Crystallography in agriculture: green and red spinach 
(*Amaranthus tricolor*) grown on soil and hydroponic

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Abstract. Red spinach is said to contain calcium oxalate based on chemical analysis. This study is an attempt to look at the calcium oxalate crystals in the leaves. Red spinach was planted on organic soil and hydroponic system using soluble inorganic nutrients. A hydroponic plant grows faster by almost two-fold compared to the on organic soil. Both methods produced the same reddish-purple stem and leaves on red Amaranth. The size of the stomata was also about the same (2.4 x 0.9 micron). However, scanning electron microscope images of the leaf showed the presence of much more and bigger calcium oxalate crystal in the hydroponic leaf than that of spinach planted on organic soil. Green spinach grew on organic soil also contained calcium crystal but with lesser amount compared with the red spinach. Energy Dispersive X-ray analysis indicates the presence of C, O, Ca, K, Mg, P, S, and Nb in the leaves. The result of this study is useful for farmers in making their selection of plants to grow and public health information as well.

1. Introduction

There are many varieties of spinach available in the market today. The small size leaf spinach, such as Malabar spinach is served directly and fresh as salads for lunch and dinner is nowadays grown by a hydroponic method in a big way. On the other hand, both green and red spinach (*Amaranthus tricolor*) are popular in ASEAN countries for making soup, fried vegetables, and noodles or bihon goreng. It can also be cut into small pieces and mixed with cucumber and other vegetables to be served as fresh salads during lunch and dinner.

*Amaranthus tricolor* usually grows in soil because of the size of the three, and the leaves are bigger than the small leave spinach. However, it is now getting more popular to grow it by hydroponic, which is more efficient and easier to manage. Spinach is quite nutritious containing proteins, fats, beta-carotene, ascorbic acid, moisture and carbohydrates. It also contains several vitamins including vitamin A, B, C and K. The presence of alkaloids, flavonoids and lutein provide the medicinal value for our health. The reddish-purple color is due to the presence of anthocyanin which is an antioxidant. The spinach also contains essential minerals such as K, Ca, Mg, Fe, P, which are useful for health.
However, it has been reported that spinach also contains oxalate which is the source of stone in kidneys and other organs. Based on the extraction of dried leaves of Indian purple (Amaranth cruentus) and green (Amaranth viridis) spinach, Radek & Savage [1] reported that the total oxalate in mg/100g dried matter (DM) was 16.72 and 19.29% DM respectively. This value is high enough to cause concern over the health issue. Furthermore, the United States Department of Agriculture (USDA) has screened spinach Spinacia oleracea germplasm (338 accessions) for oxalate concentration. They found that the value ranging from 5.3-11.6% on a dry weight basis. They also observed a significant difference among the genotypes. All S. oleracea varieties have low oxalate concentration but the two varieties of S. tetradra and four S. turkestanica have high oxalate.

The oxalate concentration is affected by moisture and no correlation with leaf types and weight per plant. From the list of oxalate content in the vegetable plants, the farmers can make a selection which spinach or vegetable to grow [2]. Another idea of the presence of oxalate is the possibility of internal conversion into carbon dioxide by oxalate oxidase [3]. In medicinal plants oxalate can act as a good diagnostic tool for identification and detection of adulterants in crude drugs. Working on powdered dried leaf samples the largest crystal size obtained was 12.98-18.82 µm length and 9.74-14.27µm in width [4]. Both green and red spinach (Amaranthus tricolor) are popular for everyday cooking in both Malaysia and Indonesia.

Our literature survey indicates that there are not many reports or detailed studies on the oxalate content in vegetables especially spinach. Therefore, it is time to look at oxalate content in our spinach grown in the local environment. In this study red spinach was grown in soil and hydroponically. The presence of oxalate crystal was studied by using scanning electron microscope directly on the leaf. Elemental analysis was determined by energy dispersive-X-ray experiments.

