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In Vivo Study of the Effect of Different Levels of Chemical Fertilizers on the Indigotin Dye in the Indigofera Tinctoria Plant Using Raman Spectroscopy

Fatemah Alharthi

In vivo study of the effect of different levels of chemical fertilizers on the indigotin dye in the Indigofera tinctoria plant using Raman spectroscopy

By

Fatemah Alharthi

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In vivo study of the effect of different levels of chemical fertilizers on the indigotin dye in the Indigofera tinctoria plant using Raman spectroscopy

By

Fatemah Alharthi

Approved:

_____

Ariunbold Gombojav
(Major Professor)

_____

Hendrik F. Arnoldus
(Committee Member)

_____

Richard L. Harkess
(Committee Member)

_____

Rick Travis
Dean
College of Arts & Sciences

Name: Fatemah Alharthi

Date of Degree: December 14, 2018

Institution: Mississippi State University

Major Field: Physics

Major Professor: Ariunbold Gombojav

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An impact of nitrogen, phosphorus and potassium fertilizers on the concentration changes of carotenoid pigments in the *Indigofera tinctoria* plant by using Raman spectroscopic techniques is studied. Three different concentration levels of the fertilizers with a normal supply as control were added to the plants at two stages. The Raman spectra were taken to determine the carotenoid concentration level changes in the plant leaves *in vivo*. The ring-stretching mode are the Raman spectroscopic signatures for the carotenoid pigment and its magnitude increased significantly (over 170%) for the case of phosphorus and potassium fertilizers. The effect from the nitrogen fertilizer was detected to be about 130% in comparison with the corresponding control plants. This study has a potential application for the increased extraction of the indigotin dye from plants for the medical and textile industries.

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CHAPTER I

INTRODUCTION

1.1 General introduction

Dye has been used for thousands of years, but the most common uses in recent history concern applications to food, dye sensitized solar cells, and textile products [1]. One of the oldest dyes in the world is indigo, a natural plant produced product that has been used for thousands of years [2]. Some of the oldest recordings of indigo come from ancient times in India when it was called Nila. One of the features of indigo that makes it an important dye is that it can be used across a wide variety of different applications [3]. One of the reasons it is ideal for textile use is because it is insoluble in water [4].

The indigo plant, *Indigofera tinctoria*, has been the most famous source for the blue dye because of its availability. This is a tropical plant that belongs to the leguminosae family and it is well-known in Africa, Asia, the East Indies and South America. However, it is also possible to extract indigo from the plant *Isatis tinctoria*, which is found in the moderate climates throughout Western Europe [4].

Indigofera tinctoria features dark green, oval leaves in a shrub like plant on a single semi-woody stem. The plant can grow from 60 cm high to 360 cm high. The plant has flowers that resemble small butterflies and usually found in a pink color, or less commonly in a purple color. Although the plant does not live in extreme temperatures, it also does not grow well in soil saturated with water (Figure1.1).

Figure 1.1 *Indigofera tinctoria* plant [3]

The feature of this plant important to this research is in its production of blue indigo dye, which is referred to as indigotin. The molecular formula of indigotin is $C_{16}H_{10}N_2O_2$ and its chemical structure is shown in (Figure 1.2).

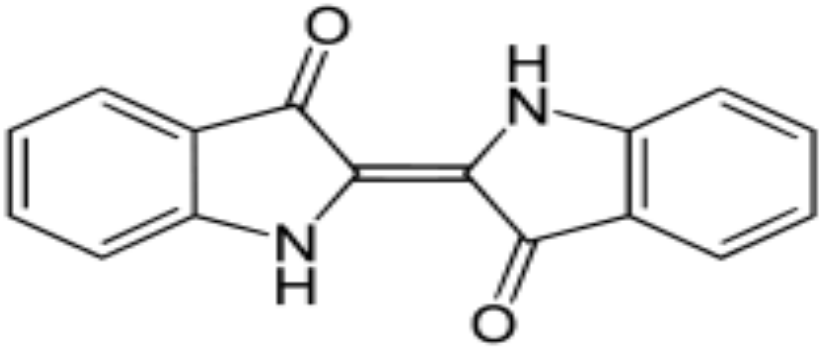

Figure 1.2 The chemical structure of indigo dye[5]

The dye is primarily found in the leaves and produced through a process of fermentation using the enzyme indimulsin [1]. This complex process occurs through multiple stages of fermentation, which begins with the extraction of leaves that contain the indicant; which is the raw material used in indigo dye. The next step in creating the dye is to add indimulsin to hydrolyze the indicant which then produces indoxyl and glucose. The resulting liquid is aerated through intense whipping so it is fully mixed and emulsified. At this point, the air will oxidize the indozyl into indigotin, generating isatin as a side product. The indoxyl will either react with an indoxyl molecule to result in indigotin, or it will react with the isatin to produce a red tinted dye product called indirubin (Figure1.3) [5].

