EVALUATION OF PLANT ROOT SYSTEM COMPLEXITY UNDER HEAVY METAL STRESS BASED ON FRACTAL ANALYSIS

（フラクタル解析に基づく重金属ストレス下の植物の根系の形態評価）

March 2019

Saitama University, Graduate School of Science and Engineering (Doctoral Program)

Supervisor: Prof. H. Kadono

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by

LI TAO

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A dissertation submitted as a partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Environmental Science and Engineering

Supervisor: Professor Hirofumi Kadono

Department of Environmental Science and Infrastructure Engineering

Graduate School of Science and Engineering

Saitama University

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Chapter 1 Introduction

1.1 Introduction

Plant growth significantly depends on the root system morphology, which includes root length, root branching density, root distribution etc.. Roots play a critical role in water and nutriment acquisition under different environment circumstances (Fitter, 1987). Extensive studies have focused on methods to determine root growth mainly through length measurement (Chaiwongsar et al., 2012), weight measurement (Xu et al., 2013) and lateral root counting. However, a challenging problem in the measurement of root morphology is quantifying the complexity of the root architecture. If quantification of root complexity could be described, it would provide a strong evidence of existing relationships between root functions and the overall plant performance. Therefore, an accurate method to estimate the complexity of the root system is required.

As a way to estimate the complexity of the root system, a different approach that was based on fractal analysis has been applied (Tatsumi et al., 1989). Fractal is a natural phenomenon or a mathematical set showing a repeating pattern that could be displayed at every scale. Despite considering root systems as fractal objects is still under debate as to the genuinely of having self-similarity (Bernstein et al., 1996) and thus non-integer dimension, application of fractal geometry to describe complexity of roots has found applications in describing the influence of environment (Tatsumi et al., 1989). For instance, effect of different phosphorous conditions on root systems of common bean genotypes by Nielsen (Nielsen et al., 1999), effect of drought stress on rice root system (Wang et al., 2009), effect of salt-stressed conditions on corn root system development (Subramanian et al., 2015). To our knowledge up to now, no study has examined the root complexity under heavy metal stress using fractal analysis.

Liao et al. (Liao et al. 2001) clearly demonstrated that the content of each diameter of the root structure had a great influence on the fractal characteristics distribution. At the same time, the density degree of the single root diameter grade also has an important influence on the fractal dimension. That was, the lower the fractal dimension, the lower numbers of the fine roots of the plant, which indicates the higher complexity of the main root of the plant. Xie et al. (Xie et al. 2002) calculated and compared the fractal structure characteristics of Salix sp., P. purdomii and Abies fabri according to the digital photos of tree roots using computational box dimension method. The results showed that the higher fractal dimension of the root system, the more complex the root structure and the multi-layered micro-branched structure; the lower the fractal dimension, the simpler the branching structure. The study on the root structures and stable soil capacity of the above three trees showed that the logarithmic dimension of roots and the logarithm of tensile resistance showed a stable exponential function, indicating that the fractal dimension of tree roots can reveal the structure of root's characteristics and developmental dynamics more accurate.

Traditional methods, such as trench method (Nemoto et al., 1998), core method (Steingrobe et al., 2001) (Lukac et al. 2001), etc., are frequently used to measure and analysis the root system. However, there are many disadvantages of these traditional methods, such as large losses of fine roots, labor- intensive by these processing. Most of the current researches have studied on root fractal analysis mainly in two-dimensional processing, as the root systems are buried deeply. So, the root systems are very difficult to perform in situ measurement. Before the analysis, there have three ways to confirm the root system from three-dimensional distribution to two-dimensional plane distribution: 1), using the glass window method to track the root system in the soil; 2), maintaining the spatial structure when removing the root system from the soil, like pin boards methods.

In the last decade, with the development of industry, the environment contamination due to heavy metal has become an increasing global concern as their widespread distribution and toxicity to living things. Heavy metal-induced effects include oxidative stress, genotoxicity, inhibition of the photosynthetic process, and inhibition of root metabolism (Andresen and Küpper, 2013). In particular, heavy metal toxicity has been found to affect root formation other than grain yield, seed formation, chlorophyll synthesis, hormone proteins and membrane functions. Cd plays a major role in inhibition of plant growth by accumulation in plant leaf and root (Street et al., 2009). Cd has been shown to affect lateral root formation and root development (Ronzan et al., 2018).

On the other hand, copper (Cu) plays important role in CO2 assimilation and ATP synthesis (Yadav, 2010). Exposure of plants to excess Cu generates oxidative stress and ROS, and Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus.et al., 2001). Further, excess Cu has a detrimental influence on plants, especially on root growth and morphology (Sheldon and Menzies,2005). Arduini et. al (Arduini et al., 1994) found that excess Cu can reduce the lateral root index. Unlike Cd, Zn has been considered as a micronutrient which is needed in small amounts by plant. Under certain optimal concentrations, ZnO (400nM) showed large root growth of peanut (Prasad et al., 2012). Zn toxicity is apparent as a reduction in the growth of the main root, fewer and shorter lateral roots and a yellowing of roots (Ren et al., 1993). Toxic concentrations of Zn causes inhibition of photosystems I and II and thus a decrease in photosynthesis (Van Assche and Clijsters, 1986). The mechanism of the action due to Zn is the displacement of Mg by Zn at the water splitting site in photosystem II (Kupper et al., 1996). Teige et al. (Teige et al. 1990) suggested that the primary toxic action of heavy metal is the inhibition of ATP synthesis and therefore energy metabolism in plants.

Further, it has been known that such root systems undergo changes with environmental conditions, for example, heavy metal pollution of the soil. Therefore, there is a need for a method to characterize the complexity of the root systems that is also sensitive to evaluate the environmental effects. To our knowledge up to now, no study has examined the root complexity under heavy metal stress using fractal analysis.

In this study, we have considered the effect of the above three heavy metals, Cd, Cu, and Zn on root system of wheat plant.

Wheat (Triticum.spp.) is the most widely grown crop in the world and is the first strategic cereal crop for a majority of populations and is the second most important food crop in the developing world after rice (Curtis et al., 2002) . It has been known that, the heavy metals such as Cd could accumulate in high amounts in the roots of wheat plants (Brunetti et al., 2012).

In this study, to investigate the effects of heavy metals on the wheat root development, we have applied fractal geometry and calculated the complexity index (CI) and defined a parameter called relative complexity index (RCI) that is based on FD. FD was calculated using the box-counting method, a popular and easy method used in calculating the fractal dimensions (Tatsumi et al., 1989). Wheat plants were exposed to three Cd concentrations of 0.001, 0.01, and 0.05mM, three different Cu concentrations of 0.016, 0.4, and 1.2 mM, and two different Zn conditions of 0.3 and 0.75mM. Under all the conditions, control was the one under 0 mM. The changes in RCI was estimated as a measure of the influence of the heavy metal stress on the root system. For comparison, conventional measures of root lengths and root weights were also measure and correlation with the RCIs were obtained.

In this dissertation, I demonstrate the effects of Cd, Cu and Zn on the complexity index (CI) changes of the wheat root development based on the fractal geometry.In chapter 2, methods to determine CI based on fractal dimension is briefly explained. In this study, Box-counting method is applied to evaluate the root complexity of root system. MATLAB program of image processing and box-counting method is demonstrated. In chapter 3, imaging processing was briefly explained. In chapter 4,5 and 6, fractal geometry was applied to characterize the complexity of the root system morphology of wheat plants under the exposure of heavy metals, namely Cd, and Zn. . Wheat roots were exposed to cadmium chloride solution ($CdCl_2$) of concentrations of 0, 0.001, 0.01 and 0.05 mM, copper sulfate solution (CuSO4·5H2O) of 0, 0.016,0.4 and 1.2 mM and zinc nitrate hexahydrate (Zn(NO3)2·6H2O) of 0, 0.3, and 0.75ppm for over three weeks. We calculated the relative complexity index (RCI), which indicates fractal dimension (FD) changes. FDs were calculated by box-counting method with digitized and skeletonized images of roots of wheat plants cultivated in hydroculture system. For comparison with RCI, relative root elongation (RRE) and relative weight (RW) were measured under different concentrations of Cd (0.001,0.01 and 0.05 mM), Cu (0.016,0.4 and 1.2mM) and Zn (0.3 and 0.75mM) for over three weeks.

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Chapter 2
Fractal Dimension

2.1 Introduction of fractal

The Fractal concept was discovered by American mathematician B.B. Mandelbrot in his book "Fractals: Form, Chance and Dimensions" in the mid-1970s (B.B. Mandelbrot, 1982). The term is defined from the Latin word fracturs, meaning fractured or broken. Fractals mean no characteristic length scale, which refers to the representative of various lengths contained in the set object under consideration, such as a ball, which can be used as its feature length).

A fractal is a natural phenomenon or a mathematical set that shows repeating pattern that display at every scale (Yan et al. 1995, Falconer 2004). Basically, fractal has two fundamental properties: Self-similarity and non-integer dimension. Fractal has fine structure at randomly small scales. The feature of self-similarity means the objects exactly or approximately similar to a reduced-size copy of itself and each part is resembled to itself. The fractal objects can be continuously divided itself into small sections each of which is resemble. Furthermore, the shape of fractal is too irregular to be easily simply described in traditional Euclidian dimension. For classical geometry, shapes have integer dimension, like the dimension of a line is 1, the dimension of an area is 2, the dimension of the volume is 3. For the irregular shape, fractal geometry as an effective tool for dealing with the dimension of non-integer shape. To explain how this can happen, it is necessary to consider the meaning of dimension.

A typical fractal example in nature is measure the dimension of a coastline along the ocean shore. As the coastline is the irregularity and basically the same at all levels of magnification, when viewed from different scale. Mandelbrot uses data from a paper by Lewis Fry Richardson (Lewis Fry Richardson et. al 1954) who showed that the length of a coastline changes with scale, or, more precisely, with the length (resolution) of the measuring stick (ruler) used. Mandelbrot (Mandelbrot et al. 1982) discussed and reported the fractal dimension of such lines. For the west coast of Britain, Mandelbrot reported that D=1.25.

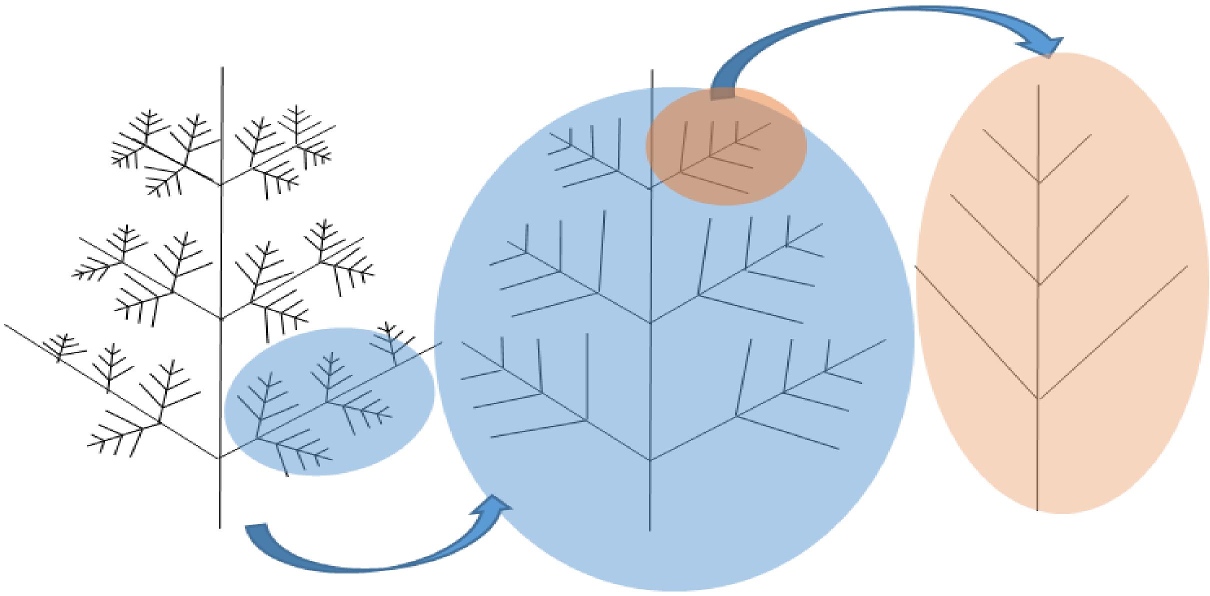


Fig.2.1 Tree branching structure

Other fractal examples in nature are rivers, cracks, mountains, and clouds. Figure 2.1 shows the tree branching structure. The repeating branch pattern that showed at different scale.

2.2 Applications of fractal geometry

Recently, fractal theory has been widely applied in various fields, and has achieved fruitful results in all aspects of physics, materials science, zoology, and economics imaging technology. As a newfound body of knowledge of the discipline of mathematics, fractal research has evolved globally and exponentially since the 1970's with profound conceptual applications in the fields of mathematics, chemistry, space science, earth science, engineering technology, environmental science, physics, computer science, medicine, and the arts.

The root structure of plants is very complicated and irregular, and the difference is very large between different plants, as it is strongly affected by environmental factors. Therefore, it is difficult to quantify the root system. With the plant growing, when the main root grows to a certain extent, the first lateral root will format. And so on, the secondary root, a third lateral root will grow. All the root branching is similar to the main root. Xia et al. (Xia et al 2011) reported that the root system of alfalfa with higher fractal dimension distributed in relative uniform soil layer, and the more biomass and well-distributed of roots in soil， the fractal dimension is comparatively larger. Ji et al. noted that with the root complexity (fractal dimension) increasing, the root growth activity as well as the length and amount of root branches are growing while the surface soil displacement is reducing. The complexity of root can reflect on the displacement field of soil slope(Ji etal. 2014). Xiao et al. through the statistical analysis on root system of Vetiveria zizanioides which have grown in poor soil for one year, the distribution laws of roots' number and cross-sectional area along the depth of soil layer were obtained. The analysis results show that both the number and the cross sectional area of roots decreased with the depth increase, but most of the roots distributed in deeper soil layer. Based on a series of indoor tests on the roots of V. zizanioides, the tensile strength characteristics which are related with the diameter and length of roots have been studied. The tested results indicate that, with the increases of diameter and length of roots, theirs tensile strength decreased gradually, and the effect of root diameter on tensile strength was more obvious. At the same time, the existing calculation model of roots tensile strength was improved, and the model parameters for V. zizanioides were given. The calculated results were in good agreement with those of the tested results. it is proved that the theoretical model is reliable, which provides a theoretical foundation for the research of shear strength of root-soil composite, so that this is also an essential prerequisite for stability analysis of plant slope. (Xiao et al. 2014). Li et al. showed that fractal dimension might be a substitutive index for complex traits such as crown and rooting characteristics (Li et al. 2014)

Further, it has been known that such root systems undergo changes with environmental conditions, for example, heavy metal pollution of the soil. Therefore, there is a need for a method to characterize the complexity of the root systems that is also sensitive to evaluate the environmental effects. To our knowledge up to now, no study has examined the root complexity under heavy metal stress using fractal analysis.

2.3 Calculating methods of fractal dimension

2.3.1 Area-perimeter

Two area-based methods can be used to determine the fractal dimension of linear features if those features form closed loops (Mandelbrot, 1975). The area-perimeter relation and Korcak's empirical relation for islands both have been used to determine the fractal dimension of lakes, contour loops, and islands. If the data is appropriate, these are relatively simple methods to use and their implementation is fairly simple, requiring few of the decisions most of the other methods demand. When using the area-perimeter relation, however, one must not confound the relation by using perimeters derived from sources of different scales (i.e., the area of a given feature will not change much when measured from sources of different scales, but the perimeter measurementscan change significantly).

Perimeters and areas of similarly shaped fractal geometries in two-dimensional space are related to one another by power-law relationships. The exponents obtained from these power laws are associated with, but do not necessarily provide, unbiased estimates of the fractal dimensions of the perimeters and areas.

Calculating the distance between each adjoining pair of pixels located around the region border. Area is determined by counting the pixels within the identified region, and the perimeter is for calculating the distance between each adjoining pair of pixels located around the region border. The relationship is expressed as follows,

$$A \propto P^{2/D} \qquad (2.1)$$

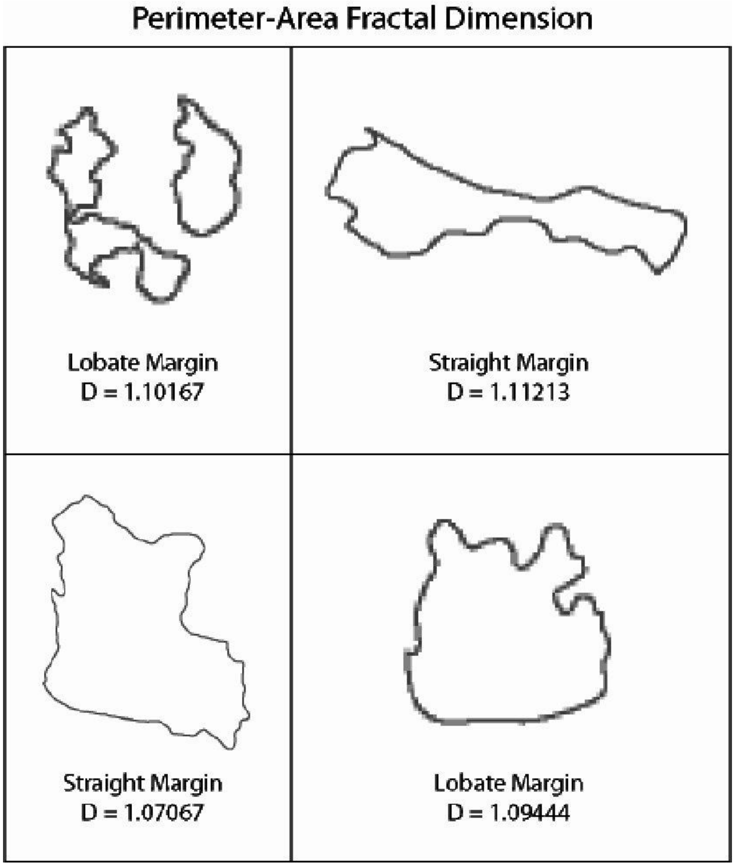


Figure.2.2 Examples of perimeter-area fractal dimensions

Figure 2.2 shows the examples of perimeter-area fractal dimensions A group of fractal shapes (landscapes, shoreline, cloud image) can be determined their dimension by perimeter-area method.

2.3.2 Power spectrum

Using spectral methods to obtain the fractal dimensions of features is another widely used method. Although a rigorous method, it is computationally difficult and computer intensive (Bartlett, 1991). This method requires much more data preprocessing than any of the other methods, all of which work with the data "as is." Spectral methods require the raw data to be detrended and tapered, and not doing so properly can greatly alter the results (Fox and Hayes, 1985; Cart and Benzer, 1991). They should only be applied to selfaffine curves (i.e,, profiles) since the method will always return a fractal dimension of one for self-similar curves (Peitgen and Saupe, 1988), Descriptions of the steps required to perform a spectral analysis for fractal purposes can be found in Huang and Turcotte (1989, 1992), Pentland (1984), Peitgen and Saupe(1988), and Turcotte (1992). An early application of the power spectrum method to the study of surface topography was reported in Sayles and Thomas (1978). Berry and Hanny (1978) subsequently placed those results into a fractal framework. It was not until Berryand Lewis (1980) that a formal link between the fractal dimension and the powerspectrum was provided, however. Finally, Mandelbrot et al. (1984) cleared upsome of the practical issues related to the method which had arisen in the literature.