2. Material and methods

Both red and green spinach was grown in ordinary soil mixed with organic compost behind the house in Bandar Baru Bangi, Selangor, Malaysia. The seed was obtained from Bali, Indonesia. Sufficient water was given to the plant every day until it was harvested after 30 days of planting. Hydroponic cultivation of spinach was carried out at the laboratory of Universitas Padjadjaran. Dissolved inorganic fertilizer A and B obtained from the agro-shop were used throughout the cultivation (figure 1). Hydroponic cultivation of red spinach uses the DFT (Deep Flow Technique) system with dimensions of 350cm x 120cm x 80cm. Red spinach is planted on gutters with a diameter of 2.5 inches at the age of 10 days after seedling. The nutrients provided are from AB Mix inorganic fertilizers. AB Mix hydroponic nutrition consists of 2 parts, Part A that contain Calcium and Part B containing Phosphate and Sulphate. Concentrated solutions of Parts A and B must be placed separately. Mixing these two concentrated solutions can cause calcium phosphate and calcium sulphate deposits to form. Nutrients A and B mixed with water with the degree of density given during growth are maintained in the range of EC values between 2-2.5 mS/cm.
Figure 1. Red spinach 20 days old after planting on the hydroponic setup.

Scanning Electron Microscopy
The spinach leaves were cut into small portions and dried on open air for two days. Each leave sample was mounted on a circular aluminium stub and platinum-coated in a vacuum using a sputter coater. The leaf morphology was examined by using scanning electron microscope energy-dispersive X-ray spectrometry FE-SEM, ZEISS SUPRA 55VP operating at 0.2-30kV beam current up to 400nA and the lowest vacuum of few pA-300nA. In the present study 3kV accelerating electron was applied for the imaging and 15kV for EDX experiment except at the husk where 10keV was sufficient.

3. Results and discussion
Both green and red spinach is sold in the market after about one month of cultivation. The trees are still young about 2 feet high. The soft stem and leaves are cooked together in the form of a soup or fried vegetable. However, the plant can grow more than 4 feet as shown in figure 2 but the stem will be too hard for food preparation.

Figure 2. A three months old red spinach plant.

The hydroponic spinach grows at a much faster rate by almost two-fold. Normally the hydroponic spinach is harvested after 15 days of cultivation where the plants are about 28 cm height. The size of the leaf depends on the time of harvesting but can be as wide as 15 cm and 19 cm length. The petiole lengths can be up to 10 cm. The upper leaf appears more reddish-purple than the bottom. The midrib and veins are much thicker at the bottom (figure 3).
Figure 3. The bottom leaf of hydroponic (a) and conventional (b) spinach. The top part of the conventional spinach (c).

Scanning electron microscope images of the midrib and veins consists of many fiber-looks like layers of xylem and phloem (figure 4) vessels that deliver water and nutrients to the leaf and eventually the cell. The lamina part between the veins is not smooth but consists of branching secondary veins. The average size of the stomata is 20.43µm in length and 9.00 µm opening. It is interesting to read a crazy idea that the complex structure of the veins is being imitated but replacing the water and nutrients by human blood with the aim to produce a functional mini heart [5].

Figure 4. The morphology of the bottom leaf of the hydroponic spinach showing the nature of midrib and vein (a) and the lamina section of the bottom leaf (b) (SEM, 500X).

For conventional spinach, at 500-time magnification, the presence of crystallites was not clearly observable. However, at higher magnification, we began to see the presence of small crystallites of the size between 2 and 4 microns in the lamina area (figure 5).
However, at 500 times magnification, the presence of crystallites was observable for the top part of the hydroponic leaf (figure 6) indicating the significant presence of oxalate in hydroponic leaf.

The size and shape of the crystal are distinctively clear (figure 7) when observed at 2500X magnification.
Figure 7. Hydroponic spinach leaf showing bigger oxalate crystals (SEM. 2500 magnification).

Green spinach also showed similar morphology as red spinach except its colour is green (figure 8) indicating the chlorophyll domination or the absence of anthocyanin.

Figure 8. Green spinach leaves.

The presence of oxalate crystal was observable at 1000-time magnification (figure 9) at few spots in the leaf. The size of the crystals is less than 2µm.
Figure 9. SEM image of the bottom part of green spinach (SEM, 1000 magnification).

*Elemental analysis by energy dispersive X-Ray (EDX)*

EDX is useful not only to detect important elements present in the plant but also its distribution. Therefore, it can also be used to identify the crystal. Other technologies such as Atomic Absorption Spectrophotometry (AAS), ICP, XRF, and NAA can be used to detect the presence of trace elements with greater accuracy. In the present study, the elements were detected at five different spots in the leaf samples and the average percentage value of the elements was calculated (table 1).