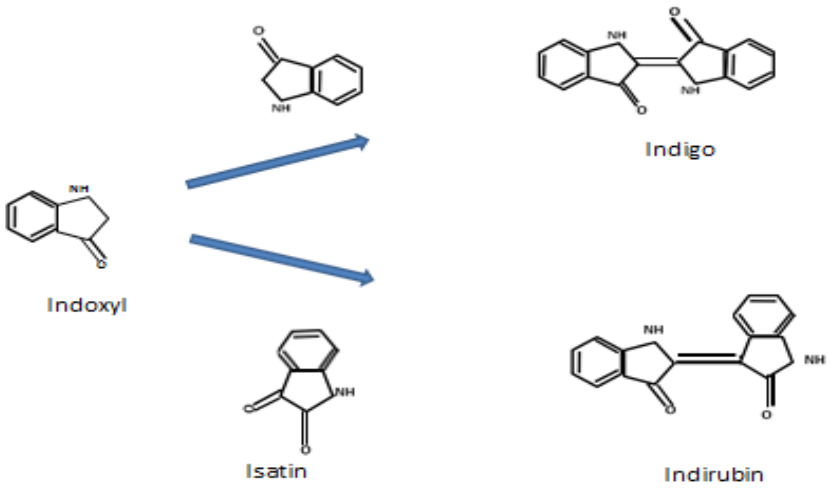

Figure 1.3 Indigo dye biochemical path[5]

Indigofera tinctoria has applications across many fields. The blue dye is used in the production of blue jeans because it is conducive to coloring common threads. However, it is also considered a medicinal plant and is used in many Indian and Chinese homeopathic medical treatments [6]. Indirubin, the active component of *Indigofera tinctoria,* has also shown some promise as an effective anticancer agent regarding chronic myelocytic leukemia [7-8]. There is also some evidence to suggest the plant can be used for treating illnesses such as liver disease, epilepsy, nervous disorders, inflammation, sores, bronchitis, mature ulcers, and hemorrhoids [9]. The potential for the plant having an effect across multiple therapeutic uses would constitute a crossover from homeopathic approaches to mainstream medicine. Evidence has been found concerning the isolated furano-flavonoids that could reduce cardiovascular risk through the decrease of blood lipid levels [10]. There is also evidence *Indigofera tinctoria* may have uses in relationship to anti-hyperglycemic

activity, antioxidant effects, cytotoxicity effects, antibacterial effects, anti-inflammatory activity, antidiabetic activity, hepatoprotective activity, or even as an Anticonvelsive agent [11].

In light of the number of ways the plant may have some influence on medical conditions, especially cancerous and chronic illnesses, the research for this study focuses on the concentration of dye that exists within the *Indigofera tinctoria* plant. Through studying the effect N, P, and K fertilizers as they are applied to the plant, it is possible to measure the relative concentrations of blue dye in the plant across the three different fertilizers using Raman spectroscopy.

1.2 Raman spectroscopy

1.2.1 Overview

The term Raman spectroscopy was applied to this technique because of the Indian physicist Sir Raman Venvata Chandrasckra who developed the concepts used for this form of spectroscopy. Raman spectroscopy is an important technique used across multiple applications in research. The purpose of the technique is to observe and identify specific molecules [12]. Raman spectroscopy is a mechanism that scatters light the purpose of which is to define simple forms through the way in which photons interact with samples. The radiation that is scattered is at a different wavelength than the incident radiation. Materials can be analyzed by measuring shifts in the light energy that scatters as a result. Raman spectroscopy is a technique that is considered to be useful for the identification of materials. Many fields use this technique, such as drug investigation, security inspection, pharmaceuticals, food safety, jewelry identification, and any field that must identify materials in a quick and effective manner. Raman spectroscopy has been found to outperform other vibrational spectroscopy methods, including FT – IR and NIR. The technique has advantages in measuring gas, liquid (no absorption due to water), and solid materials, and also has the desirable property of being able to be used through transparent plastic and glass containers [12].

While the effect was originally predicted by Smekal in 1923, Sir Raman Venvata Chandrasckra actually discovered the effect in 1928. The prediction of the effect suggested it could happen, but Sir Raman discovered that it did happen through experimentation using sunlight and filters to observe color shifts. The changes observed led Raman to investigate

the spectra of multiple liquids which included carbon tetrachloride and gasoline. This technique used a mercury lamp and a spectrograph instead of sunlight [12].