Power spectrum method is using Fast Fourier Transform, identified the frequencies that provided the maximum power. The equation is expressed as,

$$P(\omega) \propto \omega^{-(5-2D)} \qquad (2.2)$$

Where,

P: Power

ω: Frequency

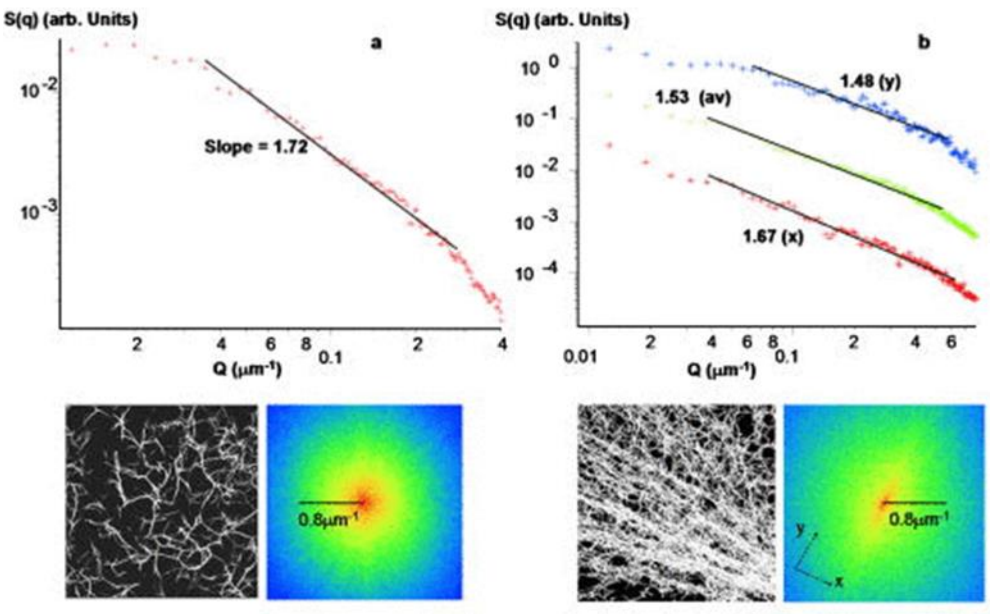


Fig. 2.3 Examples of power spectrum fractal dimensions

2.3.3 Divider relation

The divider method has long been used to determine the length of cartographic lines (Maling, 1992). Richardson's (1961) investigations into the scale dependencies of border lengths, one of the key building blocks in the development of Mandelbrot's concept of fractional dimensions (Mandelbrot, 1967),has justifiably become one of the more cited references in the divider method literature. Because of the ease with which this method can be implemented--using either physical or computational dividers--a large number of studies have used the divider method to determine fractal dimensions of features ranging from particle shapes to lava flows . This breadth of applications has resulted in a large number of independent reviews and a suite of contradicting recommendations . As an illustration of the isolated efforts which can be observed, some have even referred to this method as being a "relatively new" technique (Power and Tullis, 1991). The divider method can be implemented in a number of ways, but the basic implementation is to "walk" the divider along the line and record the number of steps required to cover the line. By systematically increasing the width of the divider and repeating the stepping process, the relation between step size and line length over a range of resolutions can be determined. Calculation of the fractal dimension follows . The equation is expressed as,

$$L(r)\,) \propto r^{-(1-D)} \qquad (2.3)$$

To begin with (Fig. 2.4), one employs a wide-range map, say 1:100 000. A map divider is then set to, say, 10 cm, fixed on one end of the line, then rotated n1 times until the other end is reached; the residual, if any, is ignored. (This is an inherent cause of error in the algorithm.) The first value of L is given by n1× 10 km. The divider is then reduced to a length of 5 cm and rotated n2 times: now L=n2× 5 km. This step is repeated M times, using increasingly shorter spreads and more accurate maps (1:50 000, 1:10 000, …).

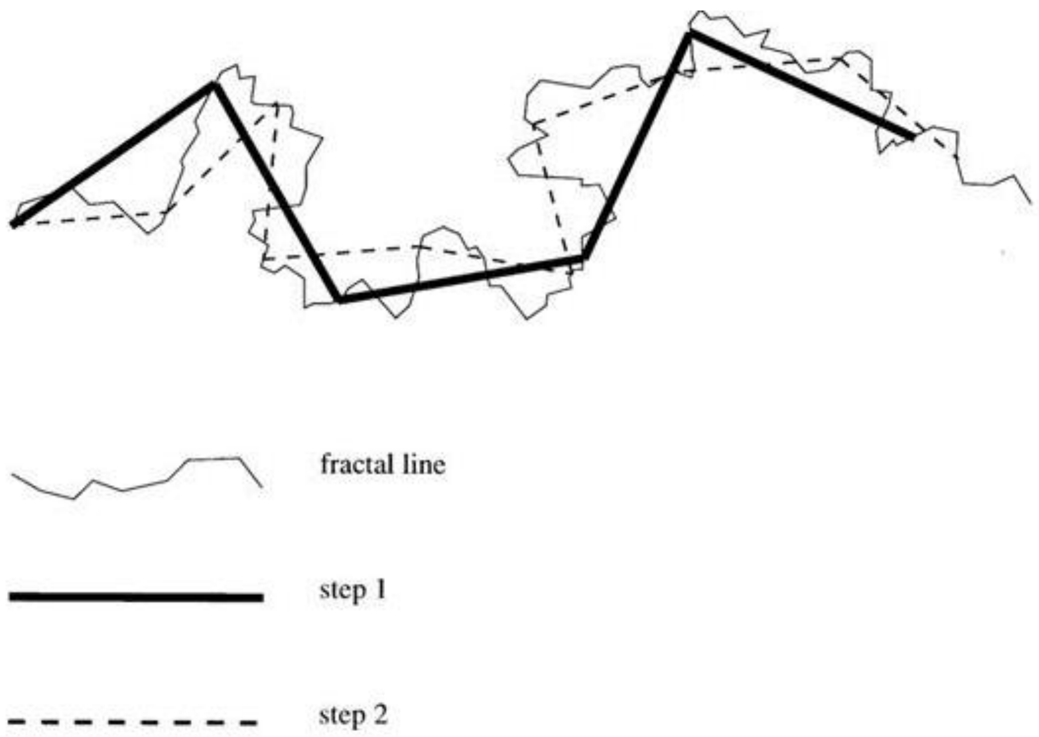


Fig.2.4 Examples of divider relation fractal dimensions

2.3.4 Box-counting

The box counting method is widely used to determine the fractal dimension of many different phenomena. Prior to its applications in fractal research, box counting was mainly used to quickly determine the area of irregular cartographic features (e.g., Gierhart, 1954; Maling, 1968). Since it can be applied with equal effectiveness to point sets, linear features, areas, and volumes, the box counting method is a widely used means of determining fractal dimensions. This method is also known as the grid or reticular cell counting method (Gagnepain and Roques-Carmes, 1986; Peitgen and Saupe, 1988), and has been shown to be equivalent to the Minkowski-Bouligond (or "sausage") dimension (Dubuc et al., 1989b). The basic implementation, using the determination of a linear feature as the example, is as follows:

1. Cover the feature with a single "box."

2. Divide the box into four quadrants, and count the number of cells occupied.

3. Divide each subsequent quadrant into four sub-quadrants and continue doing so until the minimum box size is equal to the resolution of the data, keeping track of the number of quadrants or cells occupied at each step. The fractal dimension is easily obtained from the log-log plot.

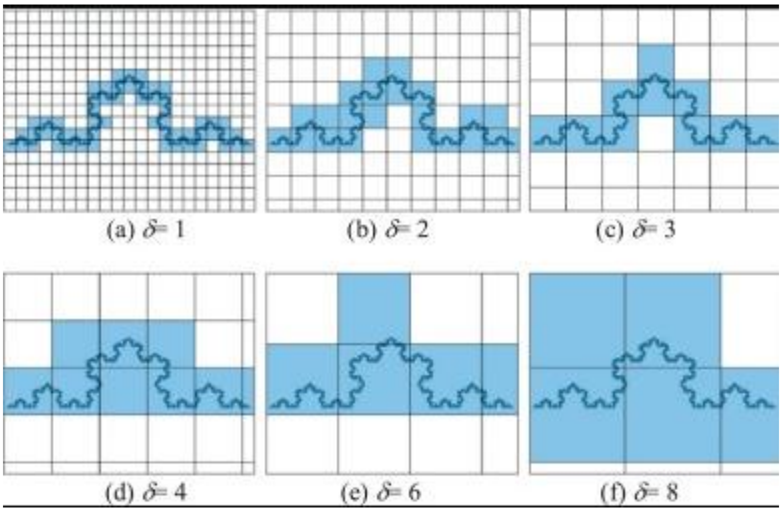


Fig.2.5 Example of box-counting method

The equation is given by :

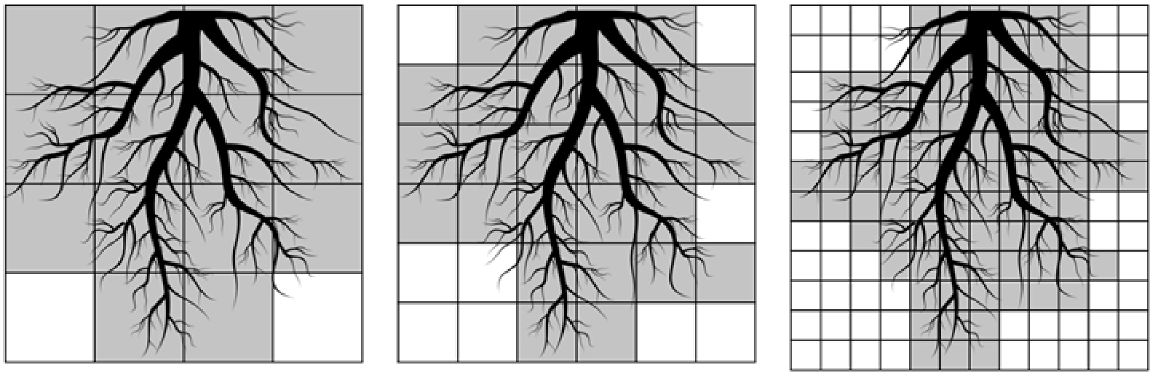
$$N \propto b^{-D} \tag{2.4}$$

Where, N: Numbers of filled box

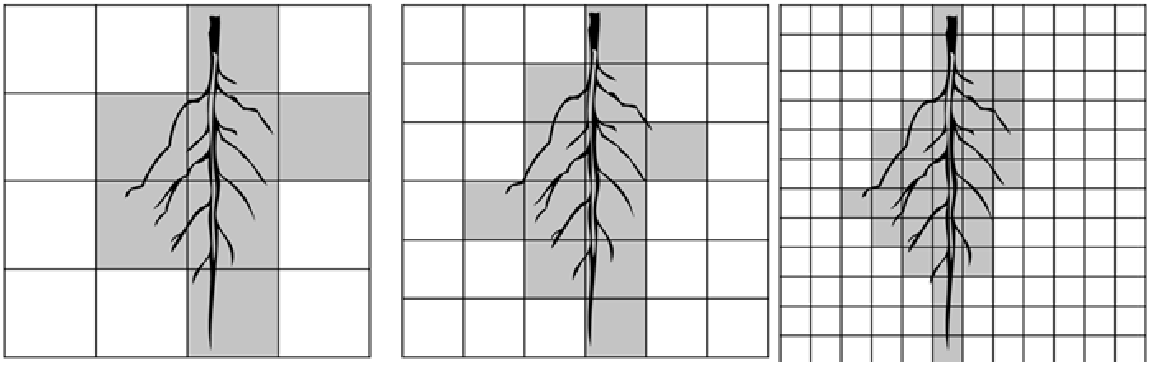
b: box size

In this study, I choose box-counting method. First, count number of occupied boxes N(r) under different r (grid size). Then, Plot log (occupied boxes) logN(r) vs log(1/grid side) log(1/r). Finally, we will get the regression line, as shown in fig 2.6. The slope of this regression line gives the fractal dimension. So, the fractal dimension is expressed as

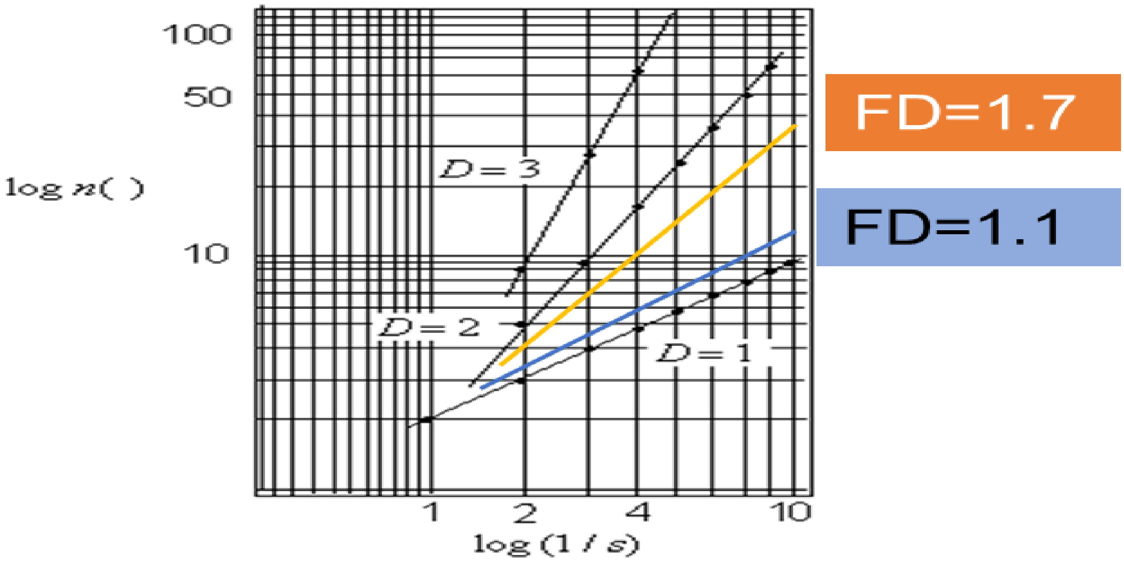
$$FD = \log(N(r)) / \log(1/r) \tag{2.5}$$



(a)



(b)



(c)

Fig.2.6 (a) Complexity root system using box-counting method;(b) Simple root system using Box-counting; (c) Regression line of all the cases

According to 1<FD<2, we defined complexity index (CI) as,

$$CI=FD-1 \tag{2.6}$$

$$RCI=\frac{\text{CI Post}}{\text{CI Pre}}x100 \tag{2.7}$$

As this way, the complexity index of line is 0, and the complexity index of area is 1.

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Chapter 3
Image processing

To characterize CI of the roots, photographs of the root systems were taken. To make photographs of the root system, at first, each of the entangled root sample was separated carefully and put in a transparent box. The box was filled with several millimeters depth of water so that the fine roots were clearly displayed. Next, the transparent box was put under a LED backlight panel. Photographs of the entire images of the root systems were taken weekly and digitized (5.72MB; 6000×4000pixels) with a digital camera (16.2 megapixels; Nikon D500, Tokyo, Japan). The images were acquired and saved in the JPEG format.

After digitization of images, they were smoothed to remove the spiky noise by applying a median filter (filter size=2), a commonly used filter to remove spiky noise while retaining the edges of the image undisturbed. Next, the images were converted into binary images through binarization processing.

The binarization involved converting grey scale images to a binary image through setting a threshold and creating output binary images to consisting of two values 1 and 0. All pixels in the input image with luminance greater than optimal threshold were set to a value 1 (white), while the remaining other pixels were set to value 0 (black). The original images scaled in the gray 0 to 255 were binarized with an adaptive threshold over the frame with pixels over the threshold value set to 1 and the rest of the pixels to 0.

The binarization was followed by the skeletonization processing. Skeletonization is an essential step to obtain the peeling off an object as many pixels as possible without affecting the general shape of the pattern. This peeling was repeated until the most interior layer was reached. All the image processing was developed in MATLAB (MATLAB R2017b).

To estimate the FD of the root systems, binarized images were skeletonized and skeletonization is an essential step. The skeletonized images were obtained according to the box-counting method as described by Tatsumi et al. During the skeletonization process, skeleton images were obtained through peeling off as many pixels as possible of the object without affecting the general shape of the object pattern. The peeling off was repeated until the most interior layer was reached. Different steps of image processing such as filtering, binarization and skeletonization were custom developed using MATLAB (MATLAB R2017b).

During estimation of FD, different scaled grids were applied over the skeletonized image, and the number of pixels that contained parts of the root image was counted as a function of the size of grid size as shown in Fig.3.1. Through this procedure two values were calculated, one the number of squares covered by the image N(r) and another is the grid size (r) or the length of the squares of the grids.

To calculate FD, first, the number of squares covering the root image N(r) was plotted against (r) in log-log scale. Next a linear regression fitting was done to calculate the slope D or the fractal dimension FD of the image, as expressed by,

$$FD= \log N(r)/\log (1/r).$$

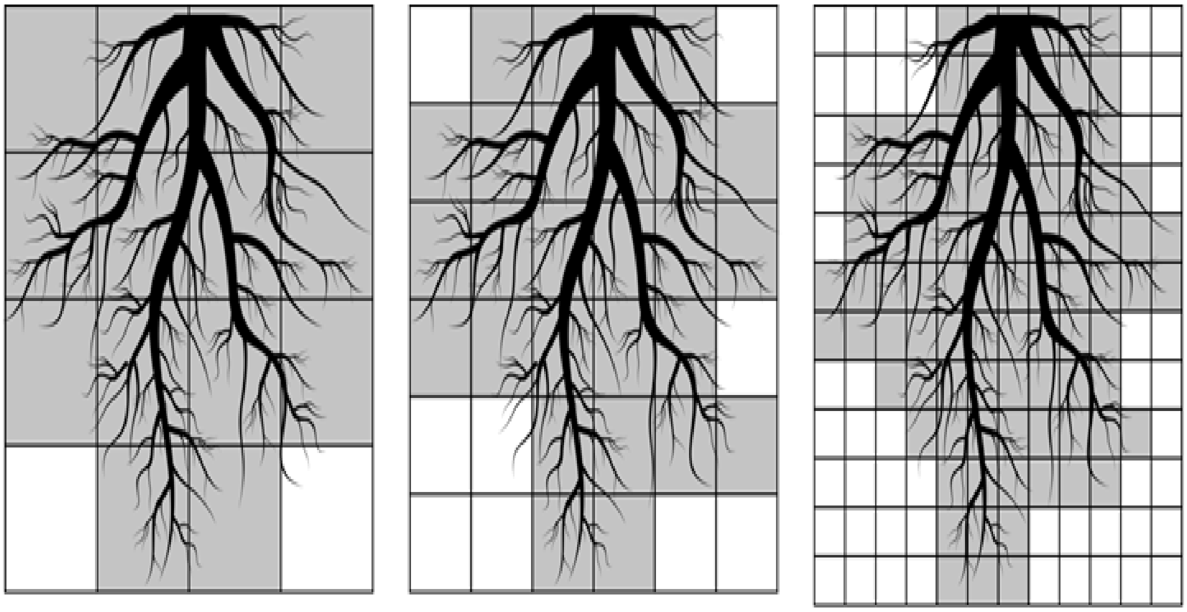

Fig. 3.1 Different size grids for root system.

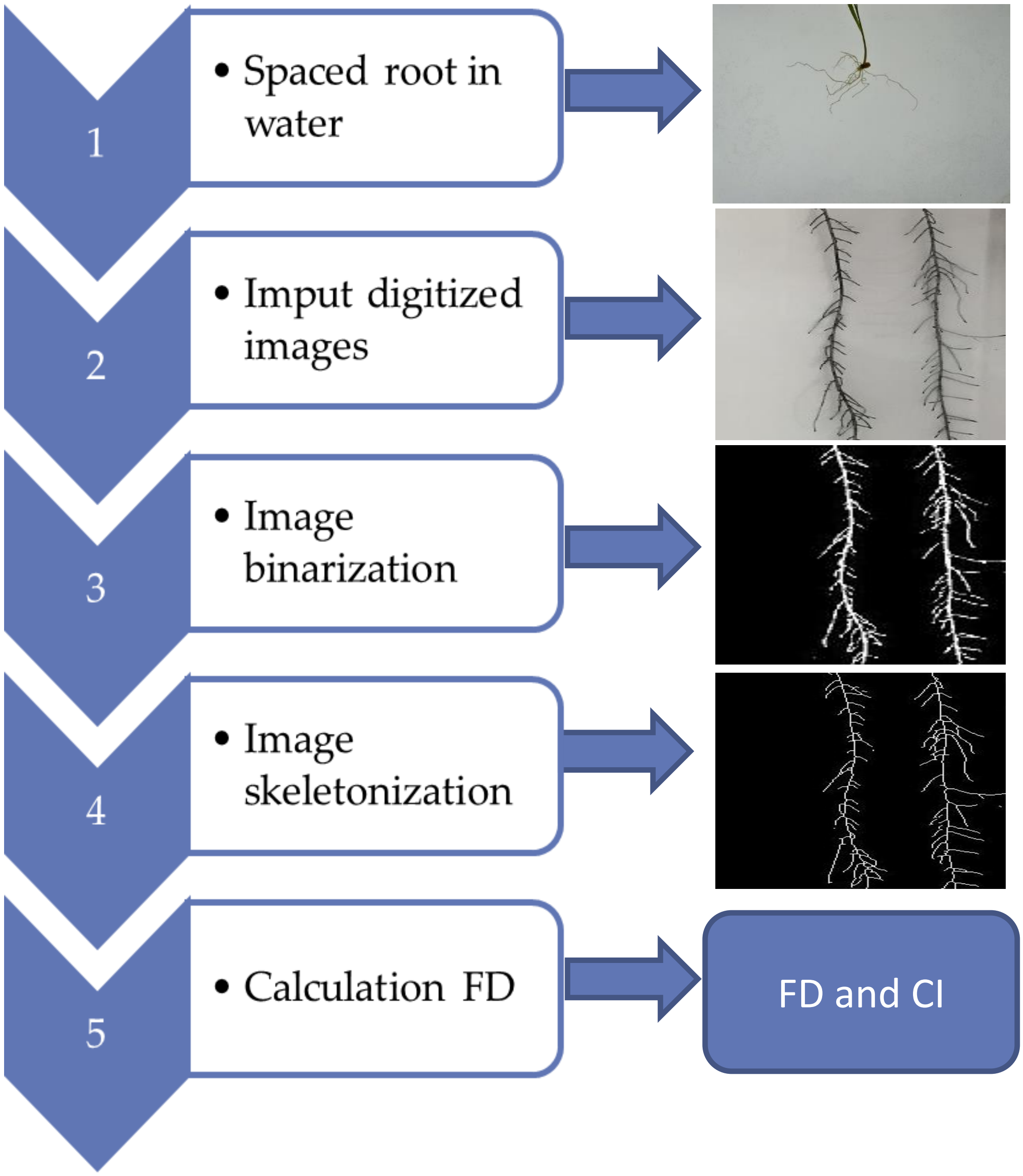

Fig. 3.2 Image processing flow chart. The images of root systems were digitized by using camera, and skeletonized following the binarization as an image preprocessing for fractal analysis.