**Table 1. Percentage of elements in the spinach leaves.**

| Spinach leaf   | C%  | O%  | Ca% | K% | S% | Mg% | P%   | Nb% |
|----------------|-----|-----|-----|----|----|-----|------|-----|
| Conventional   | 47.3| 31.5| 4.3 | 10.6| 0.1| 0.76| 0.92 | 0.40|
| Hydroponic     | 44.5| 33.9| 2.8 | 12.6| -  | 0.82| 0.93 | 0.42|
| Green leaf     | 48.9| 33.6| 1.9 | 11.9| 2.7| 0.85|-     | -   |

Potassium content is about the same in all the leaves but very much higher than calcium more than two-fold. Green spinach has the lowest calcium content. Although calcium content in the hydroponic leaf is lesser than potassium, the size and shape of the oxalate crystals are big and clear as shown by the micrograph images.
The water availability in the hydroponic system provides a better medium for slow evaporation which can lead to a bigger and well-defined crystal shape. Green spinach showed the absence or less amount of phosphorus and niobium (figure 10). The presence of niobium has been observed in many plant products including rice [6].

The mapping of the elements in the leaves is not only to know the distribution but it also helps to identify the crystal as well. Figure 11 below shows the concentration of calcium is mainly at the crystals. Hence the crystal is calcium oxalate. It is also clear that green spinach has fewer crystals compared to an alarming situation in the hydroponic leaf.

**Figure 10.** EDX spectrum of green (a) and red hydroponic spinach (b).
The crystal shape depends on many factors including medium and evaporation. The biological factor is much more complex. However, from the study, it is clear that the availability and the efficient adsorption of minerals in the hydroponic system allow the formation of significant amount and block shape that close to its crystal system as shown by the crystal images extracted from SEM micrographs (figure 12).

Figure 11. Elemental mapping of the spinach leaves (SEM, 5000 magnification).

Figure 12. Crystal shape of oxalate formed in (a) green spinach (b) red spinach and (c) red hydroponic spinach (SEM, 5000X).
It is well known that calcium oxalate can form polymorphism. The calcium oxalate monohydrate polymorph (CaC₂O₄·H₂O) can vary in shape from a dumbbell, to oval and picket fences. The structure of monohydrate was described in 1965 [7]. This polymorph is commonly found in urine. However, in some cases, both dumbbell and envelope/octahedral shape was also found in urine [8]. The octahedral shape is due to the formation of the second polymorph, calcium oxalate dihydrate (CaC₂O₄·2H₂O). In nephrolithiasis, the transformation from monohydrate to dihydrate oxalate is induced by urinary macromolecules that reduce the crystal attachment to the epithelial cell surface [9]. The sedimentation of the oxalate in urine is common and can take place at any pH. Unlike in urine, the crystal formation in the leaf occurs in the lamina section between the veins. The formation of oxalate through the biological-chemical pathways in the leaf system and the complexation with calcium is still an interesting subject for further investigation. The third polymorph is calcium oxalate trihydrate (CaC₂O₄·3H₂O) is well studied and found to crystallize in a triclinic system with space group Pī [10]. It is also important to note that in solid state the oxalate anion chelate to the calcium atom in a bidentate manner and form a network commonly based on dimeric structure.

Many reports on elemental analysis in plants and their products gave a total amount of each metal detected. The normal techniques to quantitatively determine the elements are by Atomic Absorption Spectrophotometry (AAS), Inductively Coupled Plasma-Optical Emission (ICP-OES), X-ray Fluorescence (XRF) and Neutron Activation Analysis (NAA). The samples were digested before the measurement except for XRF and NAA which are non-destructive. However, metal ions exist in plants or the human body is in the form of salts. In the plants, the counter anions are citrate and oxalate produced biologically by the plants. Among the metal salts, calcium oxalate is not soluble. Therefore, it will come out as precipitate or under the normal condition, it will slowly crystallize with different shapes and sizes. It is quite interesting to monitor the crystallization process in the plant system under different agronomic environment or climate. Such information will be valuable for future agricultural planning.

4. Conclusion
The results showed that red spinach contains oxalate at a much higher amount than other spinach varieties. A detailed study on oxalate content in all spinach verities and other vegetables grown in our local farm is necessary for the farmers guide for seed selection and public important. Hydroponic farmers have to find a better fertilizer formulation to reduce oxalate content in spinach. Organic fertilizer will be a good alternative. There is also an opportunity for agronomists and plant breeders for the possibility to come out with new spinach verities with less oxalate. It is also a challenge for the food industries to think about the process to reduce the negative effect of oxalate in food products. Calcium oxalate is less soluble in water with a solubility of 0.67 mg/L at 20°C.

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