However, Raman's effect is considered to be weak in nature and usually only results in a miniscule fraction of the intensity of the exciting radiation. Stability and intensity had hindering effects on Raman spectroscopy for several years in comparison to the use of IR spectroscopy. IR spectroscopy was developing much more quickly and consistently had a lower cost. However, a major improvement occurred in 1952 when the Toronto arc source was introduced. In the 1960s, Raman spectroscopy experienced a renaissance due to the discovery of laser beams, as well as the commercial development of continuous wave visible lasers. The use of highly monochromatic, coherent narrow beam and high intensity light were revolutionary factors that re-energized the use of Raman spectroscopy. The resulting technique allowed for spectra to be easily recorded from small samples, as well as samples with color, liquids, gases, solids, and samples that were at a high temperature. In addition, samples could be used in a dilute solution, within a vacuum, and within a variety of different types of nonstandard conditions [12-13].

1.2.2 Theoretical Background

There are many ways a monochromatic beam can be expected to behave when it is projected into a sample. The results may be that it is absorbed, reflected, or scattered. Raman spectroscopy is focused on the conditions in which a beam is scattered. Simply put, Raman's effect is a method of scattering light through inelastic methods so the material and its molecules can be excited to high levels of rotational or vibrational energy. When the light penetrates the molecule, it will create a relationship with the electron cloud in the bonds of the molecule. When the molecule relaxes, it emits a photon and returns to a

different vibrational and/or rotational state. There are three forms that can be observed through scattering. The first one is called Rayleigh scattering. This form is elastic and will return the molecule to its ground state, as well as emit a photon equal to the photon energy of the incident. The second form of scattering is inelastic and is called Stokes shifted Raman Scattering. This type of scattering results in a relaxed state of higher energy than the ground state, thus producing a photon that has a less energy than the incident photon. Finally, the third form is inelastic scattering called Anti-Stokes Raman scattering. When this occurs, the molecule is relaxed and placed back into its ground state, but will admit a photon that has more energy than the incident photon (Figure1.4).The Raman shift can be represented with the following equation:

$$\Delta\omega = \frac{1}{\lambda_{\circ}} - \frac{1}{\lambda 1} \qquad (1)$$

Where $\Delta\omega$ is the shift in wavenumber, $\lambda_{\circ}$ the excitation wavelength, and λ_1 the Raman spectrum wavelength [12].

Based on the intensity and position of the spectral peaks that can be observed in Raman spectroscopy, it is possible to identify and characterize a molecule. On the red side, which is of a lower frequency, radiation scattering of the Rayleigh line is determined to be Stokes scattering. When the radiation scattering occurs on the blue side, or the higher frequency side of the Rayleigh line, it is called anti-Stokes [14].

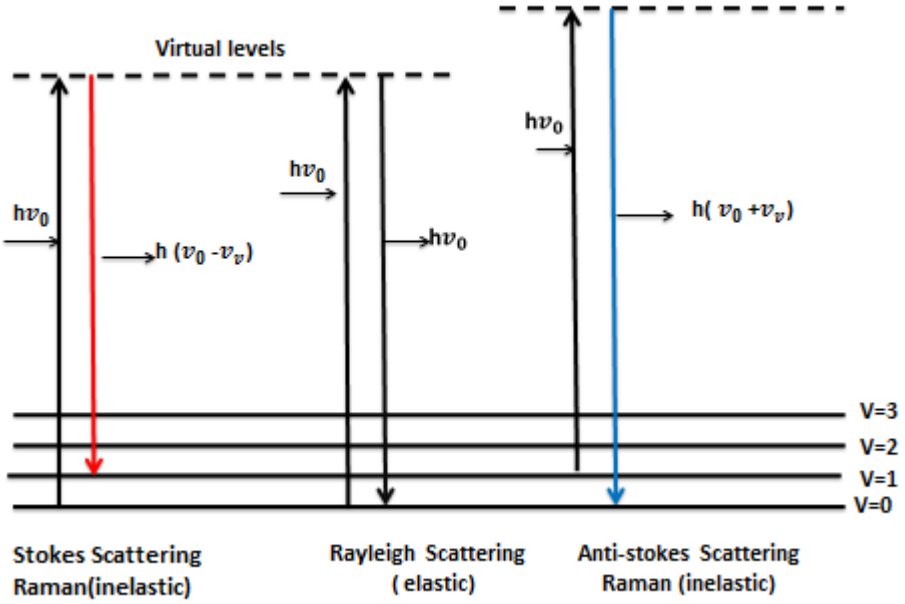

Figure 1.4 Quantum energy transitions for Rayleigh and Raman scattering [12]