CI indicates the degree of complexity of the image. The FD takes unity for a simple line object and increases towards 2 as the complexity of the line object increases, (1<FD<2). Therefore, FD being one would mean a simple root having one dimension or a line, a value larger than one would indicate the increasing complexity of the root system.

The complexity of the wheat root system under different Cd concentration were measured using this method. A flow chart of the procedure of the calculation of FD and CI is given in Fig.3.2.

Chapter 4
Negative effect measure by CI under cadmium stress

4.1 Introduction

Cd, Unlike Zn and Cu, is non-essential and potentially toxic for higher plants, animals and humans (Yadav. 2010). Cd is recognized as an extremely significant pollutant due to its high toxicity and large solubility in water (Pinto et al., 2004). Cd is toxic to most organisms and a potential threat to human health. People with long-term exposure to Cd through air, water, soil, and food will be leaded to cancer and organ system toxicity such as skeletal, urinary, reproductive, cardiovascular, central and peripheral nervous, and respiratory systems (Rahimzadeh, 2017).

Crops and other plants take up Cd from the soil or water and may enrich it in their roots and shoots. Cd-induced effects include oxidative stress, genotoxicity, inhibition of the photosynthetic apparatus, and inhibition of root metabolism (Andresen and Küpper, 2013.). Cd plays a major role in inhibition of plant growth by accumulation in plant (Emamverdian and Ding, 2017). Resources of Cd in nature are volcanic emissions and weathering of rocks (Fontanili et al., 2016). Soils contains Cd naturally at trace amounts in soil (0.8-3.5 mg kg-1 soil), however, anthrophonic activities such as mining, polluting water, and using fossil fuels, application of phosphate fertilizer and sewage sludge can input large quantities of Cd to soils, causing soil pollution (Ahmad et al., 2016). Cd is a mobile heavy metal that can be transferred easily between plants. Cd has strong toxicity and is one of the most dangerous heavy metals (Lin et al., 2016). Itai-Itai disease is one of the well-known diseases caused by accumulation of Cd in plants (Suda and Makino, 2016). Cd in plants can intervene in plant chemical synthesis processes such as ammonification, nitrification, DE nitrification, and microbiological process that affect the quantity and quantity of the crop products (Cojocaru, 2016,). Cd in leaf leads to leaf chlorosis (Lin et al., 2016), photosynthesis inhibition with the decline of pigment content, chlorophyll a, and phycobiliproteins (Simek et al., 2016), and then reduce the plant biomass. Effect of Cd on P. Flagellifera showed that the excess amount of Cd could decrease the plant growth and photosynthesis pigment, damage thylakoid membranes, and disturb the cell wall activity (Simonetti, 2016), chlorophyll content, and stomata size of Schinus molle trees (Pereira et al., 2016). With increasing 100 mg kg-1 of Cd, the plant growth approximately decreased 37.6 % in P. Orientalis and 40.6 % in J. Chinensis (Guo et al., 2016; Emamverdian and Ding, 2017). Stomatal Opening, Transpiration and Photosynthesis is caused by Cd, and Chlorosis, Leaf Rolls and Stunting are the main symptoms of cadmium toxicity in plants (Sandalio et al., 2001). Cd also reduced the absorption of nitrate and its transport from root to shoot by inhibiting the Nitrate Reductase activity in shoots (Hernandez et al., 1996). An oxidative stress may be involved in Cd toxicity, by either inducing oxygen free radical production, or by decreasing enzymatic and non-enzymatic antioxidants (Sandalio et al., 2001).

The inhibiting effect of Cd on plant mass accumulation, height, root length, leaf area, and other biometric parameters are reported in almost all investigations. The phytotoxic symptoms observed includes: root browning, leaf red-brownish discolouration, leaf epinasty and leaf chlorosis (Vassilev and Yordanov, 1997). The symptoms of phytotoxicity were expressed more clearly in roots as the significantly higher heavy metal accumulation is in roots (Foy et al., 2005). The negative effect of Cd on plant growth was accompanied by an increase in dry to fresh mass (DM/FM) ratio in all organs (Moya et al., 1993). Cd pollution is one of the most serious environmental problems and there were approximately 20 million hectares of cultivated lands contaminated by Cd (Liu et al., 2015). Although our knowledge of Cd toxicity in higher plants has increased considerably in the recent years, there are still many gaps about the basic mechanisms that control Cd movement and its accumulation in plants. More research is needed regarding the mechanism of Cd uptake by the root, translocation, and its deposition within plants. Study indicates that the barley roots were the main sites of cadmium uptakes, Cd ions initially entered elongation zone cells and were accumulated in this area. Then, it extends up to mature zone and down to meristem zone gradually. After Cd ions entered meristem zone, it disturbed the mechanisms controlling the organization of microtubule (MT) cytoskeleton and tubulin assembly/disassembly processes. The toxic effects resulted in a decrease of mitotic index and inducement of abnormal mitosis, inducing aberrant chromosomes, such as C-mitosis, anaphase bridges and chromosome stickiness. So, MT cytoskeleton can be thought to be one of target sites of cadmium toxicity in root tip cells of plants (Shi et al/ 2016). With the recent rapid development of human activities, soil contamination due to heavy metals has become a common problem in both the fields of agriculture and environment. Such heavy metal pollution was mainly caused by mining and smelting, urban industry, and the use of agrochemicals. Pollution could be harmful to the development of the root system of the plant, and therefore, there is a requirement for quantitative evaluation not only for the complexity of the root architecture but also for the changes in the complexity with changes in the environment. In particular, the soil contamination due to the heavy metals such as cadmium (Cd) could have negative effects on the root system. In the last decade, with the development of industry, environment contamination by heavy metal has become an increasing global concern as their widespread distribution and toxicity to living things. In particular, heavy metal toxicity has been found to affect root formation other than grain yield, seed formation, chlorophyll synthesis, hormone proteins and membrane functions. Cd has been shown to affect lateral root formation and root development (Ronzan et al., 2018). Cd toxicity has been found to affect growth, water stress, transpiration, photosynthesis rate, chlorophyll content, yield reduction, nutrient imbalance, and oxidative stress [4]. Such effects have been found to be largely due to the accumulation of Cd within the root tissue with a little translocated to the shoots of the plants. Therefore, with Cd toxicity largely affecting root growth and its morphology, there comes a need for evaluating the root growth and its complexity.

Wheat (Triticum.spp.) is the most widely grown crop in the world, as itis the first strategic cereal crop for a majority of populations and is the second most important food crop in the developing world after rice. It has been known that, the heavy metals such as Cd could accumulate in high amounts in the roots of wheat plants

In this chapter, the immediate influence of Cd on wheat root system is discussed with the results from CI and compare the results obtained by fractal geometry with conventional technique measurements. To investigate the negative effects of Cd on the complexity of the wheat root development, we applied fractal geometry and calculated the complexity index. CI was calculated using the box-counting method.

The objective of this experiment is

1) To examine developmental responses of the root system under Cd exposure based on fractal geometry.
2) Comparison of the CI measurements with conventional measurements.

4.2Meterials and methods

4.2.1 Plant materials and growth conditions

Wheat (Triticum.spp.) is a popular monocotyledonous plant, which is the second most-produced and largely consumed cereal worldwide. In this experiment, Norin 61 wheat cultivar waschosen as the plants for study. As samples, we chose equal-sized seedlings one week after germination and photographed every week. In order to avoid root destruction and damage when removing from the soil, the seedlings were grown in a hydroculture system (12L). This was done to avoid root destruction and reduction when removing the plant from the soil. For keeping the wheat seedlings under healthy conditions, the plants were watered with nutrient solution for three times a week. All the plants were grown in a growth chamber (Conviron, Controlled Environmental Led, Winnipeg Manitoba, Canada) under fully controlled environmental conditions of 12h photoperiod, 27℃ day, 20℃ night, relative moisture of 65%-75% and light intensity of 260-350 µmolm^{-2s-1}.

According the Environmental Quality Standards for Soil Pollution of Japan, the standard value of cadmium (Cd) concentration is 0.01 mg/l (≈ 0.000889mM Cd in the form of $CdCl_2.H_2O$) [13]. For this reason, four different concentrations, namely, 0, 0.001, 0.01 and 0.05mM of Cd in the form of $CdCl_2$ were chosen. In all of the experiments, a total of sixty samples with fifteen samples for each concentration were used. The root samples were taken weekly by exposing their root to cadmium chloride ($CdCl_2$) solution for over three weeks. Four plant sets were prepared in total with one set being control, and the other three being under Cd stress with six replicates for each of the four Cd concentrations.

4.2.2 Complexity measurements

A total of four plant systems consisting of sixty samples with fifteen samples for each concentration of Cd was prepared with one under control, and the other three under Cd stress. For Cd exposure growth conditions, root samples were exposed to cadmium chloride ($CdCl_2$) solution for over three weeks after germination and six replicates for each of the four concentrations of 0.001, 0.01 and 0.05 mM were prepared. For control condition or under Cd concentration of 0 mM, a total of fifteen samples was prepared.

To characterize FD of the roots, photographs of the root systems were taken. To make photographs of the root system, at first, each of the entangled root sample was separated carefully and put in a transparent box. The box was filled with several millimetres depth of water so that the fine roots were clearly displayed. Next, the transparent box was put under a LED backlight panel. Photographs of the entire images of the root systems were taken weekly and digitized (5.72MB; 6000×4000pixels) with a digital camera (16.2 megapixels; Nikon D500, Tokyo, Japan). The images were acquired and saved in the JPEG format.

To estimate the complexity of the root systems, binarized images were skeletonized and skeletonization is an essential step. The skeletonized images were obtained according to the box-counting method as described by Tatsumi et al [14]. During the skeletonization process, skeleton images were obtained through peeling off as many pixels as possible of the object without affecting the general shape of the object pattern. The peeling off was repeated until the most interior layer was reached. Different steps of image processing such as filtering, binarization and skeletonization were custom developed using MATLAB (MATLAB R2017b).

Fractal dimension was calculated from the root system images using box-counting methed. During estimation of FD, different scaled grids were applied over the skeletonized image, and the number of pixels that contained parts of the root image was counted as a function of the size of grid size as shown in Fig.2.6. Through this procedure two values were calculated, one the number of squares covered by the image N(r) and another is the grid size (r) or the length of the squares of the grids. The t-test was performed to analysed the statistical significance of the SD of the FD (Fractal dimension) and CI (Complexity Index)under different concentrations of Cd.

4.2.3 Traditional measurements

Root length and weight measurement are common conventional measures of heavy metal effect of plant root system. In order to make weight measurements, at first, the plants along with the root systems were removed carefully from the hydroculture solution. Next, they were washed in distilled water. After washing the roots, they were stretched and the lengths were measured. The fresh weights were then measured for each plant. Also, the dry weight of root was determined after drying at 105℃ first 30min, followed by keeping them at 70℃ for 72h until the weight became constant. The dry weights (DW) of roots and shoots were determined using a SHIMADZU AUX320 analytical balance. Relative weights (RW) and relative root elongations (RRE) were defined to evaluate the changes due to exposure to Cu. The definitions are as follow;

$$\text{RRE\%} = \frac{\text{Length after exposure}}{\text{Length prior exposure}} \times 100,$$

$$\text{RW\%} = \frac{\text{Weight after exposure}}{\text{Weight prior exposure}} \times 100.$$

Also, the length of primary root was analysed by Image J software (Version1.52) under four concentrations of 0.001, 0.01 and 0.05 mM. NeuronJ is an image J plugin to facilitate the tracing and quantification of elongated image structures. Fig.4.1(a) showed the skeletonized root image, and after manual tracing by NeuronJ step, the coloured primary root in Fig.4.1(b) will be used for root length measure.

(a) (b)

Fig.4.1 Skeletonized root image of root system (a) and tracing using Image J(b)

4.3 Results and discussion

4.3.1 Complexity index and FD

Figure 4.2 shows the changes in the skeletonized images of root system at each week for three weeks under different concentrations of Cd, 0, 0.001, 0.01, and 0.05mM. Under control or 0mM of Cd, with passing of each week, the root systems do not only get longer in length as could be seen from the root length measurement, but also there is more lateral branching of the roots. Therefore, as expected for a healthy plant, the complexity of the root structure develops with time.

In contrast, growing the plant under $CdCl_2$, there is a clear decrease in the length of the root which is prominent under high concentration of Cd. Further, there is a significant decrease in lateral branching even within a week for smaller concentration of 0.001mM Cd. This effect of decrease in lateral branching with increasing exposure and also increasing concentrations becomes larger for 0.01mM and 0.05mM and for second and third weeks. Therefore, exposure to even smaller amounts of Cd largely reduces the lateral branching while the length of roots is affected severely under large concentrations of Cd, 0.05mM and longer exposure time of three weeks. The effect of damage to roots could also be seen in the overall growth of the plant as shown by the photographs of the plant samples taken under each of the conditions.

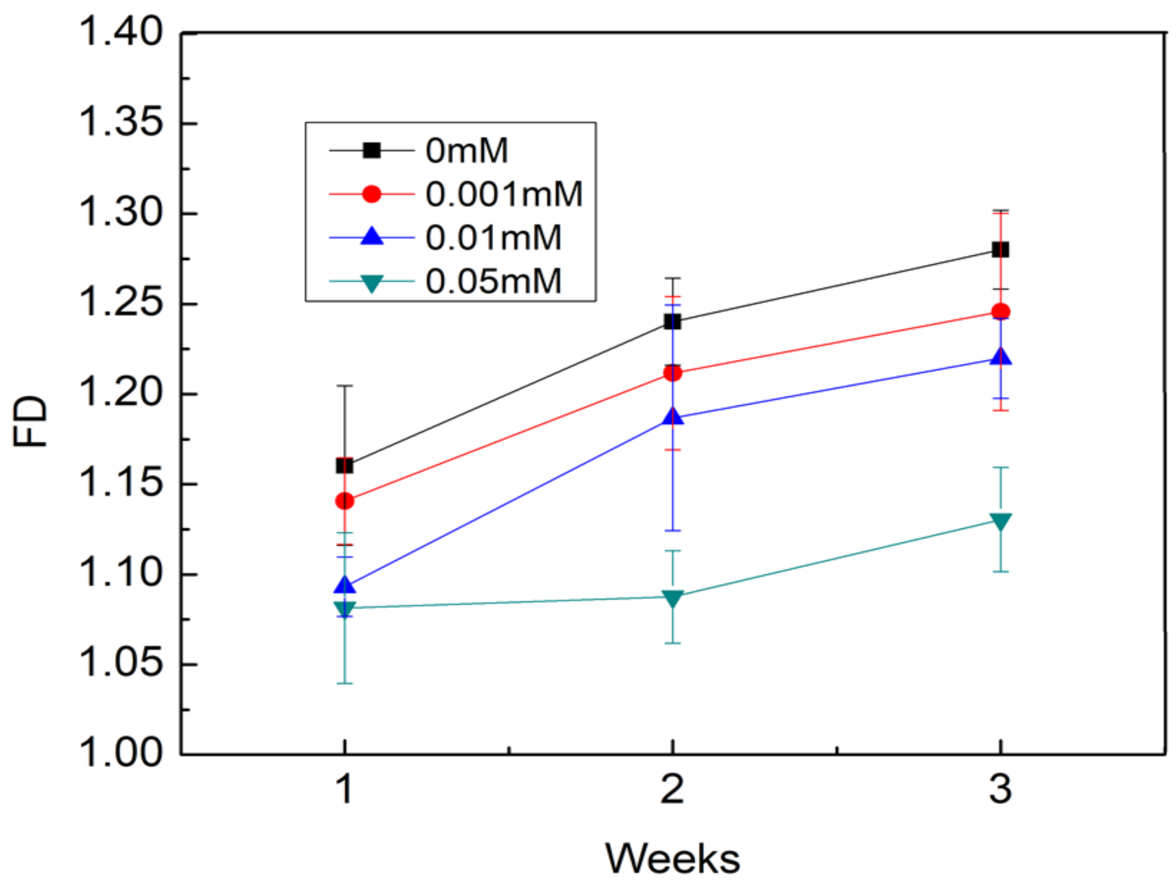

Fig.4.2 FD for wheat root skeleton images for different Cd concentration

From the skeletonized images of Fig.4.3, FD were calculated by box-counting method. For the first week, FD values ranged from 1.16 for control to 1.08 for 0.05mM concentration of Cd indicating that the changes were small even for large concentrations of Cd (Fig.4.2). However, during second week, the effects of Cd started to appear clearly. The FD value of over 1.24 for control drastically decreased to around 1.09 for 0.05mM with distinct reductions seen for other Cd concentrations also. Again, a similar reduction tendency can be seen for third week for all Cd concentrations. The reduction percentages of FD and RCI were found to be respectively, 12%, 21%, and 53% for Cd concentrations 0.001, 0.01, and 0.05mM, respectively. Further comparing second and third weeks, the reduction percentages were found to be small as compared to those between the first and second weeks. Statistical analysis showed significant difference ($p<0.05$) between the control and all of the FD and RCI under all Cd concentrations. The results imply that the fractal dimension can be an effective measure for the structural development of the root system.

Results of RCI over 3 weeks under four Cd concentrations are shown in Fig.4.4 for each week. As seen from the figure, compared with the control shown in black square, there were clear reductions in FD under the presence of Cd with increasing exposing weeks. In addition, FDs decreased with increasing concentrations. Effect of different Cd concentration of on RCI was given in Figure 4a. The RCI was maximum in control condition for each week. The significant reduction in RCIs can be seen even within the first week, that for the lowest concentration of 0.001 mM and the reduction percentage was 12.5%. For second and third week, the reduction percentages compared with control were found to be respectively, 12.4% and 10.7% for 0.001 mM concentration. The highest reduction in RCI compared to control is for Cd concentration of 0.05mM, and the reduction percentages for each week were 50.0% 62.5% and 64.3%, respectively. Therefore, the highly toxic effects of Cd can be clearly seen through the measure of RCI even for small concentrations of 0.001mM of Cd. Statistical analysis showed significant difference ($p<0.05$) between the control and all of the RCI under all Cd concentrations.

