of NBC at 0.50% enhanced the physiological parameters including fresh weight, dry weight, shoot length, chlorophyll content, leaf count and nutrient uptake (C, Ca, and P) of the *Syngonium podophyllum* plant. It was observed that the 1% treatment dose of NBC showed a significantly higher yield compared to the control but slightly lower than 0.50% in the *Syngonium podophyllum* plant. The decrease in the plant growth at a high treatment level of 1.0% might be owing to the buildup of NBC particles on the roots as they are in direct contact with the nanoparticles and might detain the uptake of minerals by the crops and therefore limit the plant growth at higher concentration of NBC. Similar results were observed by Shekhawat et al. (2021) and Ghoto et al. (2020). The electron microscope shows visible results of NBC in the soil system. Therefore, we conclude that the hydroponics system is a more efficient system for the uptake of nanoparticles and their effect on plants as an increase in concentration significantly increases plant uptake of NBC and improved its yield.

CHAPTER 4

CONCLUSIONS AND RECCOMENDATIONS

4.1. Conclusions

It is concluded from the study that exposure to NBC under hydroponics and soil systems shows a positive effect on the uptake, translocation and accumulation of NBC in *Syngonium podophyllum* plants. The compound electron microscope and SEM images in both systems confirmed the uptake, translocation and accumulation of NBC in the plants. The exposure of NBC improved the growth of plants in both systems as compared to the control that was not exposed to NBC. The NBC comprises of carbon, calcium, phosphorus, potassium, magnesium, and organic matter, which were taken up by the plants through the roots, thereby improving the growth of plants. The fresh weight, dry weight, shoot length, root length, chlorophyll content and leaf count of the *Syngonium podophyllum* plants increased with an increasing concentration rate of NBC both in hydroponics and soil systems. Specifically, an increasing trend in the plants growth rate was observed in treatment rates of 300 mg/L and 500 mg/L in the hydroponics system and 0.2% and 0.5% in the soil system. The presence of nanoparticles resulted in the formation of new capillaries or channels that increased the water uptake mechanism of plants resulting in enhanced growth. The slight decrease in all the growth parameters of 1.0% treatment in the soil system might be due to oxidative damage that may have occurred in the cell plasmids owing to the apparent linkage of carbon nanoparticles with chloroplasts, or there might be a nutrient blockage in the roots due to high NBC dose that hindered the uptake of nutrients to the aerial parts of the plants. The interaction of plants with carbon nanoparticles depends on multiple factors including plant specie, type of nanomaterial, concentrations to which nanomaterials are exposed to plants, and particular conditions of the experiment such as the method for nanoparticle uptake into the plant system. Nano-biochar retains massive prospects in agrarian practices. The size specification of nano-biochar presents enhanced surface area making them a suitable candidate for environmental remediation of inorganic and organic chemicals and improving soil quality and crop performance.

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4.2. Recommendations

- The materials at the nanoscale are difficult to handle, therefore particular care is required when dealing with nanoparticles.
- Nano-biochar holds superior improvements for agricultural applications compared to bulk biochar, however, the risks associated with nano biochar or nanoparticle to soil organisms and human health should be evaluated.
- Field trials with nano-biochar are required to recommend its use for agricultural applications.

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ANNEXURE

Chemicals and glassware

There are various instruments, glassware and chemicals that have been used during our research study. The chemicals used during the study include Ammonium bicarbonate, p-nitrophenol indicator, ferroin indicator, sodium hydroxide, ascorbic acid, potassium dichromate. Different acids including sulfuric acid, nitric acid, perchloric acid were mainly used for digestion of samples for analyzing organic matter and total calcium and phosphorus of samples. The hydroponics nutrient system was setup by using different chemicals including, Manganese Chloride, Zinc Sulphate, Boric acid, Potassium Dihydrogen Phosphate, Ammonium Phosphate, Magnesium Sulphate Heptahydrate, Calcium Nitrate, Sodium Molybdate, Copper Sulphate, Iron chelate EDTA, Potassium Nitrate.

The glassware used during experimentations include beaker, falcon tubes, stirrer, graduated cylinders, volumetric flasks, conical flasks, crucibles, spatulas, pipettes, funnels, Erlenmeyer flasks and droppers and glass bottles. Also, filter paper and aluminium foils were used during different experiments for weighing and filtration.

Instrumentation

Bones were dried using a drying oven (EQ-DHG-9140A) and to determine moisture content during proximate analysis of nano bone char. Muffle furnace (Vulcan D-550) was used for synthesis of Bone char from bone waste. Bone char was then converted into nano-sized bonchar using planetary ball mill (BKBM-V2S). Zeta sizer (ZS-90, Malvern instrument, UK) was used for determining the size of the nano bone char (NBC). Analytical weighing balance (Shimadzu ATX 224) was used for weighing of different chemicals, soil samples and NBC for different experiments. pH (ino-Lab pH-7110) and electrical conductivity (EC) meter (HANNA HI-2300) used for measuring pH and electric conductivity of the soil and NBC respectively. The presence of functional group was determined through Fourier transform infrared (FTIR SHIMADZU-8400) Scanning electron microscope (EFI S50 Inspect Netherland) was used to obtain the surface morphology of NBC. X-ray diffraction was used for detecting crystalline material in NBC. Energy Dispersive X-Ray (EDX) analysis was used for finding the elemental composition of

NBC. A flame photometer (FP910-4, UK) was used to measure calcium (Ca) content of the soil and plant. A UV spectrophotometer (Thermos Fisher Scientific 51119500) was used to measure bioavailable phosphorus of the soil and total phosphorus of the plants. Orbital shaker was also used for shaking samples. An Electron Microscope was used to observe the plant samples under microscope for detection of NBC.

Bone collection drying and grinding

Conversion of bone waste into bonechar and then nano bone char through ball milling technique

Hydroponics experiment

Soil experiment