CHAPTER II

CHEMCAL FERTILIZER

2.1 The importance of chemical fertilizers for the plants

In the last part of the 20th century and the beginning of the 21st century, industrial fertilizers became a prominent feature of the agricultural industry. Fertilizers provided increased production in the agricultural sector, as well as compensating for nutrient deficiencies found in soils that have been over produced and cultivated for a long period of time. Fertilizers are defined through the properties that provide necessary nutrients, whether they are artificial or natural [15]. Fertilizers typically have two main categories: organic and chemical. An organic fertilizer is a natural source of nutrients, while chemical fertilizers are considered industrial and not sourced from a natural nutrient producing resource. Natural fertilizers come from plant or animal residues, while chemical fertilizers are produced by human beings from chemical and mineral materials in manufacturing plants that are tooled for the purpose of producing these chemical interactions [16].

The properties of soil are critical in providing nutrients to plants. Most plants require approximately 18 elements for natural growth. Three of these elements typically essential for plant growth include carbon (C), hydrogen (H) and oxygen (O). These crucial elements are provided through the air and water that brings nutrients to the plants [16]. Plants need other nutrients, which must be found within the soil system.

Primary, or macronutrients, found in plants include N, P, and K. This means nitrogen, phosphorus, and potassium are all either primary or macronutrients for plants in the world. Other nutrients available to plants include calcium (Ca), sulfur (S) and Magnesium (Mg). These last three are considered secondary nutrients because it is less likely they will have an influence on limiting factors for growth that exist within a soil system. There are also a wide variety of micronutrients necessary to plant growth, but they are unimportant to the study at hand [17]. Table 2.1 shows the essential plant nutrients and their chemical symbols.

Table 2.1 The essential plant nutrients and their chemical symbols [17].

Nutrients Supplied by Air and Water	Nutrients Supplied Nutrients Supplied by the Soil System by Air and Water		
Non-Mineral	Primary or Macronutrients	Secondary	Micronutrients
Carbon - C	Nitrogen - N	Calcium - Ca	Zinc - Zn
Hydrogen - H	Phosphorus - P	Magnesium - Mg	Chlorine - Cl
Oxygen - O	Potassium - K	Sulfur - S	Boron - B
			Molybdenum - Mo
			Copper - Cu
			Iron – Fe
			Manganese – Mn
			Cobalt - Co
			Nickel - Ni

At times the quantities of materials in the soil needed for proper nutrition are not available. Therefore, chemical fertilizers can substitute for the natural organic fertilizer missing within the soil structure [17]. Three of the most important nutrients for crops are nitrogen, phosphorus, and potassium, which are often given to plants as a simple fertilizer, or one that has become a compound through combining any of these three nutrients. The

most commonly used nutrient compound in agriculture is NPK. Fertilizers have the capacity to be in a gas, solid, or liquid form for getting nutrients into the soil [15].

Nitrogen is an important macronutrient which has a critical role in the life span of the plant. It has the capacity to increase production and to increase the overall yield of an agricultural planting. Nitrogen is an important part of chlorophyll, the compound by which plants use sunlight to produce sugar from water and carbon dioxide in a process called photosynthesis. Nitrogen helps create the proteins, nitrogen organic compounds, and nucleic acids being composed for roles within the photosynthetic processes. Nitrogen is absorbed into a plant as a nitrate anion (NO_3^-) and a cation (NH_4^+) [16].

Phosphorus (P) is a basic element required by the plant and holds just as much importance as nitrogen. Plants need phosphorus at the beginning stages of growth more than any other nutrient. Phosphorus promotes the spread of plant roots, which in turn absorb water and nutrients from the soil promoting plant growth. Phosphorus is absorbed into the soil from its forms as an anion ($H_2PS_4^-$) and (HPO_4^{-2}). In sufficient quantities, phosphorus has the capacity to stimulate root growth in the early-stages, as well as increase seed formation and bring on maturity. Phosphorus also helps to optimize the way in which a plant will use water and nutrients. When phosphorus deficiency is clearly visible, it is likely a plant is going to either be stunted or discolored because of high levels of sugar retained by the plant. This will lead to decreased productivity [17].

Potassium sulfate is also a critical nutrient for a plant. Potassium (K^+) has a unique influence on crop yields because it has the quality of increasing the degree a plant can resist drought and disease. Therefore, the leaves and stems of the plant become more durable. It is an essential component of photosynthesis because it helps facilitate sugar movement a plant. Plants that do not have a sufficient amount of potassium will have yellow leaves, with the yellow color beginning at the edges indicating the leaves are damaged [16].