Fig.4.3 Skeleton images for different Cd concentrations obtained for three weeks

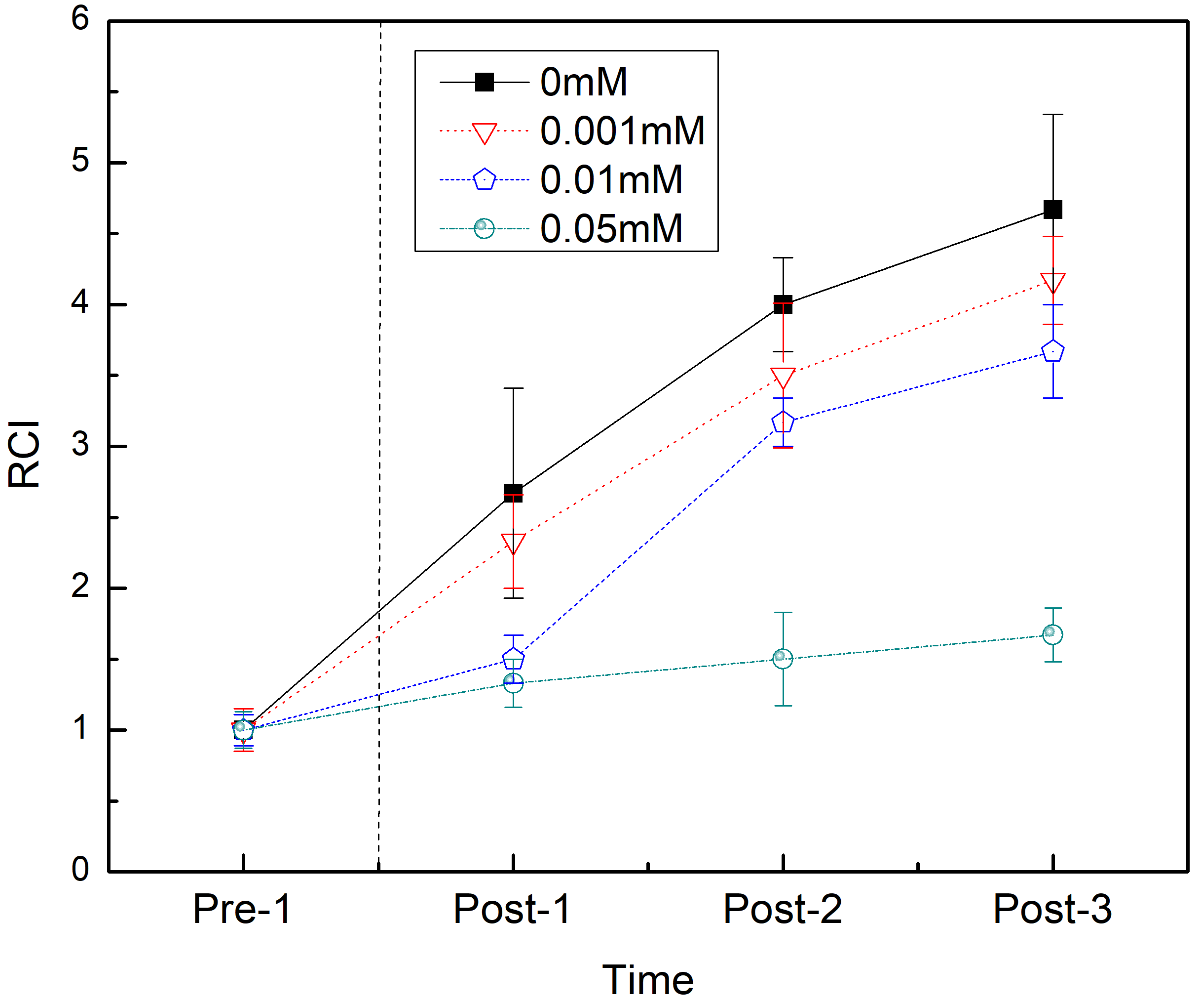

Fig.4.4 RCI for wheat root skeleton images for different Cd concentration

4.3.2 Influence of Cd on root length(Ruler and image J)

Measurement of root length by meter scale is a common measure that is conventionally used. At first, root lengths were measured on removing the roots of wheat plants out of water from the culture. The measurements were done to an accuracy of 0.1mm under both conditions of control and different concentrations of Cd. Results of length measurements over 3 weeks under four different Cd concentrations, 0, 0.001, 0.01, and 0.05mM are shown in Fig.4.5. As the Cd concentration increases, it can be seen that the root became shorter as compared to that under control (0mM) with the passage of each week due to the influence of Cd.

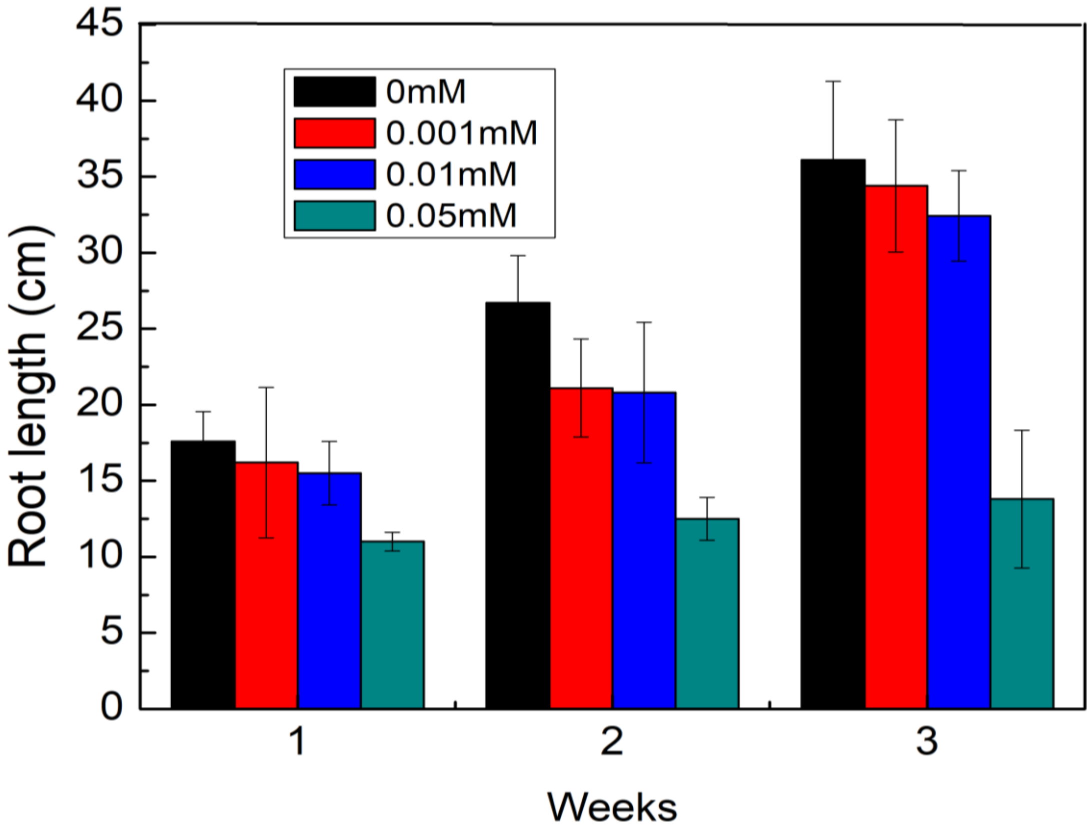

Fig. 4.5 Root length (using ruler) change under Cd stress

A significant root length difference root length change ($p<0.05$) could be observed only under 0.05mM Cd. The decrements in root length were found to be 33%, 53%, and 64% first, second and three weeks, respectively as compared to the control. Under 0.001 and 0.01mM concentrations, there were no significant differences compared with those of control for all three weeks.

The current experimental results obtained with wheat plants also indicate that root growth was strongly influenced by the presence of cadmium in the culturing medium. This agrees with the report of Arduini et al. They noted that the number and length of the lateral roots of Pinus pinaster were affected by Cd^{2+} treatment to a higher degree and higher concentrations of Cd can inhibit root growth.

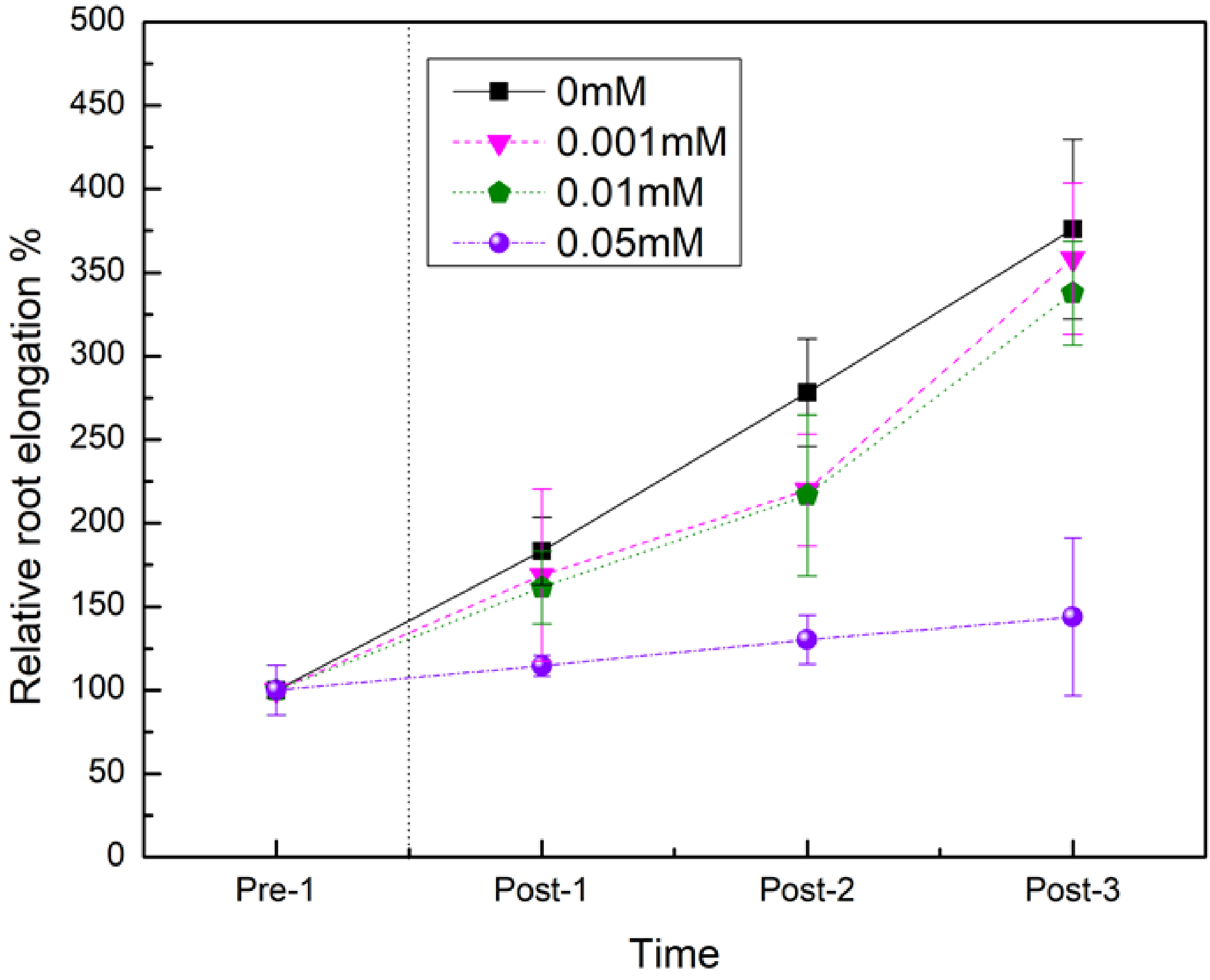

Fig. 4.6 Relative root length (using ruler) change under Cd stress

Relative root elongation (RRE) changes of wheat over 3 weeks under four Cd concentrations, 0, 0.001,0.01 and 0.05 mM are shown in Fig.4.6. Under all Cd concentrations inhibition of RRE increment could be found indicating the toxic effect of Cd on plant health. Statistical analysis showed significant difference ($p<0.05$) between control and 0.05 mM exposure for third week, the reduction percentages were 37.5%, 53.2% and 61.8% for each week, respectively.

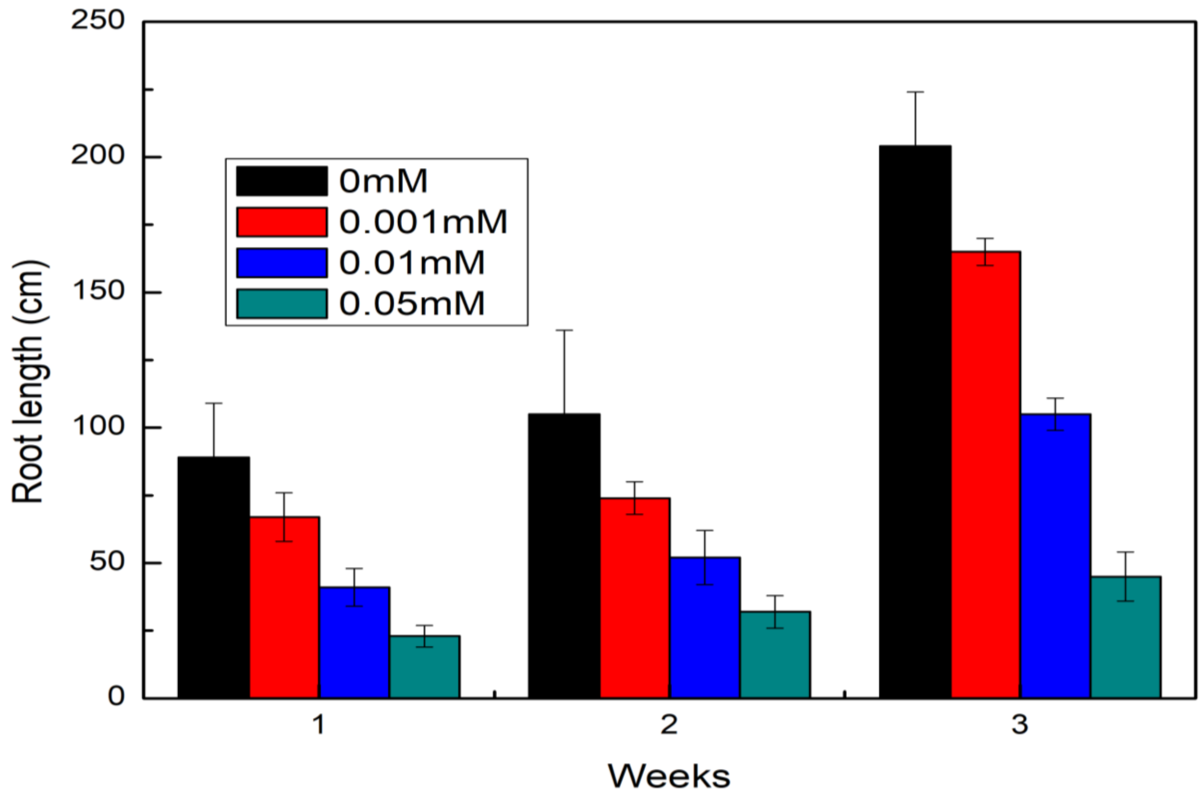

Fig. 4.7 Root length (using Image J) change under Cd stress

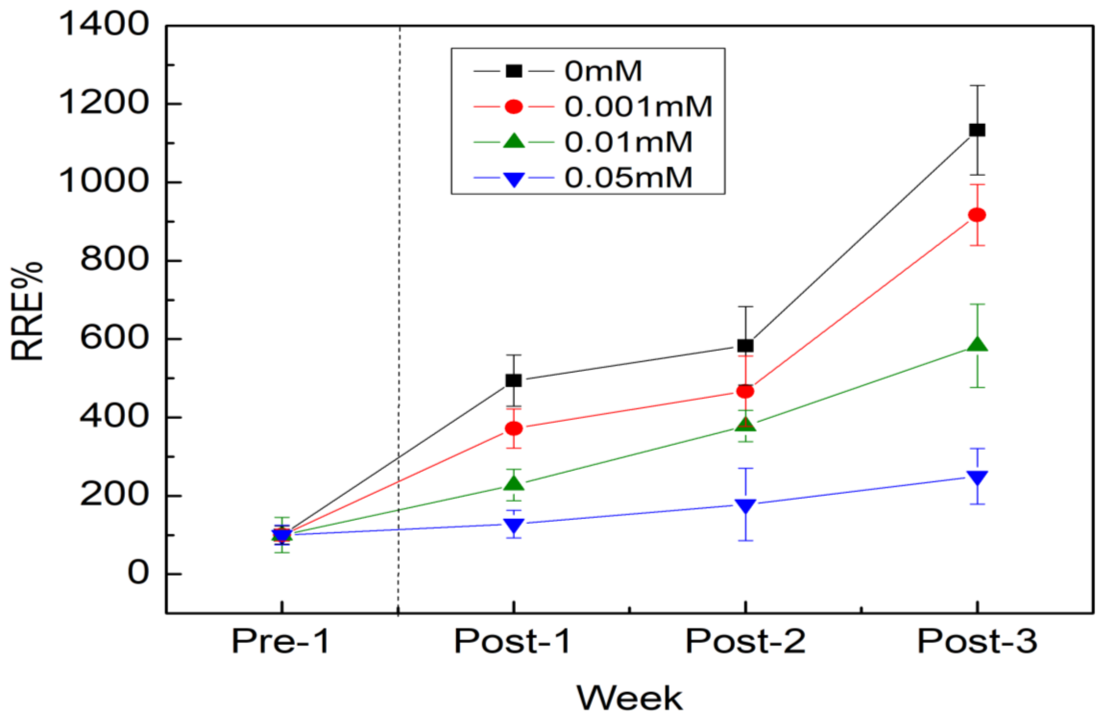

Fig. 4.8 RRE (using Image J) change under Cd stress

A significant difference of root length (using Image J) change ($p<0.05$) could be observed under all Cd concentrations(Fig 4.7 ,Fig 4.8). Under the lowest concentration 0.001mM, the decrements in root length were found to be 25%, 20%, and 19% first, second and three weeks, respectively as compared to the control. For the case of the highest concentration 0.05mM, the decrements in root length were found to be 74%, 70%, and 78%. However, the manual tracing method is tedious and time-consuming compare with fractal method. For one image it takes half an hour or hours even for primary root. When doing the process of manual tracing, the mouse-clicking by the user could be erroneous.

4.3.3 Influence of Cd on root weight

The conventional measurements of root weights were also done under different concentrations of Cd. Results of root dry weight of wheat treated with four different Cd concentrations are shown in Fig.8 for three weeks. The dry weight decreased as the Cd concentrations increased from 0.001 to 0.05mM under each of the three weeks. Similar findings that Cd significantly reduced root and stem dry weight were obtained by Zhang.

For all concentrations of Cd stress, the largest root dry weight was found under control condition, and the lowest weights were found for 0.05mM Cd. The reductions in the root dry biomass exists between treatments of Cd at second and third weeks were statistically significant ($p<0.05$) for the highest concentration of 0.05mM. At the other concentrations, the changes in the dry weight were found to be less significant. This result demonstrates that the dry weight cannot measure changes under concentrations less than 0.05mM.

In comparison, as demonstrated from Fig.4.9, FD can be sensitive to even smaller changes in Cd concentration of 0.005mM and this could be also seen in the lessening complexity of the root systems with increasing Cd concentrations.

Therefore, compared to the dry weight, FD could be used as a more sensitive measure to evaluate Cd stress on the root system of wheat plants.

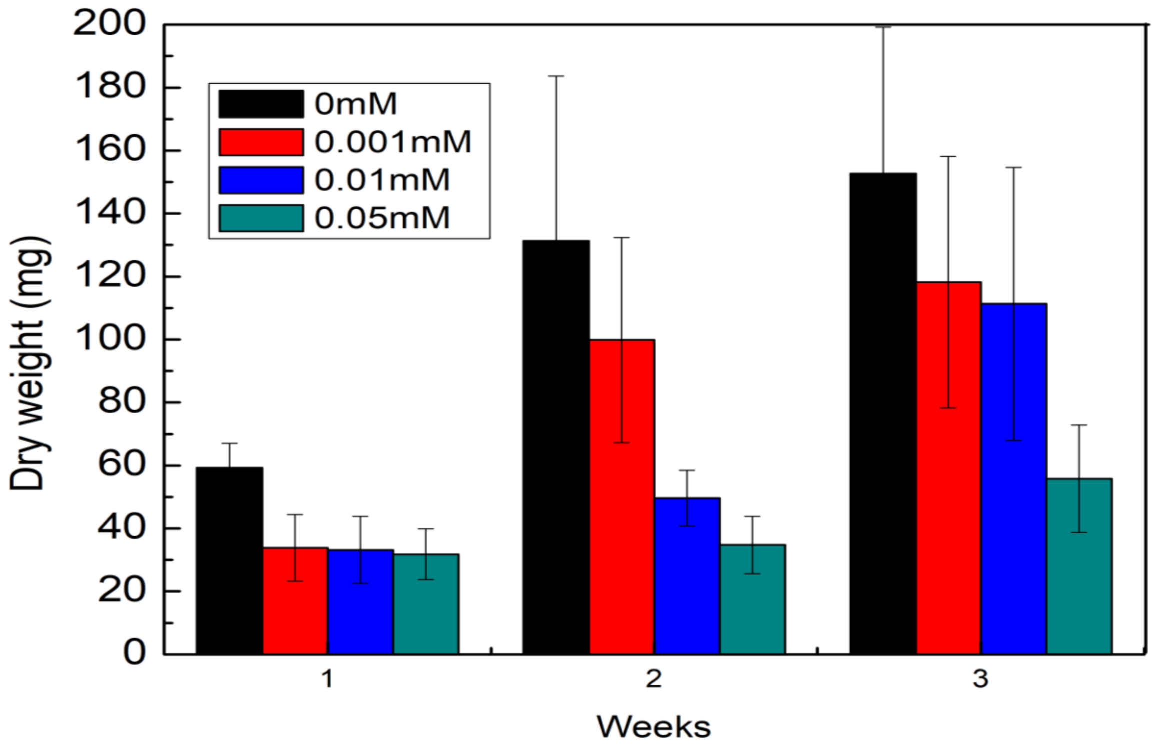

Fig.4.9 Dry weights of root for different Cd concentrations

The effect of Cd on root relative dry weight of wheat varied with four concentrations are shown in Fig.4.10. The addition of Cd 0.001,0.01 and 0.05mM, led to a decrease in relative root weight comparison to control can be seen with increasing concentrations of Cd for wheat.

For each week, the largest root biomass was observed under control condition, and the lowest root biomass was observed under highest Cd concentration (0.05 mM) after exposure to Cd. But significant changes could not be found under lower Cd concentrations of 0.001 and 0.01 mM on whole duration of experiment. The significant reductions were found to be respectively, 73.5% and 63.5% for 0.05 mM at post-2 and post-3 week.

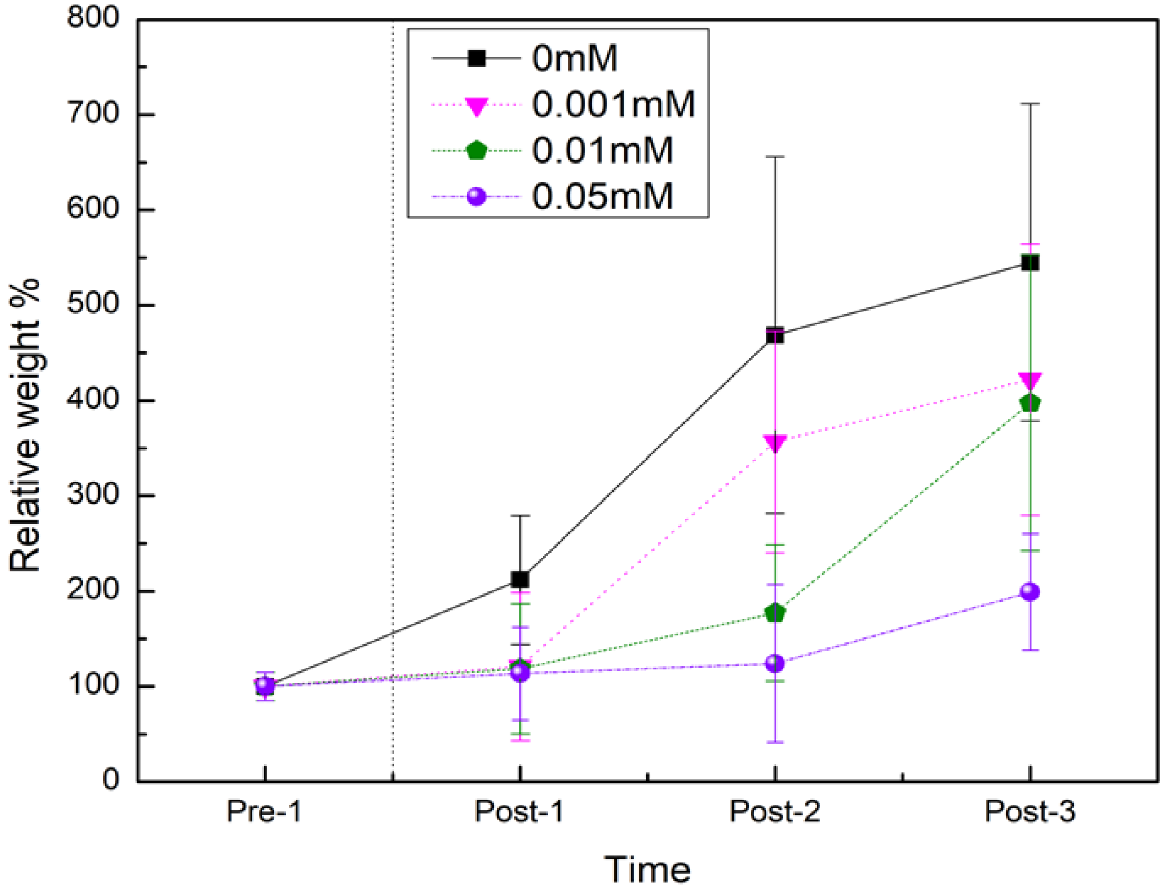

Fig. 4.10 Relative weight change under Cd stress

Our results using FD as a measure suggest that the roots were affected from an earlier stage of heavy metal exposure and start to accumulate more heavy metals in themselves than the other organs. Therefore, the root complexity or FD that includes the lateral root formation can be used as a measure for reflecting heavy metal tolerance of plants. This in line with the study of Jadia et al. who demonstrated that there was a reduction in the formation of secondary roots and number of root hairs due to exposure of Cd at 40ppm (0.36mM) and 50 ppm (0.44mM).

4.4 Conclusion

In comparison, as demonstrated from Fig.4.4, RCI can be sensitive to even smaller changes in Cd concentration of 0.005mM and this could be also seen in the lessening complexity of the root systems with increasing Cd concentrations. Therefore, compared to the dry weight, RCI could be used as a more sensitive measure to evaluate Cd stress on the root system of wheat plants.

In this study, the fractal dimension was used to evaluate the root architecture of wheat under Cd stress. The results imply that (1) fractal geometry could be a new approach to analyse the root architecture or complexity, (2) RCI/FD can be an effective measure for evaluating change in the complexity of the structural development of the root system under heavy metal stress, and (3) RCI/FD was found to be more sensitive to reflect the influence of the heavy metal than the conventional measures of length and dry weight.

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Chapter 5
Negative effect measured by CI under Cu stress

5.1 Introduction

Copper (Cu) is considered as a micronutrient for plants (Thomas et al., 1998) and plays important role in CO_2 assimilation and ATP synthesis (Yadav, 2010). Cu is also an essential component of various proteins like plantacyanin of photosynthetic system and cyto-chrome oxidase of respiratory electron transport chain (Demirevskakepova et al., 2004). But enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Cu is also added to soils from different human activities including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface. Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis et al., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS, and Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus Set al., 2001). For tissues above ground of plants, Cu toxicity often causes foliar interveinal chlorosis, the leaf becoming necrotic with increasing exposure. (S. M. Reichman, 2002.) Cu contamination is a problem both for agricultural and environment. Cu contamination is mainly from mining and smelting, agrochemicals, and industrial and agricultural wastes. Cu have many forms in soils, and free Cu2+ activity is the best indicator of bioavailability (Sauve et al. 1996). Copper (Cu) is an essential micronutrient for plant growth, and the normal range in the growing medium is from 0.05 to 0.5 mg/kg, while in plant tissues usually ranges from 3 to10 mg/kg (Carruthers, 2016). However, excess Cu has a detrimental influence on plants, especially on root growth and morphology (Sheldon and Menzies,2005). Cu tends to accumulate mainly in the root tissue (Marschner 1995), and its toxicity is mainly on root growth. Cu toxicity damages to plant roots with symptoms including disruption of the root cuticle, reduced root hair proliferation and severe deformation of root structure (Sheldon and Menzies, 2005). A solution cultural experiment find that reduction in root growth occurs at an external Cu concentration of < 1 μM, and damage evident to the root cuticle and a reduction in the number and length of root hairs at 0.2 μM of Cu concentration (Sheldon and Menzies, 2005). It is estimated that the critical concentration of Cu in the nutrient solution associated with a 10% reduction in plant growth is from 0.6 and 1.1μM (Sheldon and Menzies, 2005). Excess copper in the growing medium can also restrict root growth by burning the root tips, high levels of copper can compete with plant uptake of iron and zinc (Sheldon and Menzies 2005). The study also finds high concentration of Cu causes a reduction in plasma membrane integrity in plant roots, which is the mechanism by which Cu toxicity retards root growth (Luna et al., 1994; Arduini et al., 1995).

Cu toxicity influences photosynthesis. At Cu concentration of the elevated level, in which root symptoms were apparent without growth reductions, total chlorophyll contents and chlorophyll a to b ratios were reduced but has no effect on net photosynthesis (Rousos et al., 1989). At higher external concentrations of Cu, where growth was depressed, lower chlorophyll contents (Ouzounidou et al., 1994), a reduced photosynthetic capacity, inhibition of photosytem II (Arellano et al., 1995), and an increase in breakdown of carotenoid (Luna et al., 1994) occurred. A side effect of Cu inhibiting photosynthesis is an increase in the production of free radicals and therefore an increase in rate of leaf senescence due to oxidative damage (Luna et al., 1994). Cu toxicity has significant effects on enzyme production and metabolism. High concentration of Cu inhibits ATPase activity in the plasma membrane of Z. mays roots (Kennedy and Gonsalves, 1989), which is possibly an indirect effect of Cu toxicity resulting from the leakage of K ions. Excess Cu inhibites acid phosphatase activity in Deschampsia cespitosa (Cox and Hutchinson, 1980). Cu toxicity also associate with an increase in antioxidative enzymes as a result of Cu meditated oxidative damage (Savoure et al., 1999). Cu has a negative effect on the metabolism of N, amino acids and proteins within the shoots of plants (Weber et al., 1991).

In this chapter, the immediate influence of Cd on wheat root system is disscueeed with the results from FD and compare the results obtained by fractal geometry with conventional technique measurements. To investigate the negative effects of Cd on the complexity of the wheat root development, we applied fractal geometry and calculated the fractal dimension. Fractal dimension was calculated using the box-counting method.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

In this experiment, Norin 61 wheat cultivar was chosen as the plants for study. As samples, similar with Cd experiment, we chose equal-sized seedlings one week after germination and photographed every week. In order to avoid root destruction and damage when removing from the soil, the seedlings were grown in a hydroculture system (12L). For keeping the wheat seedlings under healthy conditions, the plants were watered with nutrient solution for three times a week. All the plants were grown in a growth chamber (Conviron, Controlled Environmental Led, Winnipeg Manitoba, Canada) under fully controlled environmental conditions of 12h photoperiod, 27℃day, 20℃night, relative moisture of 65%-75% and light intensity of 260-350 µmolm-2s-1.

Same as Cd exposure experiment, four plant hydroculture systems consisting of sixty samples for Cu experiments. The concentrations of Cu, as $CuSO4{\cdot}5H_2O$, (Wako pure chemical industries Ltd, Japan, contain not less than 95%, molecular weight 249.69) were 0, 0.016,0.4 and 1.2 mM for over three weeks after germination. Each treatment had six replicates.

5.2.2 Complexity measurements

To characterize CI of the roots, photographs of the root systems were taken. To make photographs of the root system, at first, each of the entangled root sample was separated carefully and put in a transparent box. The box was filled with several millimeters depth of water so that the fine roots were clearly displayed. Next, the transparent box was put under a LED backlight panel. Photographs of the entire images of the root systems were taken weekly and digitized (5.72MB;

6000×4000pixels) with a digital camera (16.2 megapixels; Nikon D500, Tokyo, Japan). The images were acquired and saved in the JPEG format.

To estimate the complexity of the root systems, binarized images were skeletonized and skeletonization is an essential step. The skeletonized images were obtained according to the box-counting method as described by Tatsumi et al. During the skeletonization process, skeleton images were obtained through peeling off as many pixels as possible of the object without affecting the general shape of the object pattern. The peeling off was repeated until the most interior layer was reached. Different steps of image processing such as filtering, binarization and skeletonization were custom developed using MATLAB (MATLAB R2017b).

Next, the binarized images were skeletonized. skeletonized images were obtained through peeling off as many pixels as possible of the object without affecting the general shape of the binarized object pattern. These steps of image processing, i.e., filtering, binarization and skeletonization were done using custom developed programs using MATLAB (MATLAB R2017b).

With skeletonized images, complexity of the root systems were defined based on fractal dimension (FD). FD was calculated from the skeletonized images using box-counting method (Tatsumi *et al.*, 1989). During estimation of FD, as shown in Fig.2, different scaled grids (r) were applied over the skeletonized image, and the number of boxes that contain the root image are was counted. At first, a log –log plot of N(r) against r was obtained followed by a linear regression fitting to calculate the slope D or the fractal dimension FD of the image, as expressed by, $FD= \log N(r)/\log (1/r)$. The FD takes unity for a simple line object and increases towards 2 as the complexity of the line object increases, ($1<FD<2$). Therefore, FD being one would mean a simple root having one dimension or a line and a value larger than one would indicate the increasing complexity of the root system. Thus, FD indicates the degree of complexity of the root system and the depending on the exposure to heavy metals, FD is expected to decrease or the root system getting less complex. Accordingly, we can define a complexity index (CI) as;

$$CI=FD-1$$

The complexity index of a line is 0, and the complexity index for the most complex root structure that approaches to area rather than a line is 1 and percentage of relative complexity index as:

$$RCI=\frac{CI\ Post}{CI\ Pre}x100.$$

Where CI_{Pre} and CI_{Post} are the complexity index before and after heavy metal exposure, respectively.

5.2.3 Traditional measurements

Root length and weight measurement are common conventional measures of heavy metal effect of plant root system. In order to make weight measurements, at first, the plants along with the root systems were removed carefully from the hydroculture solution. Next, they were washed in distilled water. After washing the roots, they were stretched, and the lengths were measured. The fresh weights were then measured for each plant. Also, the dry weight of root was determined after drying at 105℃ first 30min, followed by keeping them at 70℃ for 72h until the weight became constant. The dry weights (DW) of roots and shoots were determined using a SHIMADZU AUX320 analytical balance. Relative weights (RW) and relative root elongations (RRE) were defined to evaluate the changes due to exposure to Cu. The definitions are as follow;

$$RRE\% = \frac{\text{Length after exposure}}{\text{Length prior exposure}} \times 100,$$

$$RW\% = \frac{\text{Weight after exposure}}{\text{Weight prior exposure}} \times 100.$$

Also, the length of primary root was analysed by Image J software (Version1.52) under four Cu concentrations of 0.016, 0.4 and 1.2 mM. NeuronJ is an image J plugin to facilitate the tracing and quantification of elongated image structures. Fig.5.1(a) showed the skeletonized image of one sample, and after manual tracing by NeuronJ step, the coloured primary root in Fig.5.1(b) will be used for root length measure.

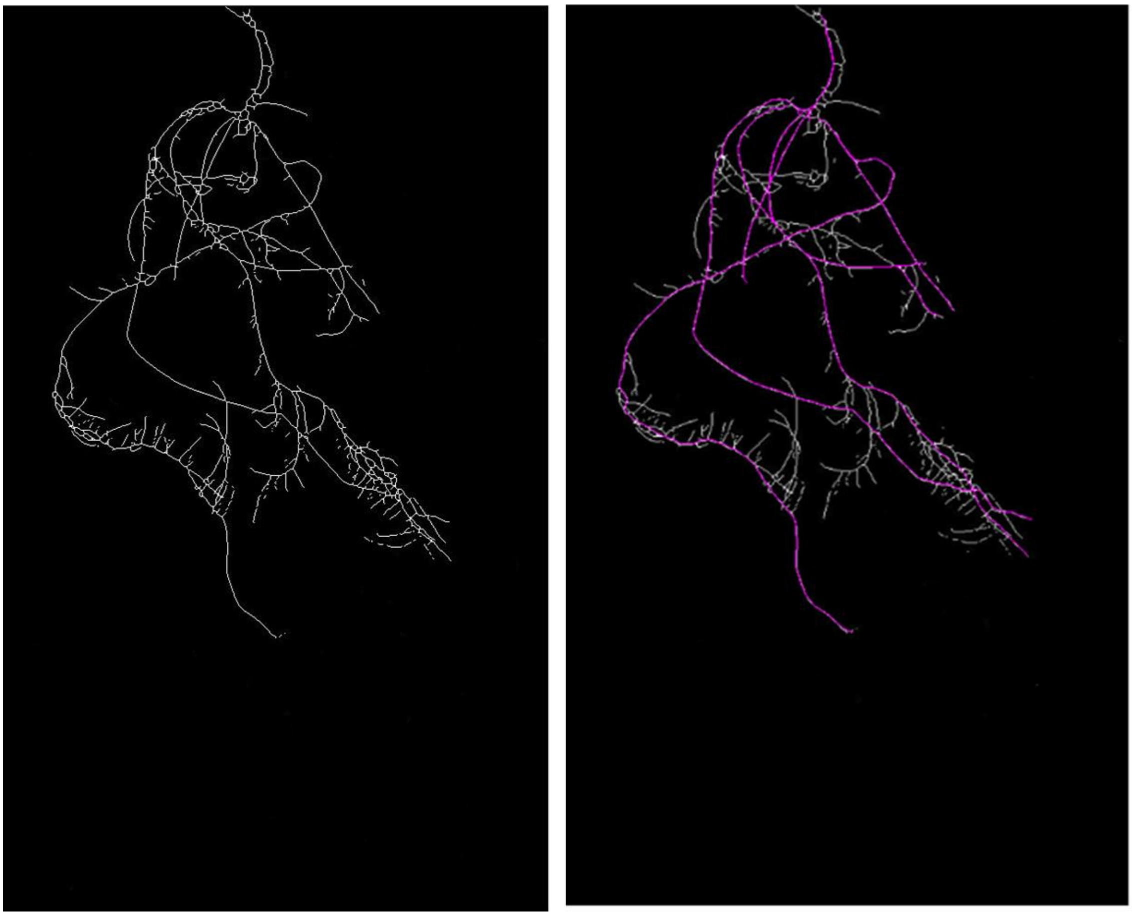

Fig.5.1 Skeletonized root image of root system (a) and tracing using Image J(b)

5.3 Results and discussion

5.3.1 Complexity and FD

Figure 5.3 showed the skeleton images of root architecture obtained under the respective exposure of Cu for three weeks and at different concentrations. Under the exposure of Cu, as seen from the skeletonized images, the root system has less lateral branching, and the number of roots or the density of roots appear to be less. These changes are pronounced with increasing concentrations of Cu and at higher concentrations, the overall root length appear to be shorter.

Table 5.1 shows the FD for wheat root under different Cu concentrations. For the first week, FD values ranged from 1.20 for control to 1.12 for 1.2mM concentration of Cu indicating that the changes were small even for large concentrations of Cu.

However, during second week, the effects of Cu started to appear clearly. The FD value of over 1.21 for control drastically decreased to around 1.12 for 0.05mM with distinct reductions seen for other Cu concentrations also. Again, a similar reduction tendency can be seen for third week for all Cd concentrations. The reduction percentages of FD and RCI were found to be respectively, 14%, 34%, and 46% for Cu concentrations 0.016, 0.4, and 1.2 mM, respectively. Further comparing second and third weeks, the reduction percentages were found to be small as compared to those between the first and second weeks. Statistical analysis showed significant difference (p<0.05) between the control and all of the FD and RCI under all Cu concentrations. The results imply that the fractal dimension can be an effective measure for the structural development of the root system.