There are multiple methods for adding chemical fertilizers to fields. In areas where nutrients are considered poor, fertilizers are typically scattered so the overall soil fertility is increased. Fertilizer is capable of having an effect before or after planting has been conducted. Large areas of *Indigofera tinctoria* can have greatly benefit from adding fertilizer to the soil. Scattering is a common method for fertilizing large areas of the *Indigofera tinctoria* plant. This allows the nutrients to have a greater chance of being absorbed through the root system. However, in this research small pots were used, and instead of being scattered, the nutrients were given to the plant. This was the most convenient method of fertilization for this research.

CHAPTER III

EXPERIMENTAL ARRANGEMENTS

3.1 Sample preparation

The experiment took place during the spring of 2018 at the greenhouses of the Plant and Soil Sciences Department and in a laboratory in the Physics and Astronomy Department. Appropriate environmental controls were applied to the experiment to control the conditions. The seeds used came from a commercial source (www.worldseedsupply.com). The count of the seeds is 150, and the measurement is 10g. The seeds are from the *Indigofera tinctoria* plant that began with a brown, hard shell. The seeds can be processed by soaking them in a solution of sulfuric acid for an entire day, or through the scratching method in which the seed coats are scratched before planting. For this experiment, the seeds were soaked in the dilute sulfuric acid for a full day.

Once the seeds had been soaked for full day, they were prepared for cultivation. The soil was prepared by placing it into 15cm farming pots so that they could be easily transported. Appropriate soil was selected for planting with the intention of making sure that it would not interfere with the fertilizing component of the experiment. The plants were watered on every other day of the experiment. The temperature and humidity levels were kept stable.

Once the plants began to experience growth that was visible, data was collected using the Raman spectroscopy from the emerging shoots. The power of the lasers was adjusted so

the sample could be tested without affecting living cells. Five examples of the Raman Spectra were collected as data from the same leaf in each of the sample plants. This provided a more consistent ability to observe changes.

3.2 Applying the fertilizers.

The application of the fertilizer was spread across three different levels of nitrogen fertilization (0g, 0.2g, and 0.4g N/L) N0, N1 and N2 which were developed in the form of urea (46%) respectively. Three different levels of phosphorus (0g, 0.05g, 0.10g P/L) P0, P1, P2 in the form of (P_2O_5 47%, 45%) were also applied. The third form was created with potassium through three different levels (0g, 0.03g, and 0.06g K/L), which were also applied in the form of potassium sulphate (K_2SO_4) and symbolized by the symbol K0, K1and K2, respectively.

3.3 Data Collection

After the plants have been cared for through two weeks of providing water and fertilization, as well as measuring plant growth, spectroscopic measurements were taken a second time. Every two weeks, Raman spectral data of the plants were obtained, with the fertilizer being applied twice at two-week intervals. The third set of data was taken without adding additional fertilizer so that the plants would not experience an excess. See (Figure3.1) for the *Indiogfera tinctora* plant from the greenhouse.

Figure 3.1 *Indiogfera tinctora* plant in the greenhouse.

Experimental setup

The experimental layout of a confocal Raman setup consisted of a continuous wave 532 nm tunable laser (LSR532H-1.5W, Lasever), optics, and spectrograph (SR-500I-A-R, Andor), and with EMCCD camera (DU970P-BVF, Andor). As is seen from Figure. 3.2, a laser beam was collimated using two silver coated mirrors (Thorlabs). The laser beam and Raman signal were separated by a dichroic mirror (DMLP500R, Thorlabs). The laser beam was focused by a 10-cm-lens. The plant sample leaf was placed at the focal plane (at 10 cm away from the lens) on the 3D translational stage (Thorlabs). The Raman signal separated from the input beam was further filtered spatially and spectrally by using two lenses and a notch filter (NF533-17, Thorlabs). The filtered signal was directed by two silver coated mirrors and focused by a 10-cm-lens into the spectrograph for creating spectrally resolved imaging with a grating (grooves 600 lines/cm) to the EMCCD camera. The spectrometer was protected from possible overexposure (saturation) by a wheel neutral

density filter (FW1AND, Thorlabs) which was placed between the spectrometer and the lens. The slit of the spectrometer was opened manually so the Raman signal could be collected and calibrated. The samples collected with the use of the spectrometer were recorded with the use of Solis software that came with the spectrometer.

The Raman spectra were collected from a leaf (not detached from the plant) placed between the glass slides at the focal plane. The glass slides prevent any systematic errors possible from misplacing the plant leaf from the focal plane. The setup was optimized first by using the pyridine chemical (liquid phase) before taking any spectra. Raman peaks were observed in the wavelength range of 431 – 510 nm, which utilized Solis software for the laser power set at 0.4 mW with an exposure time 10 sec for each spectrum. The kinetic mode records 5 spectra at each stage. Data were saved as ASCII files and processed by the MATLAB programming software provided by Mississippi State University.