Table5.1. FD for wheat root under different Cu concentrations

Week \ Cu(mM)	0	0.016	0.4	1.2
1st week	1.20	1.16*	1.13*	1.12*
2nd week	1.21	1.17*	1.14*	1.12*
3rd week	1.23	1.20*	1.15*	1.13*

*indicates significant difference at $p<0.05$

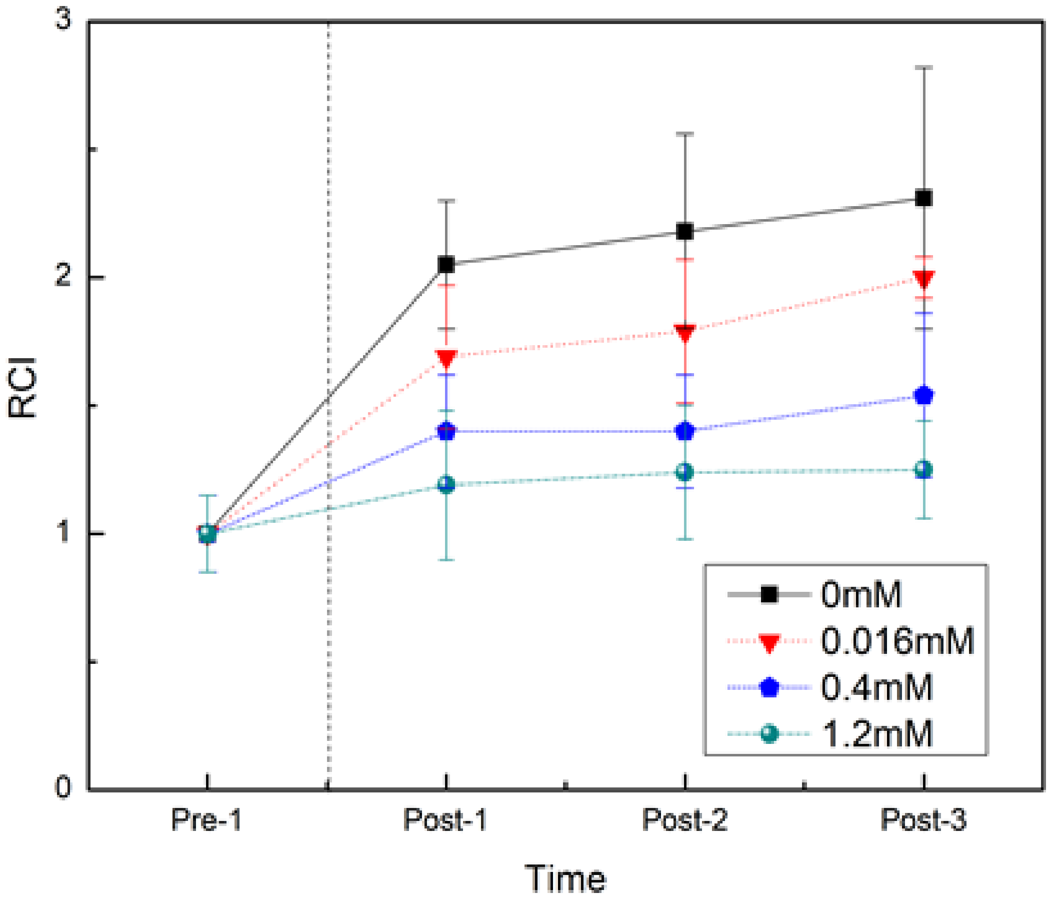

Fig.5.2 RCI for wheat root skeleton images for different Cu concentration

Similar harmful effects of Cu can be seen in Fig.5.2. The lowest RCI was obtained for the highest Cu concentration of 1.2 mM and the highest value for control condition under each week. For the first week, RCI values ranged from 2.05 for control to 1.19 for 1.2mM concentration of Cu indicating that the changes were large even at an early stage. However, with increasing weeks, the pronounced effects of Cu were clearly seen. The RCI value of over 2.18 for control drastically decreased to around 1.79 for 0.016mM with distinct reductions seen for other Cu concentrations also. Again, a similar reduction tendency can be seen for third week for all Cu concentrations. The reduction percentages for third week of RCI were found to be respectively, 14%, 34%, and 46% for Cu concentrations with 0.016, 0.4, and 1.2 mM, respectively. Statistical analysis showed significant difference ($p<0.05$) between the control and all of the RCI under all Cu concentrations.

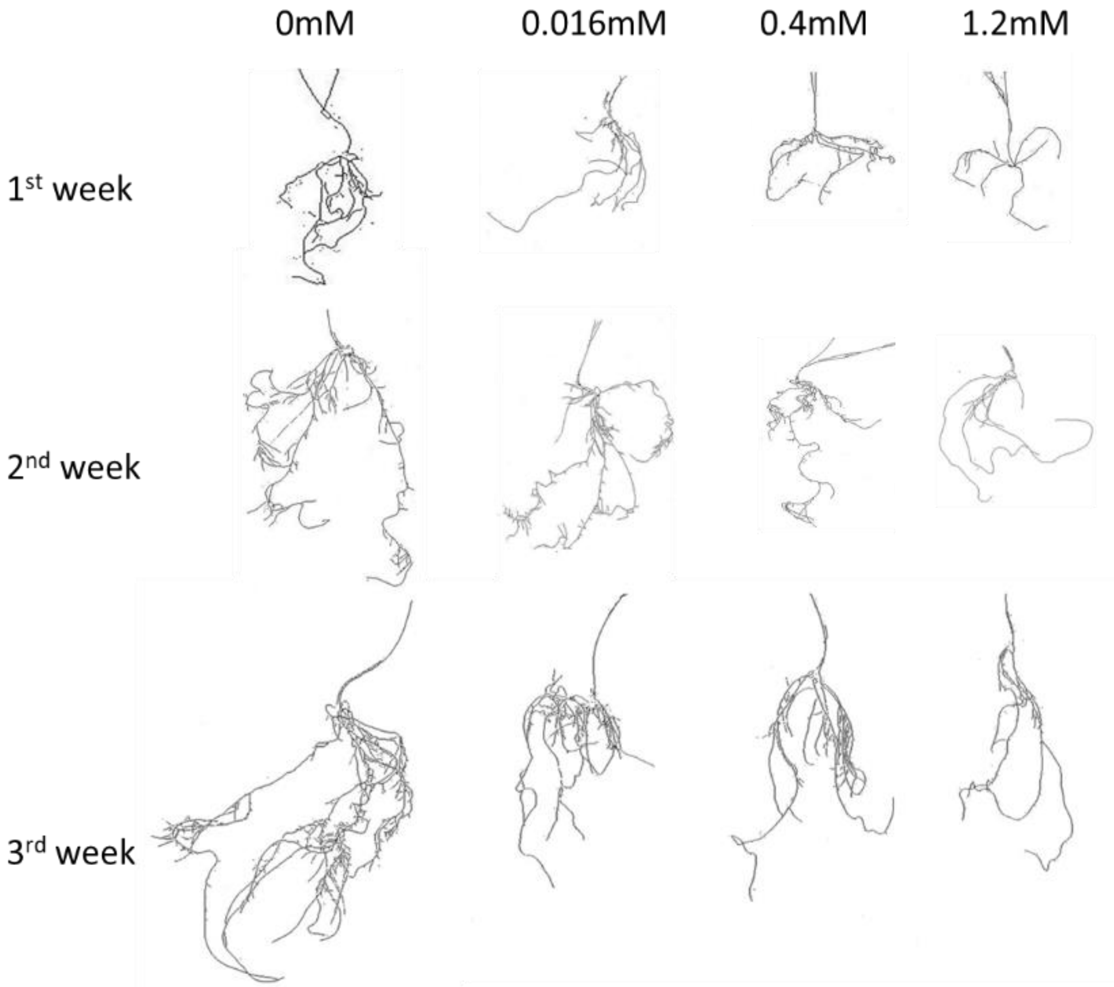

Fig.5.3 Skeleton images for different Cd concentrations obtained for three weeks

5.3.2 Influence of Cu on root length

Table5.2. Root length for wheat root under different Cu concentrations

Cu(mM) / Week	0	0.016	0.4	1.2
1st week	24.8	23.3	20.0	18.7
2nd week	27.1	24.9	22.5	21.2
3rd week	29.3	28.0	25.6	24.8

*indicates significant difference at $p<0.05$

Measurement of root length by meter scale is a common measure that is conventionally used. At first, root lengths were measured on removing the roots of wheat plants out of water from the culture. The measurements were done to an accuracy of 0.1mm under both conditions of control and different concentrations of Cu. Results of length measurements over 3 weeks under four different Cu concentrations, 0, 0.016, 0.4, and 1.2mM are shown in Table 5.2. As the Cu concentration increases, it can be seen that the root became shorter as compared to that under control (0mM) with the passage of each week due to the influence of Cu.

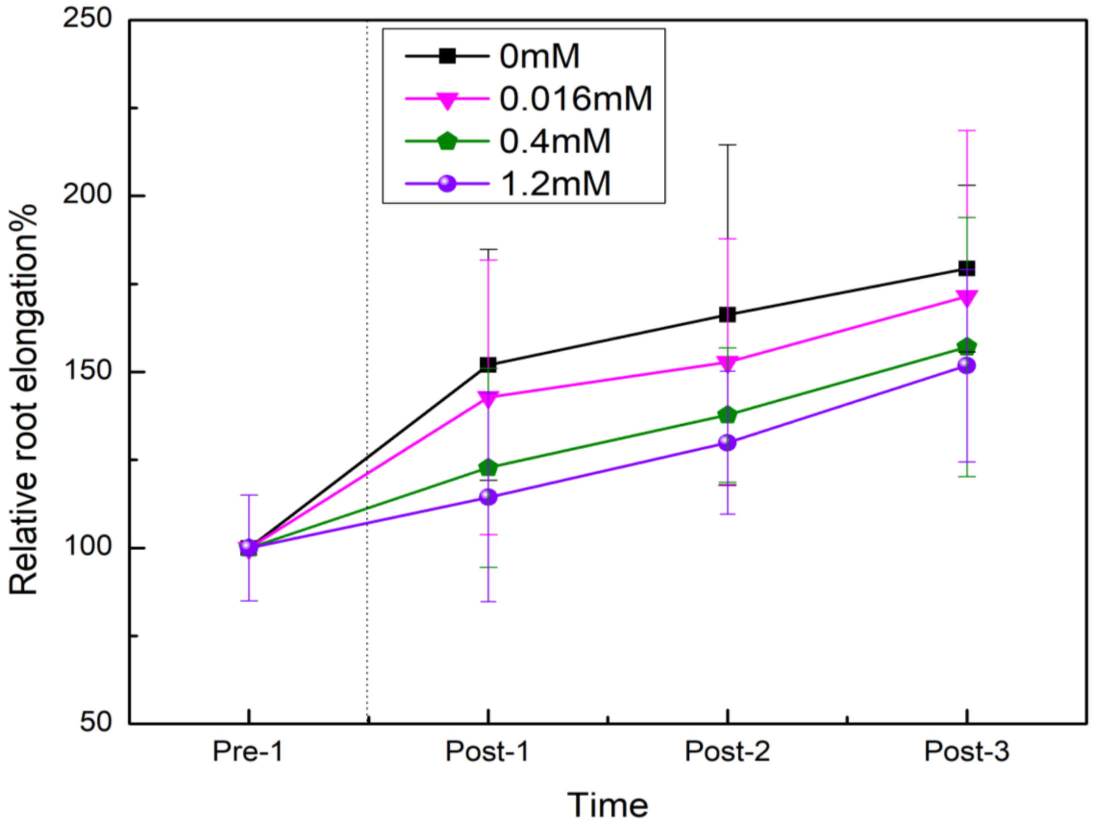

Fig.5.3 Relative root elongation for wheat root skeleton images for different Cu concentration

The results of RRE doses (0.016, 0.04, 1.2mM of Cu) on treated seeds of wheat were depicted in Fig5.3. The root elongation reduction also reveals that the Cu toxicity effect. Increasing Cu concentration with 1.2 mM decreased the RRE by 24.7% 21.8% and 15.4% for each week, but there was no significant change under all treatments. For all the experiment, no significant ($p<0.05$) difference could be obtained.

Table5.3. Root length (using imageJ) for wheat root under different Cu concentrations

Week \ Cu(mM)	0	0.016	0.4	1.2
1st week	94.3	65.5*	52.9*	43.0*
2nd week	124.2	93.5*	74.2*	67.9*
3rd week	190.8	120*	95.1*	71.1*

*indicates significant difference at p<0.05

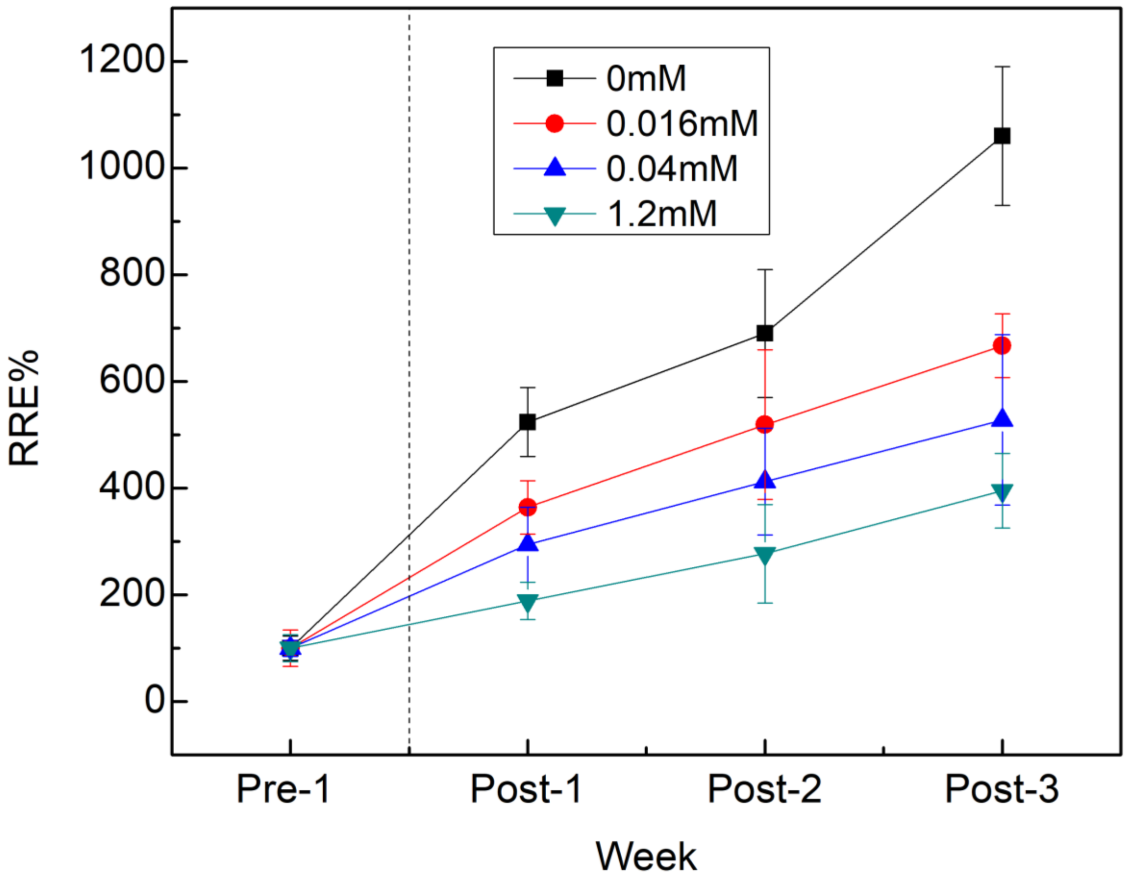

Fig.5.4 Relative root elongation (Image J) of wheat root skeleton images for different Cu concentration

The results of root length of wheat treated with four different Cu concentrations is shown in Table 5.3. The results of RRE (using Image J) of wheat treated with four different Cu concentrations is shown in Fig. 5.4. A significant root length difference in root length change ($p<0.05$) could be observed under all Cu concentrations for three weeks. Similar as Cd experiment, under the lowest concentration 0.016mM, the significantly decrements in root length were found to be 31%, 25%, and 37% for first, second and three weeks, respectively as compared to the control. However, the manual tracing method is tedious and time-consuming compare with fractal method. Comparing with our methods, time required to obtain CI using Matlab only within a few seconds.

5.3.3 Influence of Cu on root weight

Table5.4. Root weight for wheat root under different Cu concentrations

Week \ Cu(mM)	0	0.016	0.4	1.2
1st week	170.0	157.0	140.3	137.0
2nd week	187.0	170.4	151.7	142.3
3rd week	204.8	180.0	169.1	148.1

*indicates significant difference at $p<0.05$

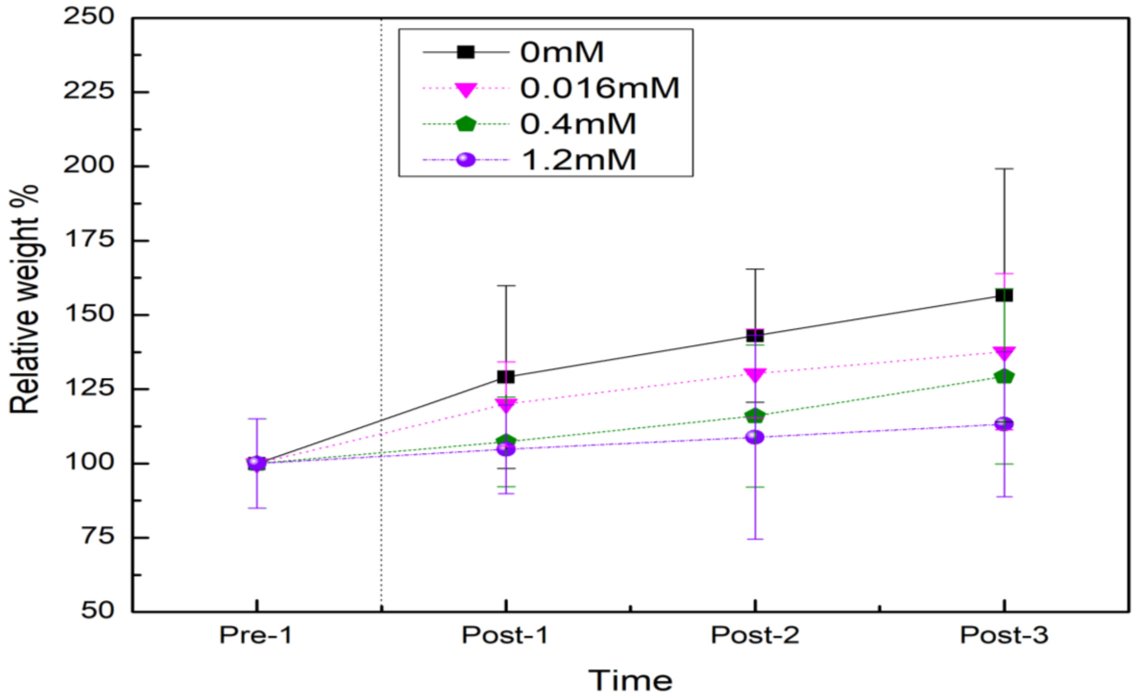

Fig.5.5 Relative weight for wheat root skeleton images for different Cu concentration

Root weight showed decrement of Cu exposure under 0, 016, 0.4 and 1.2 mM. The relative weight results after 3 weeks of experiments indicated that all the Cu treatments (0.016, 0.4 and 1.2mM) reduced the root length than control of wheat, and increasing Cu treatments reduced relative dry weights of roots. Compared to the control, application of 0.016mM Cu reduced relative weight by 16.9%, 18.8%, and 17.4% for each week. At the highest level of Cu stress (1.2mM), the reduction in relative weight for 3 weeks of post-treatment was showed about 18.9%, 23.9%, and 27.7% of controls, respectively. Statistical analysis did not show significant difference ($p<0.05$) between control and other Cu concentrations for whole duration experiment.

5.4 Conclusion

In this chapter, I explained the utilization of fractal geometry, to reveals the Cu effect on plant root growth dynamics. The quantification method (complexity) permitted a highly sensitive and accurate measurement compared with conventional techniques.

In this experiment, the complexity of system under Cu stress was evaluated. Results show that: (1) CI measurements showed significant decrement compare with control indicating the toxicity of Cu under 0.016,0.4 and 1.21mM. (2) However, no significant changes were observed in conventional measures (root weight and root length by ruler). (3) The manual tracing method is tedious and time-consuming compare with fractal method. CI measurement was timesaving, simpler and more sensitive.

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Chapter 6
Positive effect measured by CI under Zn stress

6.1 Introduction

Soil contaminated with zinc (Zn) is usually caused by the sewage sludge, urban composts, fertilizers, emissions from municipal waste incinerators, residues from met alliferous mining, the metal smelting industry, and other human activities (Yadav, 2010). Zn is an essential nutrient for living organisms, while Cd is non-essential and potentially toxic for plants. Contamination by Zn may cause phytotoxicity. High levels of Zn in soil inhibit many plant metabolic functions; result in retarded growth and cause senescence. Zinc toxicity in plants limited the growth of both root and shoot (Ebbs and Kochian, 1997; Fontes and Cox, 1998). Zinc toxicity also causes chlorosis in the younger leaves, which can extend to older leaves after prolonged exposure to high soil Zn levels (Ebbs and Kochian, 1997). Excess Zn can also give rise to manganese (Mn) and copper (Cu) deficiencies in plant shoots. Such deficiencies have been ascribed to a hindered transfer of these micronutrients from root to shoot. Another typical effect of Zn toxicity is the appearance of a purplish-red color in leaves, which is ascribed to phosphorus (P) deficiency (Lee et al., 1996).

Zinc toxicity symptoms include chlorosis and reddening of younger leaves with necrotic lesions on leaves in severe cases.Zinc is considered to have variable phloem mobility dependent on the Zn status of the plant species as well as individual plant tissues and organs (Herren and Feller, 1997). When sufficient Zn supply in one root section an average of about 30% of the Zn taken up was transported to the other root section suggesting an increasing role for phloem transport as Zn supply increases (Welch et al., 1999), indicating that a tolerance mechanism to prevent excess Zn redistribution to young developing tissues (Herren and Feller, 1997). Excess Zn supply has a negative effect on phloem loading and transport of other metals (Welch et al.,

1999) thus having the potential to induce local deficiencies and/or toxicities. Zn- may induce limitation in the supply of ATP, which is responsible for reduced phloem loading, and results in much of the toxic effects of excess Zn on plant biomass production (Rauser and Samarakoon,1980). Zinc deficiency commonly presents on young leaves as chlorosis and reddening (Davies, 1993; Lee et al., 1996).Zn Toxicity symptoms Zn toxicity is a general chlorosis of the younger leaves (Fontes and Cox, 1995). Depending on the toxicity degree, this chlorosis can progress to reddening due to anthocyanin production in younger leaves (Fontes and Cox, 1995; Lee et al., 1996a). Plants under Zn toxicity have smaller size of leaves than those of control (Ren et al., 1993). Zn toxicity also causes brown spots on the leaves of some species (Fontes and Cox, 1995).

In roots, Zn toxicity is apparent as a reduction in the growth of the main root, fewer and shorter lateral roots and a yellowing of roots (Ren et al., 1993). Effects of toxicity on physiology.Toxic concentrations of Zn causes inhibition of photosystems I and II and thus a decrease in photosynthesis (van Assche and Clijsters, 1986). The mechanism of the action is the displacement of Mg by Zn at the water splitting site in photosystem II (Kupper et al., 1996). Teige et al. (1990) suggested that the primary toxic action of Zn is the inhibition of ATP synthesis and therefore energy metabolism in plants. Excess Zn has been shown to stimulate the production of a range of enzymes in P. vulgaris (van Assche et al., 1988). This might be a compensation by the cell for the inhibition of physiological activity caused by high Zn. In O. sativa, high external Zn concentration can increase peroxidase, auxin oxidase and ascorbic acid oxidase, whereas the activity of catalase IAA oxidase, α-amylase, ATPase and phytase was inhibited (Nag et al., 1984).

High external Zn concentrations have been found to inhibit RuBP carboxylase activity in P. vulgaris however (van Assche and Clijsters, 1986). Zinc toxicity has been found to result in a decrease in amino acid accumulation in Panax quinquefolium (American ginseng) roots (Ren et al., 1993).

Zn contamination may inhibit stem cell elongation (Aidid and Okamoto, 1992), As when Zn supply increase, H. annuus cells had an increase in mitotic abnormalities and a decrease in mitotic index (Chakravarty and Srivastava, 1992).

6.2 Materials and methods

6.2.1 Plant materials and growth conditions

In this experiment, Norin 61 wheat cultivar waschosen as the plants for study. Same as Cd and Cu experiment, we chose equal-sized seedlings one week after germination and photographed every week. In order to avoid root destruction and damage when removing from the soil, the seedlings were grown in a hydroculture system (12L). For keeping the wheat seedlings under healthy conditions, the plants were watered with nutrient solution for three times a week. All the plants were grown in a growth chamber (Conviron, Controlled Environmental Led, Winnipeg Manitoba, Canada) under fully controlled environmental conditions of 12h photoperiod, 27℃day, 20℃night, relative moisture of 65%-75% and light intensity of 260-350 µmolm-$^{2s-1}$.

For Zn exposure measurement, root samples exposed to different concentrations of 0, 0.3, and 0.75ppm of Zn containing solutions as Zn(NO3)2·6H2O (Wako pure chemical industries Ltd, Japan, contain not less than 95%, molecular weight 297.49) for over three weeks after germination. Again, total of three plant systems was prepared with one system for control, and the other two under Zn stress with six replicates for each of the three concentrations. A total of thirty-six samples with twelve samples for each concentration was used.

6.2.2 Complexity measurements

After imaging processing, the binarized images were skeletonized. Skeletonized images were obtained through peeling off as many pixels as possible of the object without affecting the general shape of the binarized object pattern. These steps of image processing, i.e. filtering, binarization and skeletonization were done using custom developed programs using MATLAB (MATLAB R2017b).

With skeletonized images, complexity of the root systems was defined based on fractal dimension (FD). FD was calculated from the skeletonized images using box-counting method (Tatsumi *et al.*, 1989). During estimation of FD, as shown in Fig.1, different scaled grids (r) were applied over the skeletonized image, and the number of boxes that contain the root image were counted. At first, a log –log plot of N(r) against r was obtained followed by a linear regression fitting to calculate the slope D or the fractal dimension FD of the image, as expressed by, FD= log N(r)/log (1/r).

The FD takes unity for a simple line object and increases towards 2 as the complexity of the line object increases, ($1 \leq FD < 2$). Therefore, FD being one would mean a simple root having one dimension or a line and a value larger than one would indicate the increasing complexity of the root system. Thus, FD indicates the degree of complexity of the root system and depending on the exposure to heavy metals, FD is expected to decrease or the root system getting less complex. Accordingly, we can define a complexity index (CI) as;

$$CI = FD - 1$$

The complexity index of a line is 0, and the complexity index for the most complex root structure that approaches to area rather than a line is 1 and percentage of relative complexity index as:

$$RCI = \frac{CI_{Post}}{CI_{Pre}} x100,$$

where CI_{Pre} and CI_{Post} are the complexity indices before and after heavy metal exposure, respectively.

6.2.3 Traditional measurements

Root length and weight measurement are common conventional measures of heavy metal effect of plant root system. In order to make weight measurements, at first, the plants along with the root systems were removed carefully from the hydroculture solution. Next, they were washed in distilled water. After washing the roots, they were stretched and the lengths were measured. The fresh weights were then measured for each plant. Also, the dry weight of root was determined after drying at 105℃ first 30min, followed by keeping them at 70℃ for 72h until the weight became constant. The dry weights (DW) of roots and shoots were determined using a SHIMADZU AUX320 analytical balance. Relative weights (RW) and relative root elongations (RRE) were defined to evaluate the changes due to exposure to Zn. The definitions are as follow;

$$RRE\% = \frac{\text{Length after exposure}}{\text{Length prior exposure}} \times 100,$$

$$RW\% = \frac{\text{Weight after exposure}}{\text{Weight prior exposure}} \times 100.$$

Also, the length of primary root was analysed by Image J software (Version1.52) under two Zn concentrations of 0.3 and 0.75 mM. NeuronJ is an image J plugin to facilitate the tracing and quantification of elongated image structures. Fig.6.1(a) showed the skeletonized root image, and after manual tracing by NeuronJ step, the coloured primary root in Fig.6.1(b) will be used for root length measure.

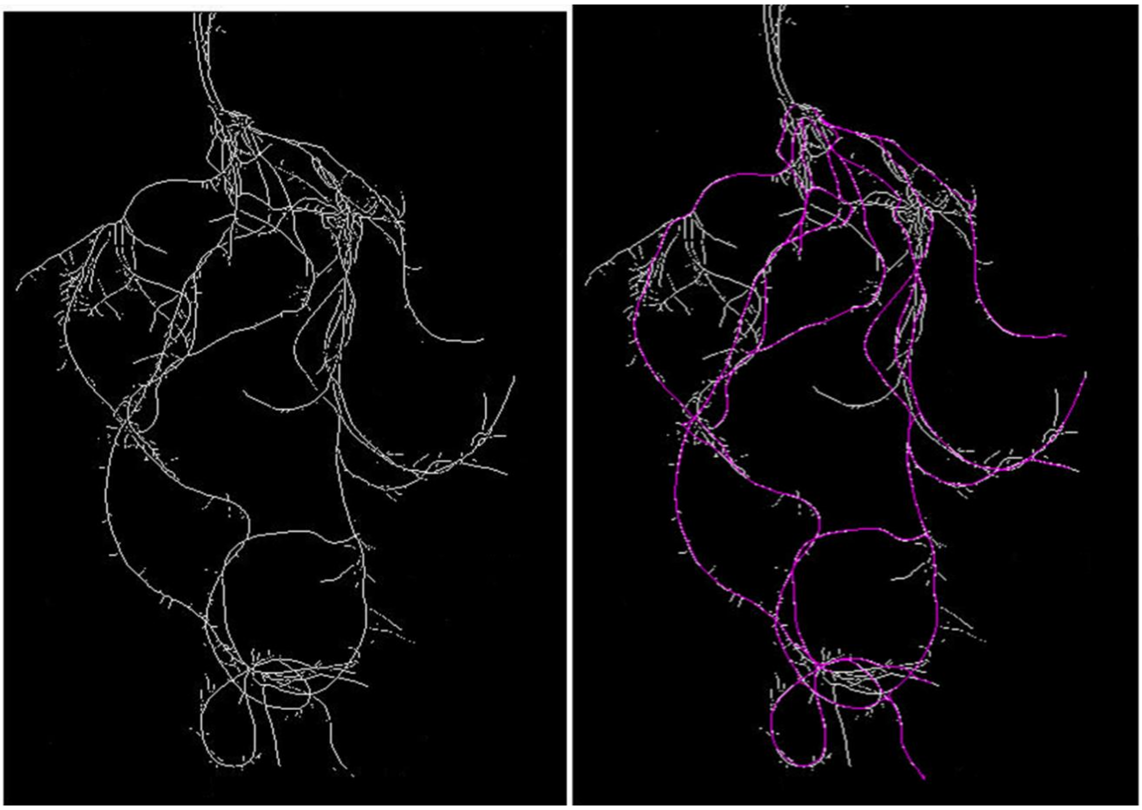

Fig.6.1 Skeletonized root image of root system (a) and tracing using Image J(b)

6.3 Results and discussion

6.3.1 Complexity and FD

Figure 6.3 showed the skeleton images of root architecture obtained under the respective exposure of Zn for three weeks and at different concentrations. For exposure under Zn, there is increased root length as well as increased root branching and there appear to be a greater number of roots or there is increased root density.

These changes are increasingly evident with increasing concentrations and also with increasing exposure weeks.

Table 6.1 indicates the FD under the different Zn concentration, 0, 0.3 and 0.75mM. Fractal dimension clearly increased over time after exposure to both of the concentrations, 0.3 and 0.75mM. Under 0.3mM, the significant increments of 12.5%, 13.8% and 16.8% could be observed over three weeks. For higher concentration of 0.75mM, the increase was larger with 30.0%, 23.0% and 26.1% for three weeks.

Contrary to the results of Cd and Cu, the promotion effect on lateral root formation could be seen clearly form the skeletonized images after Zn exposure. The root length can give the difference between control and 0.75mM, however cannot show the difference between the control and 0.3mM. The root length almost same at control and 0.3 mM. But FD value can give the large difference even at lower concentration at earlier stage.

Table6.1. FD for wheat root skeleton images under different Zn concentrations

Zn(mM) / Week	0	0.3	0.75
1st week	1.14	1.16*	1.20*
2nd week	1.22	1.25*	1.28*
3rd week	1.27	1.32*	1.36*

*indicates significant difference at $p<0.05$

Fig 6.2 showed the results of relative complexity index (RCI) under three Zn concentrations of 0, 0.3, 0. 75mM.The periods per and post correspond to Zn free and Zn exposure conditions, respectively. The two conditions are separated with broken line. Statistical analysis showed significant difference ($p<0.05$) between the control and all of the RCI under 0.3 and 0.75mM Zn stress. The results imply that the fractal dimension can be an effective measure for the structural development of the root system. The changing of fractal dimension under different condition indicates that fractal analysis is a sensitive measure of root branching intensity. Fractal dimension thus provides a useful quantitative measure for the elaboration of shape complexity during plant development.

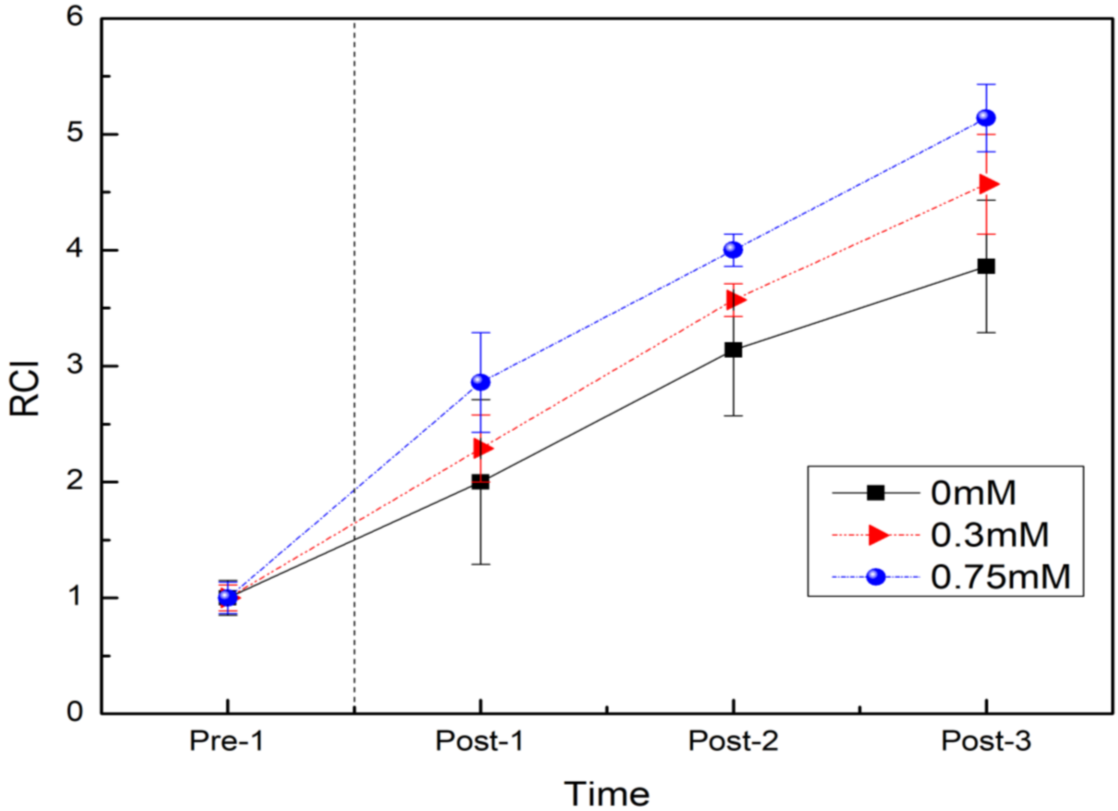

Fig.6.2 RCI for wheat root skeleton images for different Zn concentration

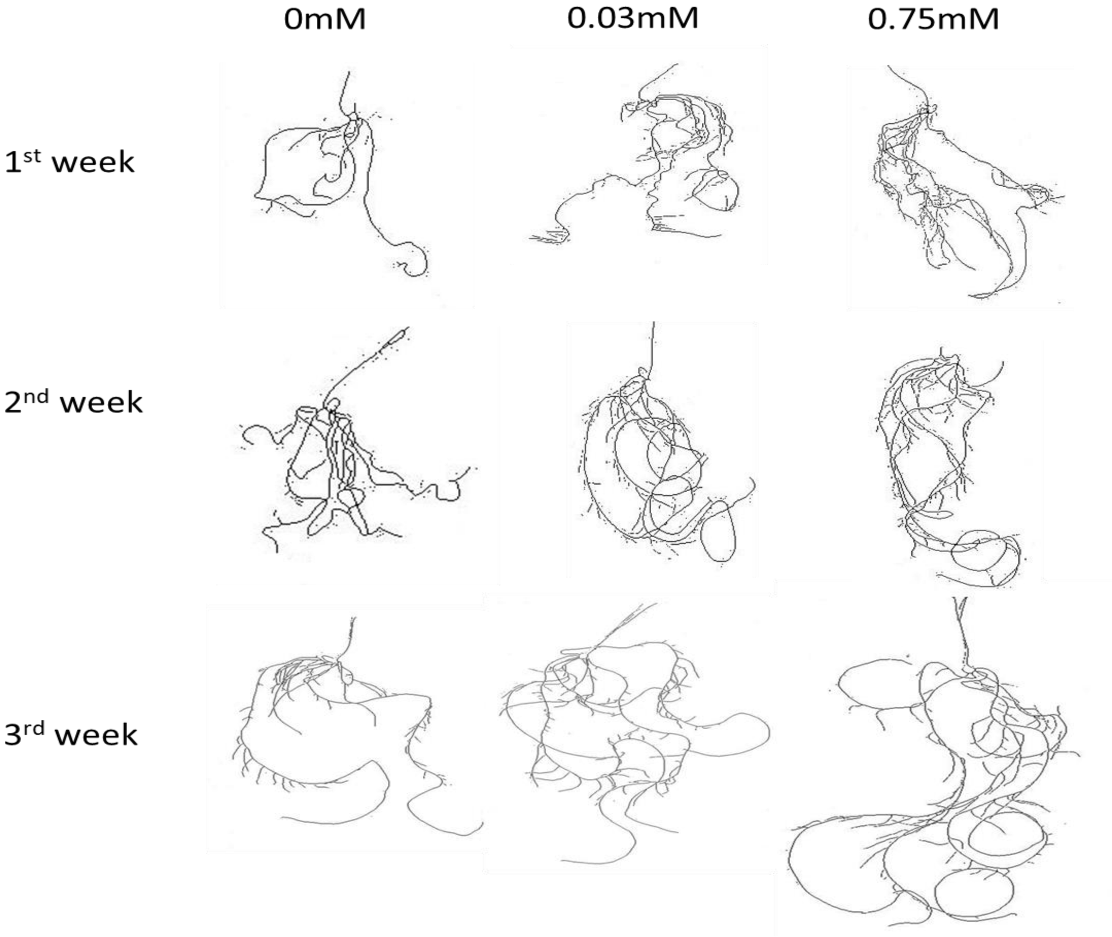

Fig.6.3 Skeleton images for different Zn concentrations obtained for three weeks

6.3.2 Influence of Zn on root length

Table 6.2 indicates the root length results under the different Zn concentrations, 0, 0.3, and 0.75 mM. Under Zn exposure, promoting effect could be observed on root length. There were gradual increments could be found with increasing concentrations of Zn for wheat for each week. Whereas there were few significant differences in element concentrations of plant roots and rhizomes, the significant increment could be found only for the highest concentration at 3rd week.

Table6.2 .Change in root length of wheat treated with four different Zn concentrations.

Week \ Zn(mM)	0	0.3	0.75
1st week	19.7	19.8	20
2nd week	25.3	25.8	26.1
3rd week	34.5	34.9	38.3*

*indicates significant difference at $p<0.05$

Fig.6.4 indicates the relative root elongation (RRE) results under the different Zn concentrations, 0, 0.3, and 0.75 mM. Under Zn exposure, promoting effect could be observed on root length. There were gradual increments could be found with increasing concentrations of Zn for wheat for each week. Whereas there were few significant differences in element concentrations of plant roots and rhizomes, the significant increment could be found only for the highest concentration at 3rd week.