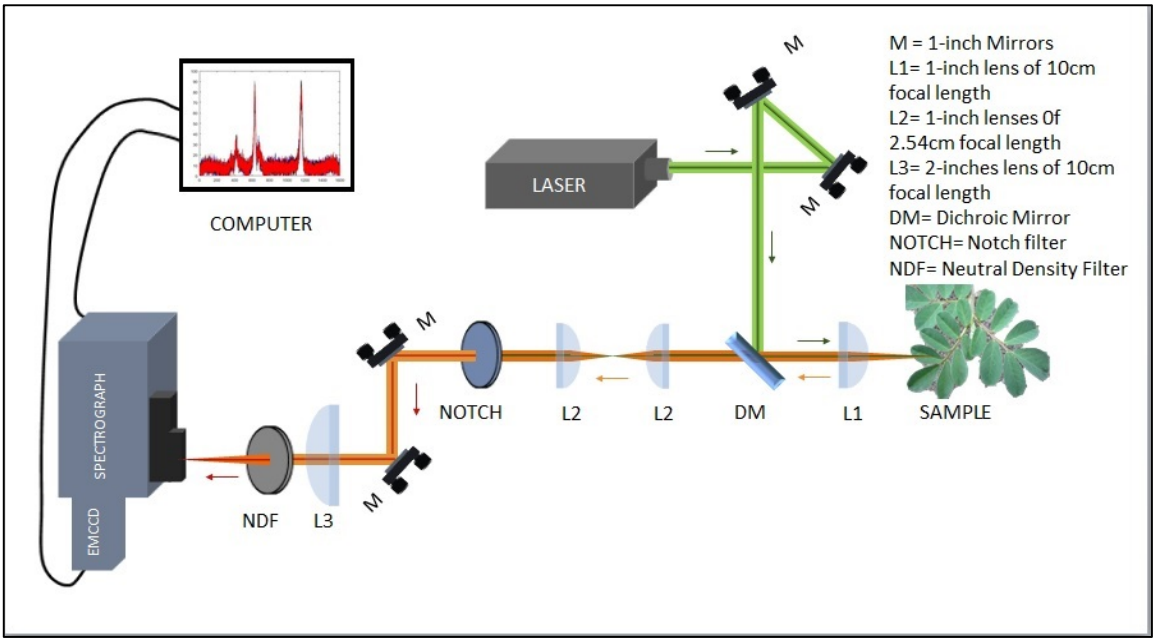

Figure 3.2 Experimental Raman setup

CHAPTER IV

DATA ANALYSIS AND RESULT

4.1 Data analysis method

It may be challenging to interpret spectra because certain laser source wavelengths may be absorbed by specific pigments. As a result, the Raman may produce weak signals that have been affected by the florescent background or through photodegradation of the sample. A continuous wave 532 nm green laser was used for the purpose of classification that allowed for good spectral analysis of the indigotin dye [18]. The ring-stretching mode is the Raman spectroscopic signature (at 1558cm^{-1}) for the carotenoids and its magnitude increased significantly. Because the carotenoid pigments increased, it is expected that the indigotin dye also increased [21]. The results of Raman spectroscopic measurements of *Indigofera tinctoria* samples will be explained in the following sections.

4.1.1 Result for nitrogen fertilizer

Research has shown that legumes are able to grow and thrive in soil that is nitrogen poor, derived from symbiotic fixation. Therefore, nitrogen fertilization is not typically necessary to grow legumes. However, when high yields of crops are desired it is possible nitrogen fertilization can help to increase the overall growth. At the early stages, the nodules will not grow fully, which leads to plants that are obtaining nitrogen from the soil as well as that which is stored within the cotyledons. This can lead to underdeveloped

growth because there are insufficient amounts of nitrogen available. A small starter dose of nitrogen can stimulate legume growth as well as nitrogen fixation [19].

It appears from the results of Raman spectroscopy that there is a notable effect of high doses of nitrogen in increasing the concentration of carotenoids in *Indigofera tinctora* plant, as N3 exceeded other factors with a peak intensity of 130% of the control peak at $1558cm^{-1}$. While N_1 showed a peak intensity of 125% of the control peak (Figure 4.1).

The result occurred because nitrogen stimulates vegetative growth because of its influence on photosynthesis and protoplasmic synthesis. In addition, nitrogen had the capacity to stimulate auxins and their transmission within the plant tissues. This has the effect of increasing levels of active substances in the plant such as indigotin. Nitrogen is also known to increase the activity within the plant tissues related to gibberellins, which are responsible for facilitating cell elongation and division. [20].

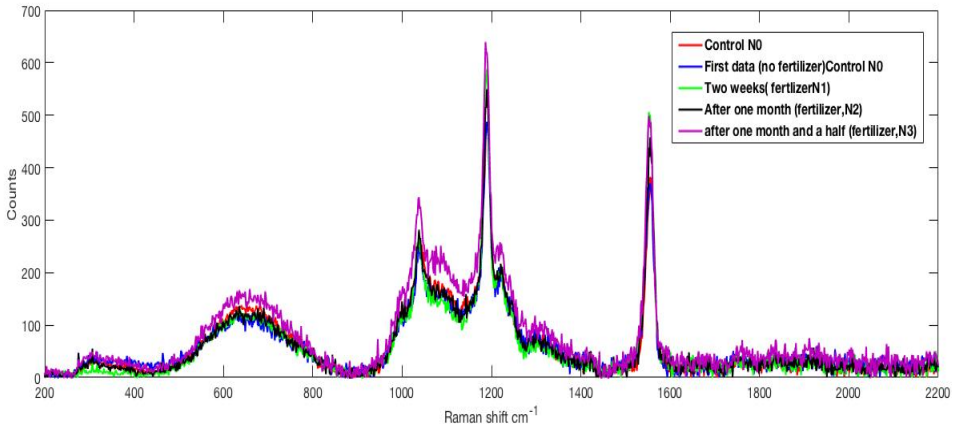

Figure 4.1 Raman spectra of carotenoids, in the Indigofera plant treated with N fertilizer
λ = 532nm; laser power= 0.4 mW; integration time = 10s

4.1.2 Result for phosphoric fertilization

Fertilization factor P3 reached a peak intensity of 170% of the control intensity at 1558 cm-1. On the other hand, P2 only reach an intensity of 120% of the control intensity at 1558 cm-1, demonstrating the effects of phosphorus fertilizer over prolonged periods (Figure 4.2). This is likely due to the role of phosphorus in supporting strong root development, which increased the levels of absorption of nutrients. In addition, phosphorus has a role in creating the molecules of ATP. Organic processes in the plant were positively affected, which resulted in increases in the concentration of carotenoids within the Indigofera plant. Phosphorus also participates in breaking down carbohydrates and converting them into energy that is essential for the growth of the plant and its various biological processes. This contributes to cell division and creation, as well as the development of amino acids and proteins, the basis of plant cell structure.

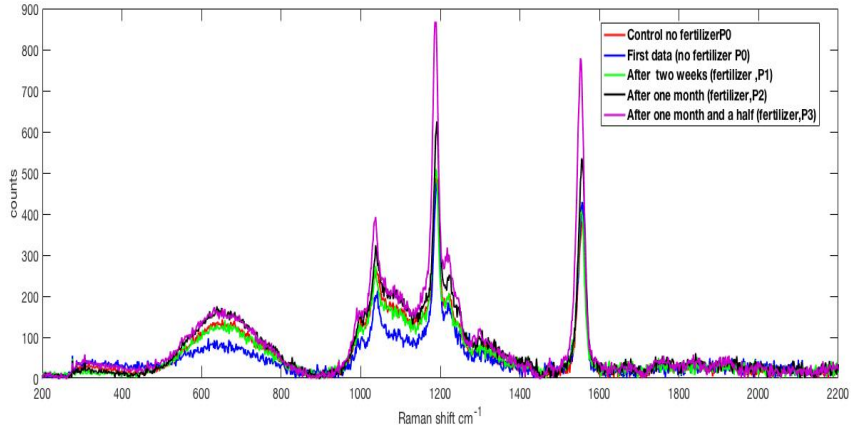

Figure 4.2 Raman spectra of carotenoids, Indigofera plant treatment with P fertilizer

λ = 532nm; laser power= 0.4mW; integration time = 10s

4.1.3 Result for potassium fertilization

Potassium fertilizer had an impact on increasing the intensity of carotenoids pigment. As figure 4.3 shows, the factor $K3$ exceeded other factors reaching an amplitude of 170% of the control at 1558 cm^{-1}. The factor $K2$ also exceeded the control K0 with a maximum height of 110% of the control at 1558 cm^{-1}. This is likely due to the role potassium plays within the plant cell. Potassium in the cell solution provides support for maintaining cells turgor pressure. In addition, potassium was indicated as important because of its role in the activation of the enzymes within the plants. Furthermore, it has a positive effect on the overall growth of the plant. Potassium is also found to regulate CO_2 uptake by controlling how the stomas would open and close.