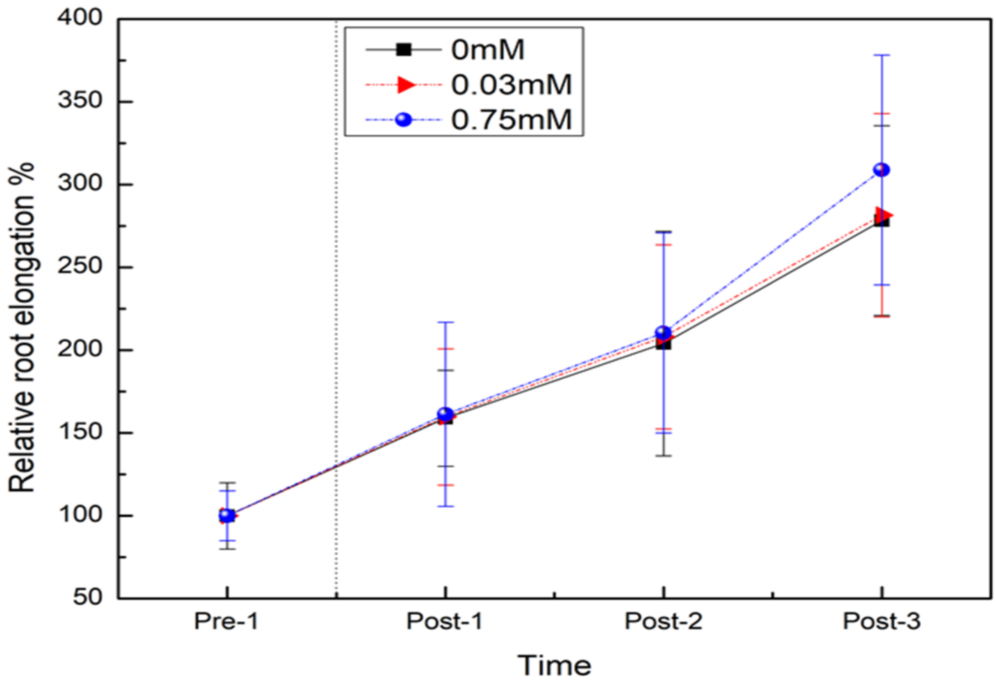

Fig.6.4 Relative root elongation for wheat root skeleton images for different Zn concentration

Table 6.3 Root length (using imageJ) for wheat root under different Cu concentrations

Zn(mM) / Week	0	0.3	0.75
1st week	84.2	106.7	132.6*
2nd week	110.8	142.6*	187.9*
3rd week	223.4	289.7*	367.1*

*indicates significant difference at p<0.05

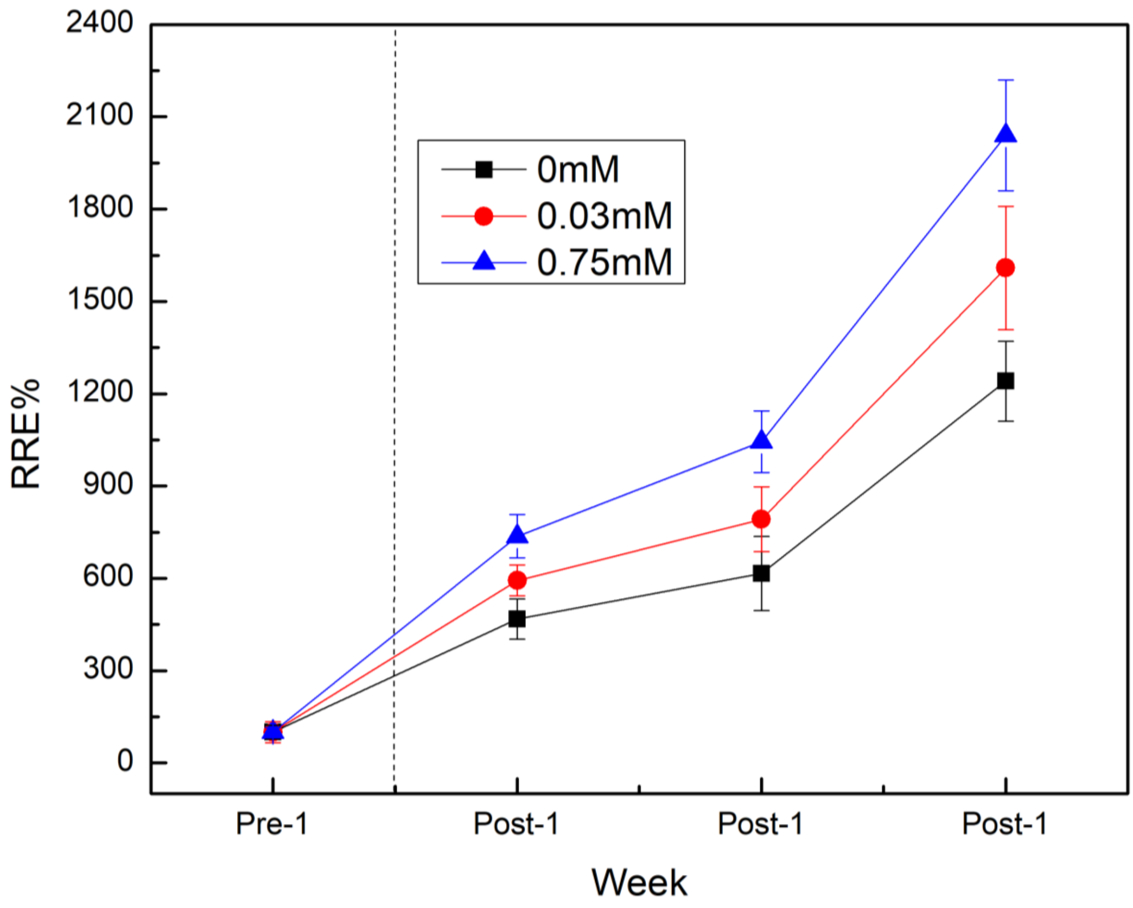

Fig.6.5 Relative root elongation(using image J) for wheat root skeleton images for different Zn concentration

Table 6.3. indicates the root length (Image J) results under different Zn concentrations, 0, 0.3, and 0.75 mM. Under Zn exposure, promoting effect could be observed on root length. There were gradual increments that could be found with increasing concentrations of Zn for each week. There were significant increment could be observed within 3 weeks. Under 0.75 mM Zn concentration, root length showed

significant increment compared with control with the rate of 37%,41% and 39% respectively.

6.3.3 Influence of Zn on root weight

Table6.4 Dry root weights (mg plant^{-1}) in wheat under four different Zn concentrations.

Zn(mM) / Week	0	0.3	0.75
1st week	54.8	56.1	60.7
2nd week	135.1	136.1	139
3rd week	166.5	173.8	195.9

*indicates significant difference at p<0.05

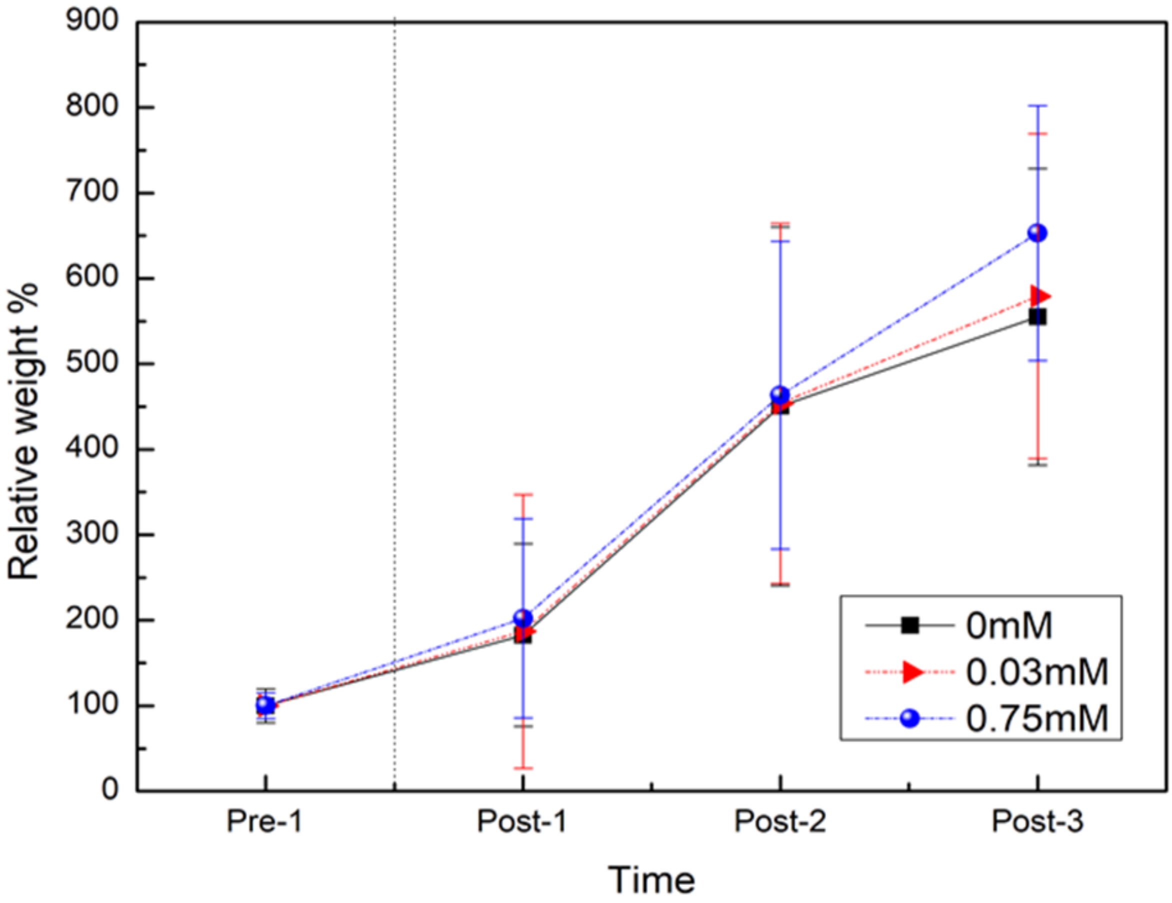

Fig.6. 6 Relative root biomass for wheat root skeleton images for different Zn concentration

Results of root biomass measurements of wheat under Zn exposure over three weeks are shown in Table2. Under all concentrations of Zn, increment of root biomass were seen. Compare with Cd results, the largest root biomass was observed under highest Zn concentration (0.75mM), and the lowest root biomass was observed under control condition. It showed 4.2% and 15.0% increment of root biomass under 0.3and 0.75 mM at third week. Under all Zn concentrations, there were no significant differences compared with control for all three weeks. The root dry weight was stimulated in Zn experiment and inhibited in Cd experiment.

Results of relative biomass changes of wheat under Zn exposure over three weeks are shown in Fig.6.6. Under all concentrations of Zn, increment of relative biomass were seen. Compare with Cd results, the largest root biomass was observed under highest Zn concentration (0.75mM), and the lowest relative biomass was observed under control condition. It showed 4.4% and 14.7% increment of root biomass under 0.3 and 0.75 mM at post-3 week. Under all Zn concentrations, there were no significant differences compared with control for all three weeks.

6.4 Conclusion

In this chapter, the fractal geometry was used to evaluate the root architecture of wheat under Zn, a micronutrient. Results show that: Zn has positive influence on root system even at early stage. Root length and weight didn't show significant changes under small Zn concentration at early stage. RCI/ FD measurement can be used to detect the immediate effect of Zn on root structural development in an early stage.

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Chapter 7
Discussion and conclusion

The present study explored the positive and negative effects under Zn and Cd, Cu treatments on roots of wheat plants by using fractal dimensions as complexity measure and compared with conventional measures of root length and root weight.

Cd stress was investigated under three different concentrations of 0.001mM, 0.01mM, and 0.05mM. The root growth inhibition was observed under all of the Cd concentrations. Similar results were also found in the wheat under Cd stress by Eker et al., which is directly related to nutrient uptake in root and shoot (Eker et al., 2013). Tai et al. referred that Cd stress significantly caused the adverse effects on plant growth and root morphology of Switch grass seedlings(Tai et al., 2017). In our investigation, Relative Complexity Index or RCI was found to be sensitive enough that it showed significant reduction ($p<0.05$) even under concentration of 0.01 mM or 0.05mM in comparison to the relative weight and relative root length. Our measure is found to be sensitive enough compared to other study which used 50mM of Cd for 15 days exposure. That study reported that Cd produced a reduction in the number and length of lateral roots on pea (Pisum sativum L.) plants with 50 mM $CdCl_2$ for 15 days (Rodríguez-Serrano et al., 2006).

Kubo et al. (Kubo et al., 2011) also reported similar results that root branching slowly or limited development was related by lower Cd uptake at seedling stage of Japanese wheat. Lux et al. researched the root structure of plant Merwilla plimbea under Cd stress and described the response of this plant under Cd stress through Cd uptake in different organs and growth parameters. Their results showed that the roots of plant exposed to Cd were significantly stunted and root formation was inhibited (Lux et al., 2011), which is similar to the observation done in this study. The reason for growth inhibition under Cd stress was mainly due to increasing Cd concentration in plant tissues resulting in decrease of chlorophyll content and thus photosynthesis rate (Miller et al., 2016)(Paunov et al., 2018)

The root characteristics that included root length and biomass were found to be highly correlated with the RCI and thus fractal dimension values in Fig.7(a) for high concentrations of Cd but not at lower concentrations whereas RCI was found to be more sensitive. As Cd concentrations increased, root length, root biomass and FD reduced with each week. FD showed a positive relationship with conventional measurements of root length (R^2=0.8536) and root dry weight (R^2=0.8621) under a Cd concentration of 0.05mM.

Copper (Cu) is an essential micronutrient for plant growth, and the normal range in the growing medium is from 0.05 to 0.5 mg/kg, while in plant tissues usually ranges from 3 to10 mg/kg (Carruthers, 2016). However, excess Cu has a detrimental influence on plants, especially on root growth and morphology (Sheldon and Menzies,2005). Cu tends to accumulate mainly in the root tissue (Marschner 1995), and its toxicity is mainly on root growth. Cu toxicity damages to plant roots with symptoms including disruption of the root cuticle, reduced root hair proliferation and severe deformation of root structure. A Culturing in solution experiment found that reduction in root growth occurs at an external Cu concentration of < 1 μM, and damage to the root cuticle was evident and there was a reduction in the number and length of root hairs at 0.2 μM of Cu concentration. It is estimated that the critical concentration of Cu in the nutrient solution associated with a 10% reduction in plant growth is from 0.6 and 1.1μM. Excess copper in the growing medium can also restrict root growth by burning the root tips. High levels of copper can compete with plant uptake of iron and zinc (Sheldon and Menzies 2005). In our experiment, significant reductions in RCI ($p<0.05$) between the control and all Cu concentrations of 0.016, 0.4 and 1.2 mM stress were found. Further, at high concentrations, FD (Fig.7(b)) also showed a positive relationship with root length (R^2=0.9345) and root dry weight (R^2=0.8291). High concentration of Cu causes a reduction in plasma membrane intensity in plant roots, which is the mechanism by which Cu toxicity retards root growth. (Luna et al., 1994; Arduini et al.,)

In addition, zinc is a plant micronutrient for plant growth which is involved in many physiological functions including auxins formation, chlorophyll formation and protein synthesis. Our results showed, as Zn concentrations increased, the complexity of root structure increased with growth parameters (length and weight) getting larger. FD also showed a positive relationship with root length (R^2=0.8142) and root dry weight (R^2=0.8481). For all the cases, as weight developed with increasing age, the complexity of root increased. From the increments in RW and RRE after exposure to Zn, we found the positive effect for both concentrations of 0.3 and 0.75 mM. A number of researchers have reported the essentiality and the role of zinc for plant growth and yield(Lucini and Bernardo, 2015) (Kisan et al, 2015). In the study conducted by Weisany et al., they pointed out that zinc (10 mg kg^{-1} as $ZnSO_4.7H_2O$) application improved shoot length, root fresh and dry weight and shoot fresh and dry weight under all salinity treatments.(Weisany et al., 2012). Efremova et al. mentioned that zinc compounds had beneficial effect on dry biomass of Saccharomyces cerevisiae CNMN-Y-11. Compared with control, the yeast biomass obtained the maximum with increasement rate of 28-38% after utilization of zinc compounds(Efremova et al., 2013). Yilmaz et al. also found that Zn treatment could lead to increases in grain yield and biomass production with 260% compare with control(Yilmaz et al., 1998). However, considering our results, the significant difference could not be shown in the increments of root length and dry weight.

The different Zn treatments affected the complexity of root system, as the overall mean RCI value for the whole root system was significantly increased. In other words, the results of fractal geometry based analysis suggested Zn application promoted lateral root formation. Nair and Chung studied the 20mg/L zinc oxide nanoparticles treatment triggered a 9% increase in lateral root formation in Arabidopsis thaliana seedlings. (Nair and Chung, 2017)

Our results using fractal geometry as a measure for the complexity of root system suggest that the roots were affected from an earlier stage of heavy metal (Cd, Cu and Zn) exposure and start to accumulate more heavy metals in themselves than the other organs. Compared to the conventional measures (dry weight and length), RCI could be used as a more sensitive measure to evaluate the positive and negative effects of heavy metal on the root system of wheat plants.

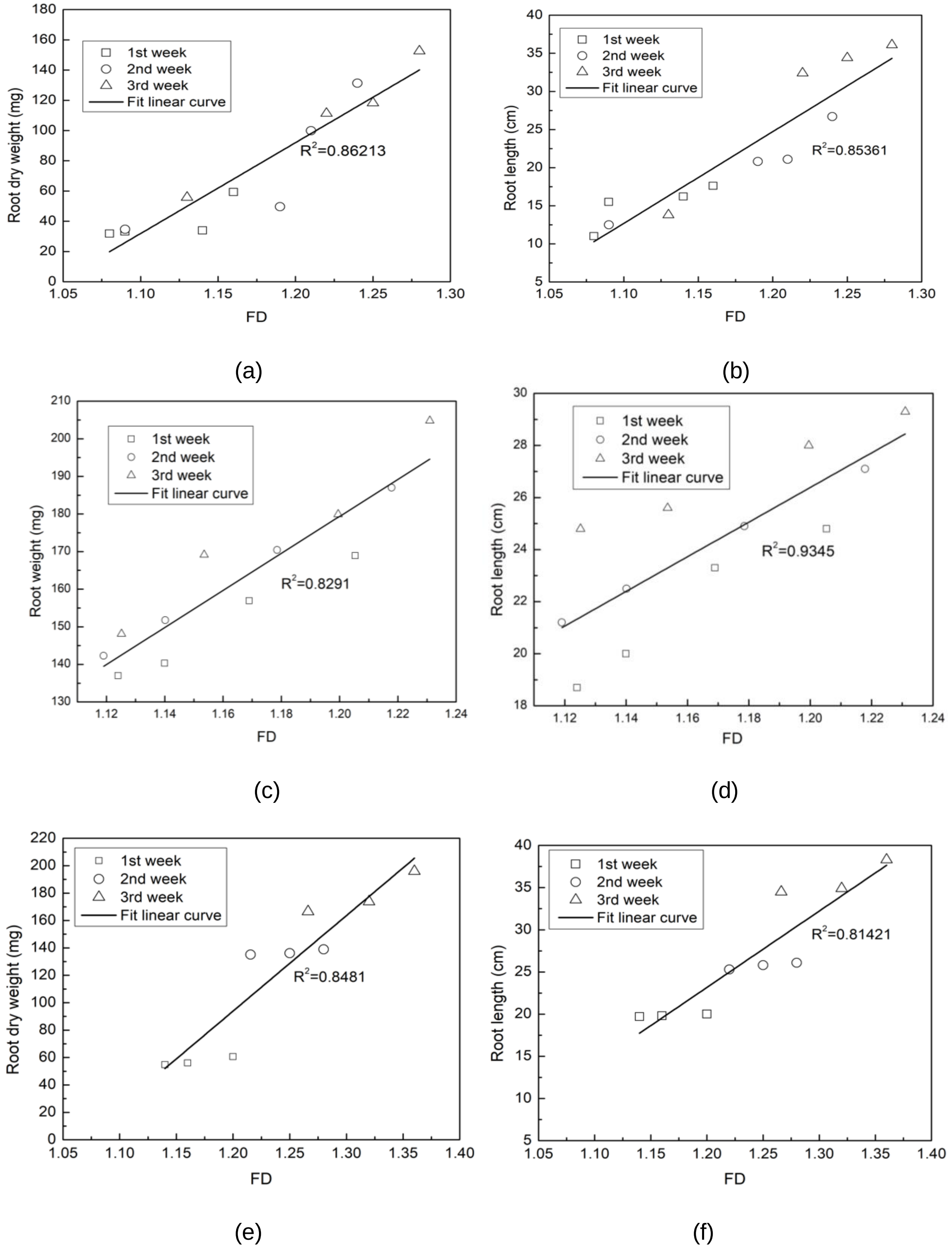

Fig.7.1 Relationship between fractal values and root weight under (a) Cd, (c)Cu, (e) Zn,

Relationship between fractal values and root length under (b) Cd, (d)Cu, (f) Zn.

The different Zn treatments affected the complexity of root system, as the overall mean FD value for the whole root system was significantly increased. According the fractal geometry, the result suggested Zn application promoted the lateral root formation. Nair and Chung studied the 20mg/L zinc oxide nanoparticles treatment triggered a 9% increase in lateral root formation in Arabidopsis thaliana seedlings. (Nair and Chung, 2017)

In addition, Zinc is a plant micronutrient for plant growth which is involved in many physiological functions include auxins formation, chlorophyll formation and protein synthesis. Our results showed, as Zn concentrations increased, the complexity of root structure increased with growth parameters (length and weight) getting larger. FD also showed a positive relationship with root length (R2=0.8142) and root dry weight (R2=0.8481). For all the cases, as weight developed with increasing age, the complexity of root increased.(Fig.7.1)

In this study, the fractal dimension was used to evaluate the root architecture of wheat under Cd, Cu and Zn exposure.

Results show that:

1)Fractal geometry could be a new approach to analyze the root complexity.

2) Complexity index can be an effective measure (both for negative and positive effect) for the structural development of the root system under heavy metal exposure.

3) Complexity index was found to be more sensitive and more reliable to reflect the influence of the heavy metal than the conventional measures.

In this study, the fractal geometry was used to evaluate the root architecture of wheat under heavy metals of Cd, Cu and Zn exposure for a period of three weeks. Root systems were photographed, binarized and skeletonized to determine the fractal dimension. Fractal dimension was used to estimate the complexity of the system. The results imply that a fractal based complexity measure could be sensitive enough that there could be significant decrease in the complexity of the root system even under smaller concentration of Cd and Cu in comparison to the traditional measures of root weight. Simultaneously, the positive effect of the micronutrient could also be assessed by the complexity measure RCI in comparison to the length and weight which failed to show any significant differences even at high concentrations. Fractal based approach to analyze the effects of heavy metals on root architecture can be an effective measure for the structural development of the root system.

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