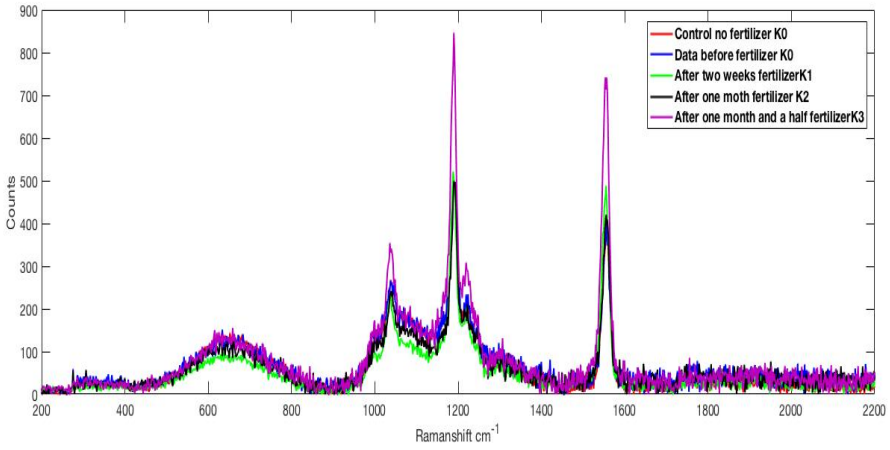

Figure 4.3 Raman spectra of carotenoids. Indigofera plant treatment with K fertilizer

λ = 532nm; laser power= 0.4mw; integration time = 10s

CHAPTER V

CONCLUSION AND FUTURE WORK

5.1 Conclusion

This research intended to examine the effect of nitrogen (N), phosphorus (P), and potassium (K) fertilizers on the carotenoids in the plant. Raman spectroscopy provided an *in vivo* method through which analysis of the dye concentration changes in *Indigofera tinctoria* plant is quantitatively evaluated. The levels of carotenoids can be used as an indicator of the level of indigotin [22]. In particular, the variations of fertilizers across time revealed that different levels of the carotenoids appeared within the plants. The fertilization increased formation of pigments. Therefore, it is expected that the indigotin dye increased. There needs more research to reveal dye concentration increase by Raman spectroscopy.

The results of the study showed that the plants treated with the chemical fertilizers nitrogen, phosphorus and potassium increased the concentration of the dye at different rates, depending on these chemicals. Although phosphorus and potassium fertilization outperformed the nitrogen fertilizer, there was still a significant effect from nitrogen fertilization levels as it was higher than the control. The treatment with (0.10g) P/L and (0.06g) K/L resulted in significantly higher concentration of carotenoid pigments (over 170%) for the case of phosphorus and potassium fertilizers. The effect of nitrogen fertilizer was detected to be about 130% in comparison with the corresponding control plants. These results are consistent with expectations because legumes do not require significant use of

nitrogen fertilizer. Thus, it makes sense the nitrogen fertilizer gave the lowest overall increase in dye concentrations when compared to the phosphorous and potassium fertilizers. The application of chemical fertilizers has potential for increasing the concentration of indigotin dye, which would in turn increase the production for medical and textile industries.

5.2 Future work

Because of the potential for wide ranging applications in the medical field, further studies of the *Indigofera tinctora* are highly recommended. The evidence also supports the idea that *Indigofera tinctoria* could have an effect on anti-hyperglycemic activity, antioxidant effects, cytotoxicity effects, antibacterial effects, anti-inflammatory activity, antidiabetic activity, hepatoprotective activity, or even as an Anticonvulsive agent [11]. Therefore, the study of increasing different types of chemicals within the plant that are related to treatments for these various diseases are recommended with the intention of supporting further medical advances. The substance indigotin is one of many active constituents within the plant. Other active constituents include flavonoids, alkaloids and glycosides, terpinoids, indigotine, indiruben, and rotenoids. Furthermore, phytochemical constituents, which may have an influence on a variety of different types of therapeutic actions, can also be found within the plant.

The *Indigofera tinctora* plants used in the research will be placed in a field in the Mississippi state university Research Station at Crystal Springs, MS so that further study and research can be conducted on these specific plants. Further research will include how these chemical fertilizers increase the production of indigotin, with increased accuracy in

techniques and in research methods. One of the methods that will be used will involve high performance liquid chromatography. This method has been used to analyze clinical medical information related to biomedical and analytical medical issues. This method is important because it helps to separate and distinguish compounds within samples.

As research continues to grow on the topic of the chemical properties of this plant; the use of a variety of different techniques will emerge. However, for the purposes of this study, Raman spectroscopy performed with accuracy, allowing the researchers to study the effects of different compounds on the increase of the chemicals within the *Indigofera tinctora* plant.

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